



Orchid bees: a new assessment on the rarity of diploid males in populations of this group of Neotropical pollinators

Douglas C. Giangarelli, Gabriele A. Freiria, Dhiego G. Ferreira, Willian M. Aguiar, Rafael E.S. Penha, Alessandra N. Alves, Maria C. Gaglianone, Silvia H. Sofia

► To cite this version:

Douglas C. Giangarelli, Gabriele A. Freiria, Dhiego G. Ferreira, Willian M. Aguiar, Rafael E.S. Penha, et al.. Orchid bees: a new assessment on the rarity of diploid males in populations of this group of Neotropical pollinators. *Apidologie*, 2015, 46 (5), pp.606-617. <10.1007/s13592-015-0350-5>. <hal-01557206>

HAL Id: hal-01557206

<https://hal.science/hal-01557206v1>

Submitted on 5 Jul 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Orchid bees: a new assessment on the rarity of diploid males in populations of this group of Neotropical pollinators

Douglas C. GIANGARELLI^{1,2}, Gabriele A. FREIRIA^{1,3}, Dhiego G. FERREIRA^{1,3},
Willian M. AGUIAR^{4,5}, Rafael E.S. PENHA^{1,3}, Alessandra N. ALVES^{1,3},
Maria C. GAGLIANONE⁴, Silvia H. SOFIA¹

¹Laboratório de Genética e Ecologia Animal, Departamento de Biologia Geral, CCB, Universidade Estadual de Londrina (UEL), Rod. Celso Garcia Cid, km 380, CP 10.011, 86057-990, Londrina, PR, Brazil

²Programa de Pós-Graduação em Ciências Biológicas, UEL, Londrina, Brazil

³Programa de Pós-Graduação em Genética e Biologia Molecular, UEL, Londrina, Brazil

⁴Laboratório de Ciências Ambientais, Programa de Pós-Graduação em Ecologia e Recursos Naturais, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego, 2000, Parque Califórnia, 28013-602, Campos dos Goytacazes, RJ, Brazil

⁵Universidade Estadual de Feira de Santana, Av. Transnordestina, s/n, Novo Horizonte, 44036-900, Feira de Santana, BA, Brazil

Received 18 March 2014 – Revised 12 November 2014 – Accepted 20 January 2015

Abstract – The incidence of diploid males among 1457 individuals of different populations of six euglossine bee species (*Eufriesea violacea*, *Eulaema cingulata*, *Euglossa annectans*, *Euglossa iopoeila*, *Euglossa pleosticta* and *Euglossa truncata*) was investigated with microsatellite markers. Bees were surveyed in Atlantic Forest fragments showing different sizes and degrees of human interference. Our analyses revealed that, although the frequencies of diploid males remained below 10 % for five out of six species studied, the frequencies of these males in three populations of *Eg. annectans* were above 15.0 %. For this species, while the average estimate of diploid males was around 11.0 %, the highest percentage (27 %) of such males was found for the population from an island. Our findings indicate that the frequency of diploid males can vary substantially among species and this fact should be taking into consideration in studies and conservation actions involving different euglossine bees.

microsatellites / Euglossini / euglossine bees / *Euglossa annectans* / forest fragments

1. INTRODUCTION

Bees and other hymenopteran insects are characterized by haplodiploidy, with arrhenotokous parthenogenesis as the common mode of reproduction (Cook 1993; Harpur et al. 2013). Among these insects, sex is determined by ploidy, by

which unfertilized eggs develop into haploid males and fertilized eggs usually develop into diploid females (Cook 1993; Cook and Crozier 1995; Harpur et al. 2013).

At genic level, the single locus complementary sex determination (sl-CSD), which depends on allelic variation at a sex locus, is one of several mechanisms of sex determination in hymenopterans. Considered widespread in bees, sl-CSD is present among social and non-social bees (van Wilgenburg et al. 2006; Zayed et al. 2004; Zayed 2009). Heterozygotes at the CSD locus develop into diploid females; however, when homozygous at this locus, instead of females, diploid males (2N males) are produced from fertilized

Electronic supplementary material The online version of this article (doi:10.1007/s13592-015-0350-5) contains supplementary material, which is available to authorized users.

Corresponding author: S. H. Sofia,
shsofiabelh@gmail.com
Manuscript editor: Yves Le Conte

eggs (Unruh and Messing 1993; Zayed et al. 2004; van Wilgenburg et al. 2006). Although, several recent studies have shown that 2N males are viable (Cowan and Stahlhut 2004; Elias et al. 2009; Harpur et al. 2013), they usually exhibit low fitness (Unruh and Messing 1993) and are thus considered very costly to populations of haplo-diploid insects (Crozier 1977; Unruh and Messing 1993; Beye et al. 2003; Heimpel and de Boer 2008; Zayed 2009; Harpur et al. 2013). In addition, considering that for each diploid male produced a functional female ceases to be raised, diploid males inflict a significant cost on the reproductive success of their parents (Unruh and Messing 1993; Zayed and Packer 2005; Zayed 2009).

In bees, diploid males have been observed in four families and in at least 27 species of both solitary and social bees (Zayed 2009). In the last two decades, several studies have investigated the frequency of diploid males in Euglossini (Roubik et al. 1996; Zayed et al. 2004; López-Urbe et al. 2007; Souza et al. 2010), a Neotropical group of bees popularly known as orchid bees considered to be important pollinators throughout the tropical Americas (Dressler 1982; Roubik and Hanson 2004).

Roubik et al. (1996) reported high frequencies of these males in populations of some species of Euglossini (e.g. *Euglossa imperialis* Cockerell) in Panama. This finding was corroborated by a second study that showed high levels of diploid male production and low effective population sizes (Zayed et al. 2004). Both of these studies were based on allozyme analyses (Roubik et al. 1996; Zayed et al. 2004), as was a study carried out by Takahashi et al. (2001), which found very different results. More recently, Souza et al. (2010), using microsatellite markers, analysed 1010 euglossine males, belonging to 27 different species across the Neotropics, and also detected very low frequencies of 2N males for all samples studied. Based on their findings, Souza et al. (2010) suggested that diploid males are rare in orchid bees. However, it should be noticed that about 50 % of the species surveyed by Souza et al. (2010) were represented by a restricted number of individuals ($n < 20$) in the samples analysed. Thus, further studies involving larger samples would be

interesting to confirm whether diploid males are in fact rare among orchid bees and if this hypothesis can be generalized for the group.

Some euglossine species are apparently sensitive to environmental disturbances (Powell and Powell 1987; Tonhasca et al. 2002; Giangarelli et al. 2009; Nemésio 2013), such as forest fragmentation and reduction in fragment size (Powell and Powell 1987; Sofia and Suzuki 2004; Giangarelli et al. 2009). Apart from reductions in the abundance of orchid bees in forest fragments of reduced size, studies have shown that some species may have stronger dispersal limitations in fragmented landscapes, and the type of matrix may also affect the dispersal of some species of the group differently (Powell and Powell 1987; Milet-Pinheiro and Schlindwein 2005).

Considering that declines in bee populations could result in genetic diversity loss (Cook 1993; Zayed 2009) and that in euglossine bees, the sI-CSD system has been proposed as the sex-determining mechanism for different species of this group of bees (Zayed et al. 2004; van Wilgenburg et al. 2006; Souza et al. 2010), it would be expected that in small populations of these bees, a higher frequency of 2N males should occur (Zayed et al. 2004; Zayed 2009). Despite this, to date, information regarding diploid male frequency in forest fragment showing different sizes and/or different degrees of disturbance is still scarce in the literature. Aiming to provide some information concerning this issue, the current study investigated the frequency of diploid males in populations of six species of orchid bees in Atlantic Forest fragments with different sizes and levels of preservation.

2. MATERIALS AND METHODS

2.1. Study areas

The 29 study sites where euglossine bees were collected are distributed throughout five different Brazilian states (Figure 1) located in southern and south-eastern Brazil as follows: in the southern region [States of Rio Grande do Sul (RS), Santa Catarina (SC), and Paraná (PR)]; and in the south-eastern region [States of São Paulo (SP) and Rio de Janeiro (RJ)]. The study sites consisted of areas that have different types of vegetation

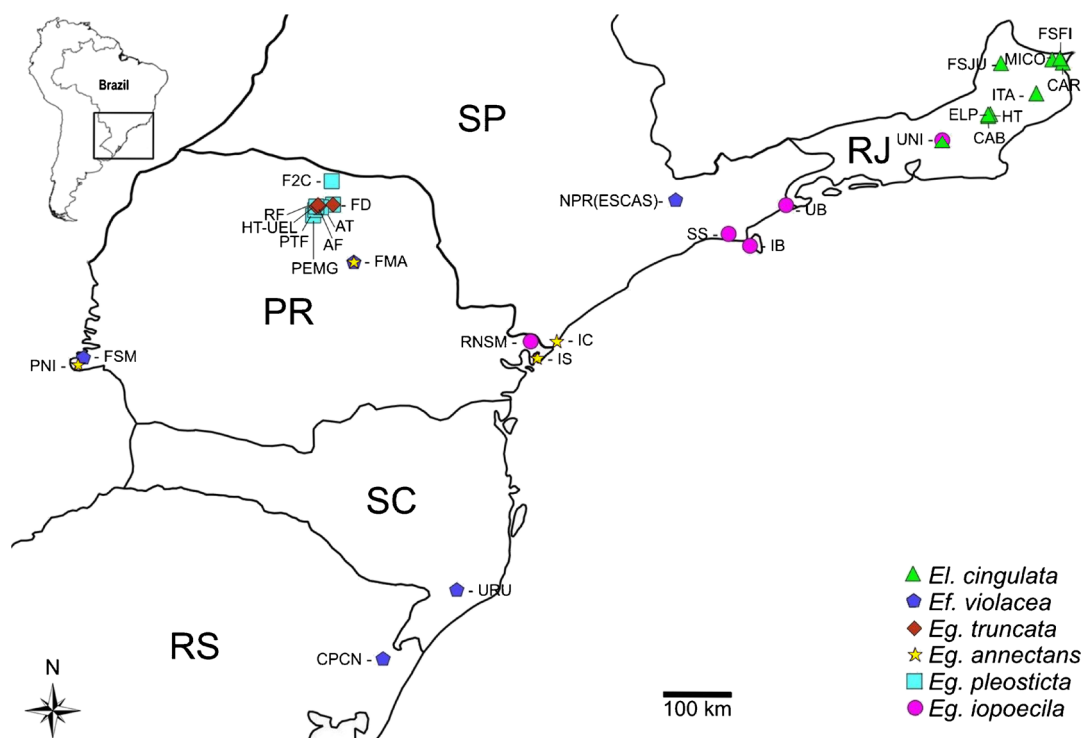


Figure 1. Map of Brazil, in South America, in detail, the 29 localities distributed in five Brazilian states (RS=Rio Grande do Sul, SC=Santa Catarina, PR=Paraná, SP=São Paulo and RJ=Rio de Janeiro) where males of the species *Eufriesea violacea*, *Eulaema cingulata*, *Euglossa annectans*, *Euglossa pleosticta*, *Euglossa iopoecila* and *Euglossa truncata* were sampled. Additional information on the 29 study sites, herein represented by codes (FSM, PEMG, FMA, ESCAS, CPCN, URU, CAB, UNI, ITA, FSJU, HT, MICO, CAR, ELP, FSFI, FD, F2C, PTF, AT, HT-UEL, RNSM, IS, PNI, IC, SS, IB, UB, AF and RF) are presented in Table I.

and degrees of preservation (Table I). Bees were sampled in remnants of dense forest (DF), seasonal semi-deciduous forest (SF) and *Araucaria* mixed forest (MF), which represent important types of Atlantic Forest ecosystems, located in private and governmental areas. Also, a few forest fragments located in urban areas, one of them representing a governmental park (AT), were surveyed for euglossine bees (Table I). The number of sites where bees were collected varied from four (*Eg. truncata*) to nine (*El. cingulata*).

While some study areas had very well preserved native vegetation characterized by large areas with primary forest, others were forest fragments with vegetation in different stages of regeneration (mainly ranging from intermediate to advanced successional stages), referred to as 'secondary forests'. Thus, in an attempt to characterize all of these areas, they were classified in one of three main categories of degree of disturbance:

low, medium and high. For these three categories we took into consideration both the vegetation successional stage and the level of anthropogenic disturbance of the area.

2.2. Bee sampling

The analysis involved males of six species [*Eufriesea violacea* (Blanchard), *Eulaema cingulata* Fabricius, *Euglossa annectans* Dressler, *Euglossa iopoecila* Dressler, *Euglossa pleosticta* Dressler and *Euglossa truncata* Rebêlo and Moure] which are strongly associated with the Atlantic Forest (Nemésio and Silveira 2007; Nemésio 2009; Mattozo et al. 2011). Hereafter in the text the following abbreviations are used for different genera and commonly employed in studies on Euglossini: *Ef.* = *Eufriesea*; *Eulaema*=*El.* and *Eg.* = *Euglossa*.

Table 1 Name of the six euglossine species surveyed, number of polymorphic microsatellite loci analysed (N_L), collection site, characterization of the 29 forest fragments sampled for orchid bees [including the following information: size (in hectare), type of vegetation, degree of disturbance and type of the surrounding matrix, private or governmental reserve], number of males analysed (n males), observed number of diploid males (2N males), expected heterozygosity (H_e), probability of a male, if diploid, being heterozygous for at least one polymorphic locus (P_{het}), probability of detecting a diploid males per locality adjusted for putative null alleles ($P_{het-null}$), number of diploid males. H_{eL} =intra-locus heterozygosity; H_{eP} =heterozygosity per population. Brazilian States of Paraná (PR), São Paulo (SP), Rio Grande do Sul (RS), Santa Catarina (SC) and Rio de Janeiro. FDM=Frequency of diploid males. 95 % CI=confidence interval of FDM.

Bee species (N_L) and Collection site	Size (ha)	Vegetation type	Type of reserve (degree of disturbance/ surrounding matrix)	2N/n males	FDM (95 % CI)	H_p	H_{eL}	P_{het}	null alleles	$P_{het-null}$
<i>Eufriesea violacea</i> ($N_L = 6$)										
FSM - 25°25'S 54°25'W - PR	280	SF	Private reserve (medium to high/ matrix: pastures and agricultural area)	2/53	0.0377 (0.005-0.130)	0.74	0.59-0.82	0.99974	2	0.999547
PEMG* - 23°27'S 51°14'W - PR	2,200	SF	Governmental reserve (low; matrix: pasture and agricultural areas)	1/60	0.0167 (0.0004-0.090)	0.70	0.53-0.84	0.99957	1	0.998804
FMA - 24°12'42"S 50°33'04"W - PR	217	SF	Private (medium/ reforested areas of <i>Pinus</i> and <i>Eucalyptus</i>)	3/60	0.0500 (0.010-0.140)	0.73	0.57-0.86	0.99976	1	0.999244
NPR (ESCAS) - 23°14'S 46°20'W - SP	2,809	SF	Private (medium/reforested areas of <i>Pinus</i>)	0/60	-	0.72	0.53-0.83	0.99968	10	0.995822
CPCN-Pró Mata* - 29°28'S 50°9'W - RS	1,778	SF and MF	Private (low/native vegetation)	1/60	0.0167 (0.0004-0.090)	0.77	0.60-0.85	0.99988	4	0.999430
URU - 28°32'S 49°18' W - SC	630	DF	Private (medium/urban area)	0/60	-	0.76	0.54-0.85	0.99989	1	0.999646
Total per species				7/353	0.019 (0.008-0.040)	0.74	0.59-0.84	0.99982	19	0.998296
<i>Eulaema cingulata</i> ($N_L = 7$)										
CAB - 22°05'51" S 42°05'38" W - RJ	870	DF	Type of reserve (degree of disturbance/ surrounding matrix) Private reserve (medium/pastures, <i>Eucalyptus</i> and banana plantation)	0/40	-	0.64	0.23-0.80	0.99972	6	0.99869
UNI* - 22°25'44"S 42° 42'59"W - RJ	3,600	DF	Governmental reserve (low to medium/native vegetation, pasture, reforested areas of <i>Eucalyptus</i>)	0/40	-	0.65	0.30-0.83	0.99966	2	0.99901
ITA - 21°47'34"S 41°26'30"W - RJ	630	SF	Private (medium/sugar cane and pastures)	1/40	0.025 (0.001-0.130)	0.64	0.32-0.83	0.99967	7	0.99835
FSJU - 21°23'10"S 41°55'15"W - RJ	6.5	SF	Private (high/pastures and agricultural areas)	0/40	-	0.67	0.33-0.84	0.99984	1	0.99973
HT - 22°04'27"S 42°3'55"W - RJ	26	SF	Private/Municipal Garden (high/pasture and reforested areas of <i>Eucalyptus</i>)	0/20	-	0.60	0.09-0.82	0.99919	0	0.99919
MICO - 21°20'07"S 41°13'37"W - RJ	200	SF	Private (medium/ pasture)	0/26	-	0.65	0.23-0.84	0.99964	4	0.99765

CAR* - 21°22'40" S 41°51'1" W - RJ	SF	1,200	Governmental reserve (low/pasture and sugar cane)	0/39	-	0.66	0.34-0.80	0.99975	7	0.99831
ELP - 22°04'43" S 42°05'45" W - RJ	DF	141	Private (low/ reforested areas of <i>Eucalyptus</i>)	0/34	-	0.64	0.25-0.81	0.99965	6	0.99834
FSFI - 21°19'10" S 41°07'15" W - RJ	SF	55	Private (high/ pasture, sugar cane, agricultural areas)	2/40	0.050 (0.006-0.170)	0.64	0.13-0.85	0.99980	6	0.99859
Total per species				3/319	0.009 (0.002-0.030)	0.64	0.30-0.82	0.99966	39	0.99828
<i>Euglossa pleosticta</i> ($N_L=6$)	Vegetation type	Size (ha)	Type of reserve (degree of disturbance/ surrounding matrix)	2N/n males	FDM (95 % CI)	He _p	He _L	P_{het}	null alleles	$P_{het-null}$
FD - 23°18'15.68"S 50°58'51.81" W - PR	SF	170	Private (medium to high/agricultural areas)	0/74	-	0.69	0.60-0.96	0.99999	2	0.99999
PEMG* - 23°27'S 51°14'W - PR	SF	2,200	Governmental (low/matrix: pastures and agricultural areas)	0/45	-	0.49	0.27-0.95	0.99914	0	0.99914
F2C - 22°58'55"S 51°00'W - PR	SF	180	Private (high/pastures)	0/55	-	0.55	0.19-0.90	0.99929	1	0.99929
PTF - 23°22'30"S 51°13'15" W - PR	SF	14.6	Private (high/agricultural areas)	0/20	-	0.61	0.03-0.89	0.99843	0	0.99806
AT - 23°20'S 51°08'W - PR	SF	66	Governmental park (medium/urban matrix)	0/24	-	0.51	0.17-0.94	0.99921	0	0.99921
HT-UJEL - 23°19'05"S 51°12'10" W - PR	SF	13.4	Urban area (high/urban matrix)	0/51	-	0.65	0.45-0.95	0.99991	2	0.99908
Total per species				0/269	-	0.58	0.18-0.93	0.99954	5	0.99811
<i>Euglossa amnectans</i> ($N_L=9$)	Vegetation type	Size (ha)	Type of reserve (degree of disturbance/ surrounding matrix)	2N/n males	FDM (95 % CI)	He _p	He _L	P_{het}	null alleles	$P_{het-null}$
FMA - 24°12'42"S 50°33'04" W - PR	SF	217	Private (medium/ reforested areas of <i>Pinus</i> and <i>Eucalyptus</i>)	4/26	0.154 (0.040-0.350)	0.61	0.33-0.81	0.99970	1	0.99942
RNSM* - 25°09'36"S 48°18'02" W - PR	DF	2,340	Private reserve (low/native vegetation in different successional stages)	4/60	0.067 (0.020-0.160)	0.78	0.54-0.89	0.99999	4	0.99999
IS* - 25°23'11"S 48°13'57" W	DF	6,738	Governmental (low/native vegetation)	7/46	0.152 (0.060-0.290)	0.76	0.56-0.86	0.99999	0	0.99999
PNI* - 25°37'46"S 54°26'47" W	SF	185,262	Governmental (low/ native vegetation)	5/60	0.083 (0.030-0.180)	0.63	0.41-0.82	0.99983	1	0.99979
IC* - 25°08'48"S 47°56'47" W	DF	22,500	Governmental reserve (low/native vegetation)	3/11	0.272 (0.060-0.610)	0.74	0.48-0.84	0.99997	0	0.99997
Total per species				23/203	0.113 (0.070-0.170)	0.70	0.52-0.84	0.99995	6	0.99988
<i>Euglossa iapoecila</i> ($N_L=10$)	Vegetation type	Size (ha)	Type of reserve (degree of disturbance/ surrounding matrix)	2N/n males	FDM (95 % CI)	He _p	He _L	P_{het}	null alleles	$P_{het-null}$
RNSM* - 25°09'36"S 48°18'02" W - PR	DF	2,340	Private (low/ native vegetation in different successional stages)	0/60	-	0.65	0.45-0.93	0.99999	29	0.99914
SS* - 23°46'11"S, 45°36'24" W - SP	DF	>30,000	Governmental (low/native vegetation)	0/57	-	0.72	0.52-0.92	0.99999	13	0.99993
IB* - 23°50'39"S 45°21'23" W - SP	DF	>30,000	Governmental (low/native vegetation)	1/57	0.017 (0.0004-0.090)	0.70	0.49-0.91	0.99999	6	0.99998

Table 1. (continued)

UB* - 23°21'51"S 44°49'25"W - SP	DF	>30,000	Governmental (low /native vegetation)	0/37	-	0.66	0.46-0.93	0.99999	6	0.99996
UNI - 22°25'44"S 42°42'59"W - RJ	DF	3,600	Governmental reserve (low to medium/ native vegetation, pasture, reforested areas of <i>Eucalyptus</i>)	0/60	-	0.68	0.28-0.91	0.99999	15	0.99994
Total per species				1/271	0.004 (0.0001-0.020)	He _p	He _L	P _{het}	null alleles	P _{het-null}
<i>Euglossa truncata</i> (N _L =7)	Vegetation type	Size (ha)	Type of reserve (degree of disturbance/ surrounding matrix)	2N/n males	FDM (95 % CI)	He _p	He _L	P _{het}	null alleles	P _{het-null}
FD - 23°18'15.68"S 50°58'51.81"W - PR	SF	170	Private (medium to high/ pastures)	0/7	-	0.56	0.56-0.81	0.99577	1	0.99346
HT-UEL - 23°19'05"S 51°12'10"W - PR	SF	13.4	Urban area (high/urban matrix)	0/10	-	0.69	0.27-0.77	0.99958	3	0.99814
AFF- 23°19'26.27"S 51°10'20.63"W - PR	SF	18	Urban area (high/ urban matrix)	0/14	-	0.68	0.46-0.86	0.99931	7	0.99226
RF - 23°18'32.32"S 51°11'0.78"W - PR	SF	20	Urban (high/ urban matrix)	0/11	-	0.62	0.19-0.84	0.99910	5	0.99028
Total per species				0/42	-	0.64	0.37-0.82	0.99884	16	0.99053
ALL				34/1457	0.023 (0.016-0.030)				154	

*Reserves showing large areas of primary forests

^F Samples analyzed by Freiria et al. (2012) through of genotyping in bis-acrylamide gel, and re-analyzed herein in an automated sequencer SF (Seasonal Semi-deciduous Forest); DF (Dense Forest); MF (Mixed Forest)

Males were collected in different forest fragments in the states of Rio de Janeiro, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul (Table 1, Figure 1). Other descriptions of the areas of the sampling sites are showed in Table 1.

Individuals (n=1457; Table 1) of these six species analysed here were sampled in different studies, with some variation in methodologies between these studies. In the case of males of *El. cingulata*, odour-baits were used with the following fragrances: methyl cinnamate, vanillin, eucalyptol, benzyl acetate and methyl salicylate. Samplings of this species occurred between May 2007 and May 2009, and the males were collected using aromatic traps (for details, see Aguiar and Gaglianone 2012). To sample the other euglossine species, aromatic traps and/or insect nets were used. To attract males of *Ef. violacea*, *Eg. pleosticta* and *Eg. truncata*, only three chemicals (eucalyptol, eugenol and vanillin) were used during the sampling, while in the case of *Eg. annectans* and *Euglossa iopoeila*, in addition to these three fragrances, another five were used to attract males: beta-ionone, benzyl acetate, methyl salicylate, benzyl benzoate and methyl cinnamate. The period of sampling varied among these five species and from site to site: a) Males of *Eufriesea violacea* were collected in November and December of 2008 and 2009 (see Freiria et al. 2012); b) *Eg. iopoeila*: between 2009 and 2013; c) *Eg. truncata* in 2008; d) *Eg. pleosticta* from 2004 to 2009; and e) *Euglossa annectans*: between 2008 and 2013. Particularly in the case of this latter species, the samplings in the five study sites occurred as follows: FMA (Monte Alegre Farm)=March/2008 - March/2010; RNSM (Salto Morato Reserve)=April/2009 - October/2010; IS (Superagui Island)=November/2011-February/2013; PNI (Iguaçu Park)=March/2012; and IC (Cardoso Island)=February/2012.

It is important to mention that the 353 males of *Ef. violacea* examined herein were those also studied by Freiria et al. (2012), but for the confirmation of diploid males, new analyses were performed on an automated sequencer, which confirmed only part of 2N males previously identified by these authors.

In general the collecting effort per area was greater than 20 hours, distributed over several days of collecting and usually involving a minimum of five days of sampling. However, for *Euglossa annectans*, in the Ilha do Cardoso State Park (IC), *Euglossa iopoeila*, in São Sebastião (SS) and Ubatuba (UB),

and, for *Eufriesea violacea* in NPR (Nazaré Paulista Reserve), CPCN-Pró Mata (CPCN Reserve) and Urussanga fragment (URU), sampling was carried out on only one or two days, resulting in a sampling effort less than 15 hours per area.

2.3. DNA extraction and molecular analysis

DNA extraction from *Eufriesea violacea* (n=353), *Eg. annectans* (n=203) and *Eg. pleosticta* (n=269) was performed as in Freiria et al. (2012), while DNA from males of *Eulaema cingulata* (n=319), *Euglossa iopoeila* (n=271) and *Euglossa truncata* (n=42) was obtained according to Almeida et al. (2001), with minor modifications, described as follows: parts of the body (legs or thorax) were placed in micro-centrifuge tubes containing 300 µL extraction buffer (500 mM Tris-HCl, 20 mM EDTA, 10 mM NaCl, pH 8.0 and 1 % SDS) and 5 µL K proteinase (20 µg/µL). Using small scissors, the body parts of bees were cut, held in a water bath at 63 °C for two hours and then subjected to phenol-chloroform washes. The DNA obtained was re-suspended in 30 µL TE (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20 °C. The concentration of DNA was measured using a DyNA Quant 200 fluorometer (Hoefer), following the quantification methodology proposed by the manufacturer. After reading, the DNA samples were diluted in water to a concentration of 5 ng/µL to then be used in the amplifications.

The genetic analyses of euglossine males from the six species herein surveyed involved genotyping at a minimum of six (*Ef. violacea* and *Eg. pleosticta*) and a maximum of 10 microsatellite loci (*Eg. iopoeila*) (Table 1), using primers developed for *Eg. cordata* (Souza et al. 2007), *Eg. annectans* (Paxton et al. 2009) and *Eg. iopoeila* (Penha et al. 2014). Amplifications of the different microsatellite loci of *Ef. violacea*, *El. cingulata*, *Eg. pleosticta* and *Eg. annectans* were based on protocols of Souza et al. (2007), Paxton et al. (2009) and Freiria et al. (2012), with some modifications. PCR reactions were produced in a final volume of 5 µL, including: 1x GoTaq® Colorless Master Mix 2x (Promega), 0.5 µM of tag-F primer+0.5 µM of R-primer mixture, 5 µM M13- primer labelled with fluorochrome, 1 % of glycerol, 5 ng of DNA and 1.15 µL nuclease-free water (Promega).

Samples were amplified in thermal cyclers (PCT-100 Peltier Thermal Cycler, MJ Research Inc. and Multigen LabNetBios), according to the protocol described

below: 94 °C for 4 minutes followed by 35 cycles of: [94 °C denaturation for 40 seconds, 1 min for primer annealing at temperatures varying according to the primer and species, and extension at 72 °C for 1 minute], ending with an extension at 72 °C for 10 minutes. Furthermore, in the case of *Eg. iopoeila* and *Eg. truncata*, we employed a second amplification protocol: 94 °C for 4 min, followed by 10 cycles de 94 °C/30 s, 56 °C/1 min, 72 °C/1 min, followed by 25 cycles 89 °C/30 s, 56 °C/1 min, 72 °C/1 min, with a final extension at 72 °C for 30 minutes. The primers and annealing temperatures employed in the amplification of microsatellite loci of the different species are shown in Table S1.

Sample genotyping was conducted in an automated sequencer (Applied Biosystems® 3500 XL Genetic Analyzer) and GeneScan 600-LIZ (Applied Biosystems) was used as the standard marker. The sample genotypes were read using the GeneMarker 1.85 software (SoftGenetics) and checked visually. All samples that failed to amplify at any locus and all those indicating diploid males were re-amplified at least two more times. In our analyses we considered as diploid males only those cases for which heterozygous status of diploid males was confirmed in all three PCRs.

In all amplification reactions for analysis in the automated sequencer, the forward primers were modified at the 5' end with a tail (5'-TGTAACGACGGCCA GT-3'), according to the method proposed by Schuelke (2000), to which the M13 primer labelled with 6-FAM fluorophore (blue), NED (yellow) and HEX (green) was ligated. This methodology eliminates the need for individually labeling the forward primers of all loci (Schuelke 2000).

2.4. Data analysis

The expected heterozygosity (H_e) - intralocus and per population – were estimated with the software package TFPGA 1.3 (Miller 1997).

The calculation of P_{het} , i.e. a probability of the molecular marker to detect a diploid individual as heterozygous, was performed according to Souza et al. (2010). However, putative null alleles can cause difficulties in microsatellites scoring and can lead to an overestimation of P_{het} (Souza et al. 2010; Boff et al. 2014). Like these authors, to account for putative null alleles we assumed that a male lacking an allele at a locus was caused by a null allele. Thus, we performed

the calculation of $P_{het-null}$ applying the formula of Boff et al. (2014). In Table S2 we show the number of samples that failed to amplify for different loci surveyed for the different species and populations.

The binomial 95 % confidence interval of the proportion of diploid males was calculated according to Souza et al. (2010) and Boff et al. (2014) (on-line tool at <http://statpages.org/confint.html>).

For species in which more than five study areas were analysed for the occurrence of 2N males, the Pearson correlation index was used to test for a correlation between the size of the study areas and frequencies of diploid males. We used a Chi-square test to test for significant difference in the frequency of diploid males sampled in our study and by Souza et al. (2010); P -values < 0.05 were considered statistically significant.

3. RESULTS

From the total of 1457 males analysed, belonging to six different euglossine species, 34 were diploid, which were found only among four species distributed as follows: *Ef. violacea*=7; *El. cingulata*=3; *Eg. annectans*=23 and *Eg. iopoeila*=1 (Table I).

Considering the frequency of diploid males in the total sample of each species, the highest frequency (11.3 %) was observed for *Eg. annectans* (Table I). Actually, diploid males were found in all five populations of this species surveyed in our study. Whereas the number of 2N males of *Eg. annectans* varied only from three to seven, their frequencies varied across a larger range: from 6.7 % (RNSM) to 27.3 % (IC=Cardoso Island). Remarkably, among the five areas surveyed for this species, three showed frequencies of 2N males above 10 %. However, for *Eg. annectans* as well the other euglossine species studied, our analyses showed no significant correlations ($P > 0.05$) between the size of the study area (forest fragment) and the number of 2N males.

Finally, with regard to P_{het} calculations, our analyses revealed that, even when null alleles were considered in our analyses ($P_{het-null}$), the power of detecting diploid males was very high (>0.99) for all species and populations surveyed (Table I), even for *Eg. annectans*.

4. DISCUSSION/ CONCLUSION

Considering the variation in frequencies of diploid (2N) males found for the six euglossine species surveyed in this study, our findings show that generalizations concerning the frequencies of such males among this group of bees should be avoided. This view is reinforced by both in the substantial number of males of each species from different populations analysed (except for *Eg. truncata*) and by the number of microsatellite loci used in the analyses, which varied from six to ten. Contrary to Souza et al. (2010), who found only five diploid males in a total sample of 1010 individuals belonging to 27 euglossine species, we detected 34 diploid males among the 1457 analysed. Considering that both studies employed the same microsatellite molecular markers and based their analyses mainly on the genotyping of samples in an automated sequencer, it is possible to see that a substantial difference was found between our results and those found by Souza et al. (2010). Indeed, through the Chi-square test, we found significant difference between the frequencies of 2N males of these both studies ($\chi^2=12.96$, $d.f=1$; $P<0.0004$). Except for *Eg. annectans*, the frequencies of diploid males described in our study for the different species tended to be higher than those reported by Souza et al. (2010). Although it is difficult to explain such dissimilarities between both studies, the large number of individuals analysed per species in each study is a factor to be considered. In general, although Souza et al. (2010) analysed 1010 males, for many species these authors analysed only a few individuals. In fact, the number of bees surveyed by these authors varied from 1 (e.g. *Eg. cognata*, *Eg. moure* and *Eg. viridis*) to about 150 (e.g. *El. bombiformes*). From the 27 studied by Souza et al. (2010), 13 were represented by less than 20 individuals in the samples analysed. Also, for some species Souza et al. (2010) analysed only two or three microsatellite loci.

According to Owen and Packer (1994), in panmictic natural populations of bees the expected frequencies of 2N males are low, 10 % or less. In our study, populations of five of six euglossine species surveyed revealed frequencies of diploid

males lower than 10 % and, thus, they would be in accordance with the expected for panmictic populations. Conversely, three of the five populations of *Eg. annectans* showed estimates of 2N males above 10 %. Regarding this species Souza et al. (2010) found a diploid male in a sample of 17 males analysed, i.e., at a frequency equal to 5.9 %. It should be emphasized here that our findings regarding the high frequency (27.3 %) of diploid males of *Eg. annectans* found to IC, an island population, must be viewed with caution, since only 11 individuals of this island were captured and analysed.

Although it has been shown that some euglossine bees are able to cross water bodies (Dressler 1982), a recent study showed that the sea seemed to act as a barrier to gene flow to *Eg. cordata* populations (Boff et al. 2014). These authors detected that island isolation reduced genetic diversity in populations of this species, but did not significantly elevate diploid male production. Considering our data, it is worth noting that two of three *Eg. annectans* populations which showed frequencies of diploid males above 10 % were island populations.

One of the causes for an increase of diploid males in populations of Euglossini and other groups of bees is the reduction in population size, with consequent loss of allelic diversity at the sex locus (Cook 1993; Roubik et al. 1996; Zayed et al. 2004; van Wilgenburg et al. 2006; Paxton et al. 2009). Thus, apparently, except for *Eg. annectans*, the frequencies of diploid males detected for populations of euglossine species analysed in our study do not suggest any declines in populations of these species, since all of them showed frequencies of 2N males within the range expected for panmictic populations (see Owen and Packer 1994). We should also consider that, in addition to null alleles, our estimates of diploid male may be elevated slightly because of overestimates of marker variability and unknown mating structure for majority of euglossine species herein studied.

All euglossine species surveyed in our study are endemic to the Atlantic Forest or show a strong association with this threatened South American forest (Faria Jr and Melo 2007; Nemésio and Silveira 2007; Nemésio 2009).

With less than 10 % of its original cover, the Atlantic Forest is today totally fragmented, with most of its remnants having sizes less than 100 ha and under significant human interference (Ribeiro et al. 2009). It is within this highly threatening scenario that populations of different euglossine species seek resources for their survival. Undoubtedly, those most sensitive species that depend more closely on more preserved forest areas are more susceptible to loss of genetic diversity. When considering the results especially related to *Eg. annectans* and its intimate distribution with the Atlantic Forest, this species merits the focus of further studies on population genetic structure, taking also into account variation in the frequencies of 2N males.

Based on our findings, we suggest that, in addition to other euglossine species that have been pointed out as potentially more sensitive to habitat disturbance (Powell and Powell 1987; Tonhasca et al. 2002; Giangarelli et al. 2009), *Eg. annectans* is another Euglossini species sensitive to anthropogenic interference. However, since without a more complete knowledge about different factors which can affect diploid male production in bees, such as sex allele diversity, effective population size and dispersal rate, it is difficult to suggest whether the relatively higher frequencies of diploid males of *Eg. annectans* could be due to an increased incidence of inbreeding in the studied populations of this species. Based on that, we recommend further genetic studies involving a larger number of populations of this orchid bee species to corroborate or not the hypothesis that this orchid bee species is sensitive to habitat fragmentation.

Our results for *Eg. annectans*, which revealed the occurrence of diploid males in all remnants of Atlantic Forest where this species was sampled, suggest that we should pay more attention to populations of this species. Moreover, the results presented here indicate, regarding to frequency of diploid males in natural populations of orchid bees, that generalizations should be avoided since our findings highlighted that they are not invariably rare within this group of bees.

ACKNOWLEDGMENTS

The authors thank: Coordination of Improvement of Higher Education Personnel (CAPES)/Procad (158/2007), Brazilian Council for Scientific and Technological Development (CNPq), Fundação O Boticário de Proteção à Natureza (FBPN) and Klabin S.A. and Biota-FAPESP for financial support; IBAMA and IAP for environmental license; Instituto Florestal de São Paulo for the license for the collections and staff of State Parks of São Sebastião, Ilhabela, Ilha do Cardoso, Ubatuba (Picinguaba), for the infrastructure and help in field work; Isabel Alves-dos-Santos, Guaraci Duran Cordeiro and Samuel Boff by donation of the specimens of *Euglossa iopoeila* from Ilhabela; and owners of private reserves for permission for the collection of bees. D.C. Giangarelli thanks CAPES for the scholarship. Silvia H. Sofia and Maria C. Gaglianone are research fellows from the CNPq. The authors are also grateful to two anonymous referees for their valuable comments, which contributed to improve the quality of this paper.

Abeilles à orchidée : une nouvelle évaluation de la rareté de mâles diploïdes dans les populations de ce groupe de pollinisateurs néotropicaux

microsatellite / Euglossini / *Euglossa annectans* / fragments forestiers

Prachtbienen : Neue Daten zur Seltenheit diploider Männchen in Populationen dieser Gruppe neotropischer Bestäuber

Mikrosatelliten / Euglossini / Euglossine Bienen / *Euglossa annectans* / Waldfragmente

REFERENCES

- Aguiar, W.M., Gaglianone, M.C. (2012) Euglossine bee communities in small forest fragments of the Atlantic Forest, Rio de Janeiro state, southeastern Brazil (Hymenoptera, Apidae). *Rev. Bras. Entomol.* **56**, 210–219
- Almeida, F.S., Fungaro, M.H.P., Sodré, L.M.K. (2001) RAPD and isoenzyme analysis of genetic variability in three allied species of catfish (Siluriformes, Pimelodidae) from the Tibagi river. *Brazil. J. Zool.* **253**, 113–120
- Beye, M., Hasselmann, M., Fondrk, M.K., Page, R.E., Omholt, S.W. (2003) The gene *csd* is the primary signal for the sexual development in the honeybee and encodes as SR-type protein. *Cell* **114**, 419–429

- Boff, S., Soro, A., Paxton, R.J., Alves-dos-Santos, I. (2014) Island isolation reduces genetic diversity and connectivity but does not significantly elevate diploid male production in a neotropical orchid bee. *Conserv. Genet.* . doi:10.1007/s10592-014-0605-0
- Cook, J.M. (1993) Sex determination in the Hymenoptera: a review of models and evidence. *Heredity* **71**, 421–435
- Cook, J.M., Crozier, R.H. (1995) Sex determination and population biology of the Hymenoptera. *Trends Ecol. Evol.* **10**, 281–286
- Cowan, D.P., Stahlhut, J.K. (2004) Functionally reproductive diploid and haploid males in an inbreeding hymenopteran with complementary sex determination. *Proc. Natl. Acad. Sci. USA* **101**, 10374–10379
- Crozier, R.H. (1977) Evolutionary genetics of the Hymenoptera. *Annu. Rev. Entomol.* **22**, 263–288
- Dressler, R.L. (1982) Biology of orchid bees (Euglossini). *Annu. Rev. Ecol. Syst.* **13**, 373–394
- Elias, J., Mazzi, D., Dorn, S. (2009) No need to discriminate? Reproductive diploid males in a parasitoid with complementary sex determination. *Plos One* **4**, e6024
- Faria Jr., L.R.R., Melo, G.A.R. (2007) Species of *Euglossa* (*Glossura*) in the Brazilian Atlantic forest, with taxonomic notes on *Euglossa stellfeldi* Moure (Hymenoptera, Apidae, Euglossina). *Rev. Bras. Entomol.* **51**, 275–284
- Freiria, G.A., Ruim, J.B., Souza, R.F., Sofia, S.H. (2012) Population structure and genetic diversity of the orchid bee *Eufriesea violacea* (Hymenoptera, Apidae, Euglossini) from Atlantic Forest remnants in southern and southeastern Brazil. *Apidologie* **43**, 392–402
- Giangarelli, D.C., Freiria, G.A., Colatreli, O.P., Suzuki, K.M., Sofia, S.H. (2009) *Eufriesea violacea* (Blanchard) (Hymenoptera: Apidae): an orchid bee apparently sensitive to size reduction in forest patches. *Neotrop. Entomol.* **38**, 1–6
- Harpur, B.A., Sobhani, M., Zayed, A. (2013) A review of the consequences of complementary sex determination and diploid male production on mating failures in the Hymenoptera. *Entomol. Exp. Appl.* **146**, 156–164
- Heimpel, G.E., de Boer, J.G. (2008) Sex determination in the Hymenoptera. *Annu. Rev. Entomol.* **53**, 209–230
- López-Uribe, M.M., Almanza, M.T., Ordóñez, M. (2007) Diploid male frequencies in Colombian population of euglossine bees. *Biotropica* **39**, 660–662
- Mattozo, V.C., Faria, L.R.R., Melo, G.A.R. (2011) Orchid bees (Hymenoptera: Apidae) in the coastal forests of southern Brazil: diversity, efficiency of sampling methods and comparison with other Atlantic forest surveys. *Pap. Avulsos Zool.* **51**, 505–515
- Milet-Pinheiro, P., Schlindwein, C. (2005) Do euglossine males (Apidae, Euglossini) leave tropical rainforest to collect fragrances in sugarcane monocultures? *Rev. Bras. Zool.* **22**, 853–858
- Miller, M.P. (1997) Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author
- Nemésio, A. (2009) Orchid bees (Hymenoptera: Apidae) of Brazilian Atlantic Forest. *Zootaxa* **2041**, 1–242
- Nemésio, A. (2013) Are orchid bees at risk? First comparative survey suggests declining populations of forest-dependent species. *Braz. J. Biol.* **73**, 367–374
- Nemésio, A., Silveira, F.A. (2007) Diversity and distribution of orchid bees (Hymenoptera: Apidae) with a revised checklist of their species. *Neotrop. Entomol.* **36**, 874–888
- Owen, R.E., Packer, L. (1994) Estimation of the proportion of diploid males in populations of Hymenoptera. *Heredity* **72**, 219–227
- Paxton, R.J., Zobel, M.U., Steiner, J., Zillikens, A. (2009) Microsatellite loci for *Euglossa annectans* (Hymenoptera: Apidae) and their variability in other orchid bees. *Mol. Ecol. Resour.* **9**, 1221–1223
- Penha, R.E.S., Gaglianone, M.C., Almeida, F.S., Boff, S.V., Sofia, S.H. (2014) Mitochondrial DNA of *Euglossa iopoeila* (Apidae, Euglossini) reveals two distinct lineages for this orchid bee species endemic to the Atlantic Forest. *Apidologie* . doi:10.1007/s13592-014-0329-7
- Powell, A.H., Powell, G.V.N. (1987) Population dynamics of male euglossine bees in Amazonian forest fragments. *Biotropica* **19**, 176–179
- Ribeiro, M.C., Metzger, J.P., Martensen, A.C., Ponzone, F.J., Hirota, M.M. (2009) The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biol. Conserv.* **142**, 1141–1153
- Roubik, D.W., Hanson, P.H. (2004) Orchid bees of tropical America, 1st edn. INBio, Santo Domingo de Heredia, Costa Rica
- Roubik, D.W., Weight, L.A., Bonilla, M.A. (1996) Population genetics, diploid males, and limits to social evolution of euglossine bees. *Evolution* **50**, 931–935
- Schuelke, M. (2000) An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* **18**, 233–234
- Sofia, S.H., Suzuki, K.M. (2004) Comunidades de machos de abelhas Euglossina (Hymenoptera: Apidae) em fragmentos florestais no sul do Brasil. *Neotrop. Entomol.* **33**, 693–702
- Souza, R.O., Cervini, M., Del Lama, M.A., Paxton, R.J. (2007) Microsatellite loci for euglossine bees (Hymenoptera, Apidae). *Mol. Ecol. Notes* **7**, 1352–1356
- Souza, R.O., Del Lama, M.A., Cervini, M., Mortari, N., Eltz, T., Zimmermann, Y., Bach, C., Brosi, B.J., Suni, S., Quezada-Euán, J.J.G., Paxton, R.J. (2010) Conservation genetics of neotropical pollinators revisited: microsatellite analysis suggests that diploid males are rare in orchid bees. *Evolution* **64**, 3318–3326
- Takahashi, N.C., Peruquetti, R.C., Del Lama, M.A., de Oliveira, C.L.A. (2001) A reanalysis of diploid male frequencies in euglossine bees (Hymenoptera: Apidae). *Evolution* **55**, 1897–1899

- Tonhasca, A., Blackmer, J.L., Albuquerque, G.S. (2002) Abundance and diversity of euglossine bees in the fragmented landscape of the Brazilian Atlantic Forest. *Biotropica* **34**, 416–422
- Unruh, T.R., Messing, R.H. (1993) Intraspecific biodiversity in Hymenoptera: implications for conservation and biological control. In: Gauld, I.D. (ed.) Lasalle, J, pp. 27–52. *Hymenoptera and Biodiversity*, CAB International, Wallingford
- van Wilgenburg, E., Driessen, G., Beukeboom, L.W. (2006) Single locus complementary sex determination in Hymenoptera: an "unintelligent" design? *Front. Zool.* **3**, 1–15
- Zayed, A. (2009) Bee genetics and conservation. *Apidologie* **40**, 237–262
- Zayed, A., Packer, L. (2005) Complementary sex determination substantially increases extinction proneness of haplodiploid populations. *Proc. Natl. Acad. Sci. USA* **102**, 10742–6
- Zayed, A., Roubik, D.W., Packer, L. (2004) Use of diploid male frequency data as an indicator of pollinator decline. *Proc. R. Soc. B* **271**, S9–S12