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CONSERVATION GENETICS OF NEOTROPICAL POLLINATORS REVISITED: MICROSATELLITE ANALYSIS SUGGESTS THAT DIPLOID MALES ARE RARE IN ORCHID BEES

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Allozyme analyses have suggested that Neotropical orchid bee (Euglossini) pollinators are vulnerable because of putative high frequencies of diploid males, a result of loss of sex allele diversity in small hymenopteran populations with single locus complementary sex determination. Our analysis of 1010 males from 27 species of euglossine bees sampled across the Neotropics at 2–11 polymorphic microsatellite loci revealed only five diploid males at an overall frequency of 0.005 (95% CIs 0.002–0.010); errors through genetic nondetection of diploid males were likely small. In contrast to allozyme-based studies, we detected very weak or insignificant population genetic structure, even for a pair of populations >500 km apart, possibly accounting for low diploid male frequencies. Technical flaws in previous allozyme-based analyses have probably led to considerable overestimation of diploid male production in orchid bees. Other factors may have a more immediate impact on population persistence than the genetic load imposed by diploid males on these important Neotropical pollinators.

KEY WORDS: Complementary sex determination, *csd*, Euglossini, Hymenoptera.

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Single locus complementary sex determination (slCSD), in which homozygosity at the sex locus leads to the production of effectively sterile diploid (2N) males, is thought to be ancestral to the haplodiploid Hymenoptera and has been considered widespread within the order (van Wilgenburg et al. 2006; but see Cowan and Stahlhut 2004; de Boer et al. 2007, 2008; Heimpel and de Boer 2008; Verhulst et al. 2010). The frequency of 2N males theoretically increases with inbreeding, small population size, and reduced gene flow due to lack of allelic diversity at the sex locus (Cook 1993; Cook and Crozier 1995; van Wilgenburg et al. 2006). slCSD may itself lead to lower effective population size (N_e) compared to diploidy (Zayed 2004).

All bees appear to be slCSD haplodiploids (van Wilgenburg et al. 2006; Zayed 2009) and there is growing evidence for decline in many groups (Brown and Paxton 2009; Potts et al. 2010); unequivocal evidence is seen in solitary bees in England and the Netherlands (Biesmeijer et al. 2006), bumblebees in Ireland (Fitzpatrick et al. 2007), and honey bees (*Apis mellifera*) in the USA (Oldroyd 2007; vanEngelsdorp et al. 2009). This is cause for concern because bees are important pollinators in natural and agro-ecosystems (Klein et al. 2007). Pollination is an important ecosystem service that is being degraded by anthropogenic changes (Kremen et al. 2002; Steffan-Dewenter et al. 2005), including habitat destruction, pollution, and facilitation of invasive species (Mooney et al. 2005). Degradation of habitat may result in a loss of genetic diversity, so the frequency of 2N males has been proposed to be a sensitive measure of pollinator decline for bees (Zayed et al. 2004). Zayed and Packer's (2005) theoretical modeling concluded that diploid males exert a high genetic load on populations, which could potentially drive a genetic extinction vortex in slCSD haplodiploids.

The Euglossini comprise ca. 200 species of Neotropical bees that are the sole pollinators of around 700 orchid species (Dressler 1982; Cameron 2004; Roubik and Hanson 2004). Males collect perfumes from orchid blossoms and other sources in their hind tibiae and later release them at mating sites, possibly to attract females (Eltz et al. 2005, 2007). To date the conservation genetics of orchid bees has relied on the use of allozymes as genetic markers to study 2N male frequency and determine ploidy (a male heterozygous at one or more loci is a 2N male). An early study of seven Panamanian orchid bee species suggested that 2N males comprised 12–100% of males per species (Roubik et al. 1996). In contrast, Takahashi et al. (2001) found very low (mean 0–2% per species) frequencies of 2N males in 14 Brazilian species. Zayed et al. (2004) subsequently detected 13–56% (across populations) of Panamanian *Euglossa imperialis* males to be diploid and inferred extremely limited gene flow and low N_e in the species, supporting Roubik et al.'s (1996) view that orchid bees exhibited low diversity at the sex locus. More recently, López-Uribe et al. (2007) also found high 2N male frequencies in five Colombian orchid bee species; across species, 8–32% of males were estimated to be diploid. Although all these studies employed substantial sample sizes ($n = 142$–695 males per study), confidence intervals of 2N male frequencies were large due to the low variability of allozymes, the only polymorphic markers then available for orchid bee population genetics.

The notion that orchid bees suffer high 2N male production is at odds with other aspects of the taxon's biology. For example, males of many species are common at chemical baits and hence are employed in Neotropical biodiversity inventorying (e.g., Brosi 2009) whereas both sexes are thought to be extremely mobile (Janzen 1971, 1981; Dressler 1982; Cameron 2004; Dick et al. 2004). This contradiction between biological observations and allozyme-based genetic analysis prompted our re-assessment of 2N male frequency and gene flow in orchid bees. Using three suites of recently developed microsatellite markers, we genotyped 1010 males from 27 species of euglossine bees, each at 2–11 polymorphic loci, sampled from across the Neotropics and including *Eg. imperialis* from Panama, to reveal extremely low (0.5%) frequencies of 2N males and very weak population genetic structure even across 500 km.

Material and Methods

In Brazil and Colombia, 483 males from 23 species were collected across multiple years at odor baits (1,8-cineole, skatole and vanillin) at 14 sites in seven Brazilian states and one site in Colombia (Table 1, Fig. 1). These included 143 males already genotyped using allozymes and reported by Takahashi et al. (2001). In Panama, 257 males from three species were collected at odor baits; *Eg. imperialis* was collected from three sites across March–May 2005, *Eg. tridentata* from two sites across 16 days in March-April 2006 (both at 1,8-cineole baits) and *Euglossa hemichlora* from one site in September 2007 (at p-dimethoxybenzene baits, Fig. 1). In Mexico, 73 *Euglossa aff. viridissima* males (the lineage with three mandibular teeth, 3D, to be described as a new species; Eltz et al. unpubl. ms) and 57 *Eg. viridissima* males (the lineage with two mandibular teeth, 2D; see Eltz et al. 2008) were collected at odor baits (p-dimethoxybenzene) from one site in March 2006 and May 2007. Finally, in Costa Rica, 140 *Eulaema bombiformis* males were collected from 19 forest fragments around Las Cruces Biological Station (maximum site separation 13.5 km) in June–September 2004, as described in Brosi (2009). Insects were stored in ethanol at -20°C or were dried and stored at room temperature.

DNA was extracted from legs or thoraxes using a high salt protocol (Paxton et al. 1996) or a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California) following manufacturer's recommendations. Individuals were genotyped at 2–11 polymorphic microsatellite loci (male haplotypes/genotypes in Table S1), developed for *Euglossa cordata*, *Eulaema nigrita* (Souza et al. 2007),

Table 1. Species name, collection site, number of males sampled (*n* males), number of polymorphic loci used (*n* loci), range of expected intralocus allelic diversity (H_{ina}, adjusted for putative null alleles; see Tables S1 and S2), mean allelic diversity across loci (H_{exp}, adjusted for putative null alleles), probability of detecting a heterozygous male if diploid (P_{het}), observed number of diploid (2N) males and 95% binomial confidence intervals of the observed frequency of 2N males in 27 orchid bee species from Brazil, Colombia, Costa Rica, Mexico, and Panama. See Figure 1 for sampling locations; Brazilian state codes are: Amazonas—AM; Espírito Santo—ES; Minas Gerais—MG; Mato Grosso—MT; Paraíba—PB; Rio de Janeiro—RJ; and São Paulo—SP.

Species	Collection Site	*n* males	*n* loci	H_{ina}	H_{exp}	P_{het}	2N males	95% CIs of 2N frequency
Euglossa annectans	São Carlos – SP, Brazil	17*	6	0.17–0.75	0.48	0.988	1*	0.002–0.288
Eg. chalybeata	Manaus – AM, Brazil	19	6	0.28–0.72	0.60	0.998	0	
Eg. cognata	Villavicencio, Colombia	1	9[2]				0	
Eg. cordata	Caraguatatuba – SP, Brazil	37*	8	0.30–0.88	0.63	>0.999	0	
	São Carlos – SP, Brazil	30*						
Eg. fimbriata	São Carlos – SP, Brazil	7*	8	0.25–0.86	0.56	>0.999	0	
Eg. hemichlora	Santa Rita, Panama	43	3	0.62–0.84	0.75	0.987	0	
	Manaus – AM, Brazil	30	6	0.44–0.81	0.67	>0.999	0	
Eg. imperialis	Barro Colorado, Panama	47					0	
	Fort Clayton, Panama	23	5	0.02–0.83	0.45	0.983	0	
	Gigante Peninsula, Panama	28						
Eg. intersecta	Manaus – AM, Brazil	1	6[2]				0	
Eg. mandibularis	Viçosa – MG, Brazil	95*[1]	8	0.08–0.87	0.56	>0.999	1*	0–0.057
Eg. melanotricha	Analândia – SP, Brazil	8*	9	0.38–0.88	0.66	>0.999	0	
Eg. mixta	Villavicencio, Colombia	3	5	0.44–0.67	0.49	0.968	0	
Eg. moure	Manaus – AM, Brazil	1	7[2]				0	
Eg. pleosticta	São Carlos – SP, Brazil	4*	9	0.38–0.75	0.63	>0.999	0	
	Camburí – SP, Brazil	2						
Eg. securigera	Rifaina – SP, Brazil	3	9	0.22–0.78	0.57	>0.999	0	
	São Carlos – SP, Brazil	3*						
Eg. townsendi	Araras – SP, Brazil	3	8	0.38–0.75	0.56	0.999	0	
	Rifaina – SP, Brazil	1						
Eg. tridentata	Barro Colorado, Panama	60	2	0.67–0.89	0.78	0.964	1	0–0.049
	Parque Natur. Metro., Panama	56						
Eg. truncata	São Carlos – SP, Brazil	10*	7	0.42–0.78	0.65	>0.999	0	
Eg. viridis	Villavicencio, Colombia	1	9[2]				0	
Eg. aff viridissima 3D[3]	Xmatkuil, Mexico	73	2	0.85–0.89	0.87	0.984	0	
Eg. viridissima 2D[4]	Xmatkuil, Mexico	57	2	0.59–0.87	0.73	0.948	0	
Eulaema	Manaus – AM, Brazil	21	11	0.58–0.89	0.79	>0.999	0	
bombiformis	Las Cruces, Costa Rica	140	9	0.16–0.61	0.34	0.981	2	0–0.051
El. cingulata	Manaus – AM, Brazil	8	7	0.47–0.81	0.63	>0.999	0	
El. meriana	Manaus – AM, Brazil	26	10	0.27–0.89	0.69	>0.999	0	
	Cuiabá – MT, Brazil	4						
	Manaus – AM, Brazil	4						
	Marliéria – ES, Brazil	5						
	Mimoso – MG, Brazil	4						
El. nigrita	Poconé – MT, Brazil	3*	11	0.61–0.91	0.77	>0.999	0	
	Rifaina – SP, Brazil	5						
	S. J. Campos – SP, Brazil	5						
	São Carlos – SP, Brazil	5*						
	Viçosa – MG, Brazil	5						
Eufriesea violacea	São Carlos – SP, Brazil	16	10	0.37–0.85	0.59	>0.999	0	
	Viçosa – MG, Brazil	37						
Exaerete frontalis	João Pessoa – PB, Brazil	8	3	0.66–0.78	0.74	0.983	0	

Continued.

Table 1. Continued.

Species	Collection Site	*n* males	*n* loci	H_{ina}	H_{exp}	P_{het}	2N males	95% CIs of 2N frequency
Ex. smaragdina	João Pessoa – PB, Brazil	50	3	0.79–0.83	0.81	0.993	0	
	São Carlos – SP, Brazil	1						
Grand Total		**1010**		**0.02–0.91**	**0.62**	**0.991**	**5**	**0.002–0.010**

*The same samples as analyzed by Takahashi et al. (2001);

[1]*n*=76 new samples added in addition to those of Takahashi et al. (2001);

[2]For *n*=1 male analyzed, *n* loci=number of loci employed (see Table S1);

[3]All males from the species with three mandibular teeth, 3D (see Eltz et al. 2008), to be described as a new species (Eltz et al. unpubl. data).

[4]All males from the species with two mandibular teeth, 2D (see Eltz et al. 2008).

and *Euglossa annectans* (Paxton et al. 2009); these are unlinked loci that are in Hardy–Weinberg equilibrium (HWE) in the species for which they were developed (Souza et al. 2007; Paxton et al. 2009). Genotyping and scoring were performed using autosequencers in three different laboratories (Megabace 750, ABI 310, or ABI 3100) and Genotyper or GeneMarker Version 1.71 software with internal size standards. All trace files were inspected by eye to check for potential allele miscalling due, for example, to stutter. Approximately 5% of individuals were re-amplified and alleles scored using the same autosequencer or they were genotyped in a fourth laboratory by radio-labeling and resolving on manual sequencing gels (methods in Paxton et al. 1996). Allele calling across these duplicate analyses of the same individual-locus combination was identical. We therefore estimate extremely low genotyping error rates.

Nondetection of 2N males may arise if genetic markers exhibit low allelic diversity (low heterozygosity). To compensate for genetic nondetection, we calculated the resolving power of our markers, namely the probability that a diploid individual was heterozygous at one or more loci, P_{het}, as

$$1 - \prod_{j=1}^{L} \sum_{i=1}^{N} (x_i^2),$$

where summation is across the N alleles at a locus and multiplication is across L loci. This assumes HWE, although moderate levels of inbreeding have only a slight effect on P_{het} (e.g., see Paxton et al. 2000). In estimating allelic frequencies, males carrying only one allele at all loci were considered haploid, which is a close approximation given the high allelic diversity of the loci and therefore the high probability that a diploid male is heterozygous at one or more loci (Tables S1 and S2). In addition, microsatellite analysis of four of our study species has not revealed any deviation from HWE (*Eg. annectans* in Paxton et al. 2009; *Eg. cordata* and *El. nigrita* in Souza et al. 2007; and *Eg. viridissima* in Zimmermann et al. 2009), suggesting random mating in orchid bees.

Null alleles can nevertheless cause difficulties in microsatellite allele scoring and lead to an overestimation of P_{het}. To account for putative null alleles, we assumed that a male lacking an allele at a locus was caused by a null allele, and we reduced allelic diversity (H_{ina}) and P_{het} at that locus accordingly (Table S1). We also analyzed females from seven of the 27 species at the same loci as males of the respective species (Table S2). As female euglossines are not attracted to odor baits and are therefore far more difficult to sample than males, we did not have access to females of the other 20 species. Of the seven species with females, $n > 20$ females for 5 species. Their genotypes were tested for the presence of null alleles using MICRO-CHECKER (van Oosterhout et al. 2004) and we reduced allelic diversity (H_{ina} or expected heterozygosity accounting for null alleles) and P_{het} for the three loci showing evidence of null alleles using equation (4) of Brookfield (1996; see Table S2). For the other loci, we calculated expected allelic diversity (H_{ina} or expected heterozygosity) from female genotypes using GENEPOP (Raymond and Rousset 1995). We conservatively used the lowest estimates of H_{ina} and P_{het} derived from males or females for each species-locus combination. Binomial 95% confidence intervals (2-tailed) of the proportion of diploid males were calculated using J.C. Pezzullo's Interactive Stats javascript (http://statpages.org/confint.html).

Four species were collected at two or more sites spanning 4–538 km: *Eg. cordata* (two sites), *Eg. imperialis* (three sites), *Eg. tridentata* (two sites), and *Eufriesea violacea* (two sites; see Table 2) and genotyped in the same laboratory. For each population pair, we computed estimates of genetic differentiation to infer population connectivity. Both F_{ST} and Hedrick's (2005) unbiased estimator of population differentiation, G_{ST}', were calculated with MSA version 4.05 (Dieringer and Schlötterer 2003) using the male dataset as MSA can simultaneously handle both haploid and diploid data. The significance of differentiation measures was determined using an exact test with 1000 permutations in MSA.

Figure 1. Map of the Neotropics with the 22 sampling sites highlighted as dots (five adjacent localities in Panama are given one dot).

Results

Allelic diversity accounting for null alleles (expected heterozygosity) of our loci, H_{ina}, ranged from 0.02 to 0.96 (Table 1). It was generally above 0.5 for most loci in most species (Tables S1 and S2) and averaged 0.62 (Table 1). H_{ina} differed little between males and females; it was greater by 0.044 in males versus females ($n = 5$ species and $n = 26$ locus-species combinations), suggesting that our estimates of P_{het} in species for which we did not sample females are only slightly inflated. Using 2–11 loci per species gave an average P_{het} of 0.991 (range 0.948 to >0.999), sufficient resolving power to detect the majority of diploid males as heterozygotes at one or more loci.

We detected five heterozygotes among the 1010 males that we genotyped, one each in *Eg. annectans*, *Eg. mandibularis*, and *Eg. tridentata*, and two in *El. bombiformis* (Table 1). The *Eg. mandibularis* male heterozygous at microsatellite locus Egc24 (Table S1) was the same individual that Takahashi et al. (2001) also detected by allozyme analysis as a heterozygote. We additionally detected one heterozygous *Eg. annectans* male (heterozygous at loci Egc18 and Egc24; see Table S1) that Takahashi et al. (2001)

Table 2. Geographic distances between pairs of populations of orchid bees (males) and genetic differentiation measured as F_{ST} and Hedrick's (2005) G_{ST}', with exact P values (1000 permutations) from MSA (Dieringer and Schlötterer 2003). For locations, see Figure 1.

Pair of populations	n (males)	n (loci)	Distance (km)	F_{ST} (P)	G_{ST}' (P)
Euglossa cordata (Brazil)	37	8	310	0.024 (0.005)	0.175 (0.037)
Caraguatatuba versus São Carlos	30				
Euglossa imperialis (Panama)	47	5	34	0.012 (0.176)	0.014 (0.827)
Barro Colorado versus Fort Clayton	23				
Euglossa imperialis (Panama)	47	5	4	−0.011 (0.767)	0.096 (0.422)
Barro Colorado versus Gigante Penninsula	28				
Euglossa imperialis (Panama)	23	5	32	0.004 (0.308)	0.038 (0.710)
Fort Clayton versus Gigante Penninsula	28				
Euglossa tridentata (Panama)	60	2	36	0.001 (0.354)	0.072 (0.379)
Barro Colorado versus Parque Natural Metropolitano	56				
Eufriesea violacea (Brazil)	16	10	538	−0.025 (0.991)	0.074 (0.598)
São Carlos versus Viçosa	37				

found to be homozygous by allozyme analysis. Over all males, and accounting for genetic nondetection errors (i.e., where $P_{het} < 1$), diploid male frequency averaged 0.005 (95% CI's 0.002–0.010).

Population differentiation in orchid bees was generally small and nonsignificant (Table 2), suggesting considerable gene flow. For the *Eg. imperialis* dataset comprising three Panamanian populations 4–34 km apart, global $F_{ST} = 0.001$ ($P = 0.384$) and $G_{ST}' = 0.034$ ($P = 0.786$). Pairwise measures of *Eg. imperialis* population differentiation were similarly not significantly different from zero (Table 2). The two *Eg. tridentata* populations separated by 36 km were also not significantly differentiated (Table 2). The two *Eg. cordata* populations separated by 310 km showed low, though significant, estimates of F_{ST} and G_{ST}' (Table 2). In contrast, the two *Ef. violacea* populations separated by 538 km were not significantly differentiated (Table 2), suggesting considerable gene flow between them.

Discussion

We found strong evidence for extremely low frequencies of diploid males among common and widespread orchid bees of the Neotropics. Our broad taxonomic sampling from across a wide geographic area lends weight to our analyses, while consistency in genotyping at four independent laboratories and low estimated frequencies of null alleles mean that the low 2N male frequencies we detected are unlikely to be a technical artifact. We found little or no population genetic structure over 10s–100s km; these results imply high gene flow, as also suggested by a mitochondrial DNA-based phylogeography of orchid bees (Dick et al. 2004), which could explain the apparently adequate sex allele diversity in orchid bees. Both low 2N male frequency and weak population genetic structure suggest that many orchid bees have both high gene flow and high N_e, and that they do not suffer from inbreeding through genetic drift and loss of *csd* diversity.

Why is there a discrepancy between our microsatellite-based study and all but one of the earlier allozyme-based studies demonstrating high 2N male frequencies, high population viscosity, and low N_e (Roubik et al. 1996; Zayed et al. 2004; López-Uribe et al. 2007)? We offer two explanations.

First, high frequencies of diploid males might be site or species-specific, and our sampling may not have captured sites or orchid bee species with high 2N males revealed by earlier allozyme-based studies. However, we analyzed males from four of the seven Panamanian species reported by Roubik et al. (1996) that exhibited high 2N male frequencies (Roubik et al. 1996, their Table 1), and we included two species (*Eg. imperialis* and *Eg. tridentata*) from the same sampling sites as Roubik et al. (1996). Furthermore, we did not detect any 2N males among the 98 *Eg. imperialis* males that we analyzed (95% CI's 0–3.8%) from the same three sampling sites at which Zayed et al. (2004) found 37.7% of *Eg. imperialis* males to be 2N. It is therefore unlikely that our sampling scheme was responsible for the discrepancies between our results and those of previous studies. A caveat of our interpretation is that diploid males may be produced during a specific season of the year, a period when Roubik et al. (1996) and Zayed et al. (2004) sampled but we did not.

Second, allozyme-based genotyping can suffer from allele misscoring, possibly due to protein instability, whereas DNA is more stable and therefore microsatellite genotyping more robust (Schlötterer 2004). This may have resulted in an artificial excess of male heterozygotes in allozyme studies; positive controls (diploid females) were generally lacking in allozyme-based studies. Our microsatellite loci detected high heterozygosity in females whenever they were available for analysis

(*Eg. annectans* in Paxton et al. 2009; *Eg. cordata* and *El. nigrita* in Souza et al. 2007; *Euglossa igniventris*, T. Eltz, unpubl. data; *EG. hemichlora*, *Eg. townsendi*, *Eg. viridissima,* and *Exaerete smaragdina* in Table S2) and yet frequencies of putative null alleles, a potential cause of microsatellite allele miscalling that may lead to an underestimate of 2N male frequency, were low. As we sampled females from only five of the 27 study species in sufficient number to test statistically for null alleles, we urge caution in the interpretation of our results, pending analysis of females from additional species. We nevertheless conclude that allozyme-based studies of orchid bees are probably methodologically flawed due to allele misscoring, and that this flaw accounts for the differences between allozyme-based studies and our microsatellite-based study. More direct methods of assessing diploid male frequencies and including analysis of females, for example by karyotype analysis (Eltz et al. 1998) or genome size estimation by flow cytometry (Aron et al. 2005; Cournault and Aron 2009), are needed to support our microsatellite-based conclusions.

Our interpretation of orchid bee population genetics, that they have low 2N male production, very weak population structure, high gene flow, and high N_e, fits with many independent observations of the taxon. First, individual orchid bees have been reported to travel long distances (>20 km; Janzen 1971). Second, other orchid bee species are common faunal elements in natural and disturbed habitats (Brosi 2009) and even in urban centers (López-Uribe et al. 2008). Third, census data suggest that orchid bee abundance and diversity appear to have been maintained (Roubik 2001), even within the highly fragmented Atlantic rainforest of Brazil (Tonhasca et al. 2002). Finally, results from the phylogeographic study of Dick et al. (2004) suggest that high gene flow across the South American continent is characteristic of many orchid bee species. These behavioral and genetic lines of evidence support the view that orchid bee populations are large, weakly structured and unlikely to suffer from inbreeding through loss of sex allele diversity.

Clearly, orchid bees may not be an informative test case of the idea that 2N male frequencies are a sensitive measure of bee pollinator decline (Zayed et al. 2004) as they seem to exhibit high mobility and high allelic diversity at the sex locus. For other bees, inbreeding is not necessarily associated with high frequencies of 2N males as detected by microsatellites (Paxton et al. 2000). Also, severely bottlenecked populations of the sweat bee *Lasioglossum leucozonium* with high 2N male frequencies detected by microsatellite genotyping have recently expanded across Eastern USA (Zayed et al. 2007), suggesting that high 2N male frequencies are not necessarily correlated with population decline in this invasive species. Yet for the honey bee (*A. mellifera*) with a well-characterized system of sex determination based on slCSD (Beye et al. 2003), high frequencies of 2N males have a catastrophic effect on colony survival (Woyke 1980), as in other social bees (Plowright and Pallett 1979; Carvalho 2001) and ants (Ross and Fletcher 1986). An appropriate test of the diploid male extinction vortex (Zayed and Packer 2005) and the idea that the frequency of 2N males is a sensitive measure of pollinator decline (Zayed et al. 2004) awaits analysis of slCSD populations at their range margins or of those that have been anthropogenically compromised. Eusocial Hymenoptera such as bumblebees (e.g., Takahashi et al. 2008) may be more suitable subjects for such a test than the largely solitary and subsocial orchid bees (cf. Cocom Pech et al. 2008) because hymenopteran eusociality is associated with reduced genetic diversity and low N_e (Pamilo et al. 1978, 1997; Graur 1985; Hedrick and Parker 1997; Chapman and Bourke 2001; Packer and Owen 2001).

Although bees are thought to possess slCSD (van Wilgenburg et al. 2006), the presence of a different kind of sex determination in orchid bees could explain the observed low frequencies of 2N males. A parasitoid hymenopteran has recently been shown to possess multilocus CSD (mlCSD; de Boer et al. 2008) and diploid males in hymenopterans with regular inbreeding produce fertile diploid males (de Boer et al. 2007; Cournault and Aron 2009); in one wasp with regular inbreeding, diploid males may even produce haploid sperm (Cowan and Stahlhut 2004). Sex determination through genomic imprinting has also been recently demonstrated in the haplodiploid hymenopteran *Nasonia* (Verhulst et al. 2010). The presence of occasional diploid males in otherwise haploid-male orchid bees indicates that the taxon possesses CSD. The low frequency of 2N males that we observed may be a consequence of mlCSD.

Our sampling of 26 orchid bee species from across a wide geographic range and habitat types (coastal Atlantic forest, cerrado open woodland, Amazonian tropical forest), including sites with old-growth vegetation (Barro Colorado Island) and others with highly disturbed vegetation (e.g., São Carlos; Soares et al. 2003), allow us to draw conclusions concerning the conservation genetics of this taxon. First, orchid bees currently appear to have extremely low frequencies of 2N males, suggesting that continental populations are probably not prone to the diploid male extinction vortex (Zayed and Packer 2005), possibly because of high gene flow maintaining adequate allelic diversity at the sex locus. Second, they appear to be highly mobile, again increasing N_e beyond those predicted from estimates of census size at one point in time and space. Nevertheless, we urge caution in the generalization of our results. Morphological similarity among orchid bees (Roubik and Hanson 2004; Eltz et al. 2008) may hide cryptic species diversity, and rare species or isolated populations at range margins may yet be found to suffer the genetic load of high diploid male production.

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Supporting Information

The following supporting information is available for this article:

Table S1. Male haplotypes/genotypes in base pairs, expected allelic diversity ($H_i = \sum (x_i^2)$) and presence of heterozygous males (i.e., diploid or 2N males) at microsatellite loci developed for *Euglossa cordata* (suffix Egc; Souza et al. 2007), *Eulaema nigrita* (suffix Eln; Souza et al. 2007) and *Euglossa annectans* (suffix ann; Paxton et al. 2009) in 27 orchid bee species from the Neotropics.
Table S2. Female genotypes in base pairs at microsatellite loci developed for *Euglossa cordata* (suffix Egc; Souza et al. 2007), *Eulaema nigrita* (suffix Eln; Souza et al. 2007) and *Euglossa annectans* (suffix ann; Paxton et al. 2009) for seven of 27 orchid bee species whose males were also genotyped (Table S1).

Supporting Information may be found in the online version of this article.

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