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GENETIC DIVERSITY OF TWO STINGLESS BEES, *TRIGONA NIGERRIMA* (CRESSON 1878) AND *TRIGONA CORVINA* (COCKERELL 1913), IN COFFEE DOMINATED LANDSCAPES IN SOUTHERN MEXICO

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ABSTRACT. Stingless bees (Apidae, Meliponini) are key pollinators in natural and agricultural Pantropical environments. Current human activities, however, threaten their populations, making it urgent to assess their abundance and genetic status. By a population genetics approach, we calculated the genetic diversity and estimated the number of wild colonies of two stingless bee species, *Trigona nigerrima* (Cresson) and *T. corvina* (Cockerell) in southern Mexico. Allele richness ranged from 10 to 19 (mean ± SD = 14 ± 3.5) for both species. Expected heterozygosity ranged 0.88-0.93 for *T. corvina* and 0.82-0.92 for *T. nigerrima*. The estimated number of colonies estimated was 25 on average for *T. corvina* and 21 for *T. nigerrima*. According to our results populations of both species seem not to be in danger. We also show that genetic tools can be extremely useful for colony density estimation for stingless bees as it is for bumble bees and honey bees.
Keywords: Stingless bee, microsatellites, DNA, population genetics, meliponine, *Trigona*.

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RESUMEN. Las abejas sin aguijón (Apidae, Meliponini) son especies polinizadoras clave en ambientes pantropicales, tanto naturales como agrícolas. Las actividades humanas, sin embargo, ponen en riesgo sus poblaciones silvestres, lo que hace necesario que se conozcan cuanto antes su abundancia y su diversidad genética con fines de conservación. Mediante un enfoque de genética de poblaciones se calculó la diversidad genética y el número de colonias silvestres de dos especies de abejas sin aguijón, *Trigona nigerrima* y *T. corvina*, en el sur de México. Se encontró una riqueza alélica que varió de 10 a 19 (media ± DE = 14 ± 3.5). La heterocigosidad esperada fue de 0.88-0.93 para *T. corvina* y de 0.82-0.92 para *T. nigerrima* El número de colonias estimadas para *T. corvina* fue de 25 en promedio, y para *T. nigerrima* de 21. De acuerdo con nuestros resultados las poblaciones de ambas especies parecen no estar amenazadas. También se demuestra que las herramientas de genética de poblaciones son extremadamente útiles para la estimación de colonias en abejas sin aguijón, al igual que en otros himenópteros como los abejorros y la abeja melífera.
Palabras clave: Abejas sin aguijón, microsatélites, ADN, genética de poblaciones, meliponinos, *Trigona*.

INTRODUCTION

Human population, and the amount of resources necessary to sustain it, is globally increasing. Food and housing are basic elements that demand space; therefore natural areas are incorporated into production chains to satisfy these and other necessities. As a consequence, populations of native organisms are locally reduced or might become extinct. Studies have revealed a decline in the diversity and abundance of insect pollinators in Europe (Biesmeijer *et al.* 2006) and America (Goulson *et al.* 2008, Cameron *et al.* 2011), partially driven by intensive agricultural practices (Freitas *et al.* 2009, Potts *et al.* 2010). Large extensions of land devoted to only a few crops seem to leave few resources to native insect pollinators to survive, since such management frequently results in habitat loss and pollution of the environment (Richards 2001, Carvell *et al.* 2006). A reduction in the populations of these species could actually have serious impacts on the world's economy (Kevan & Phillips 2001, Klein *et al.* 2007), and more importantly, on world's food security (Gallai *et al.* 2009). Among insect pollinators, eusocial bees (Hymenoptera, Apidae) like honey bees (Aizen & Harder 2009) and bumble bees (Hayo & Adriaan 2006) are key in crop pollination. In addition, a growing body of information reveals the importance of stingless bees (Apidae, Meliponini) as pollinators in natural and agricultural environments (Heard 1999, Cauich *et al.* 2004, Del Sarto *et al.* 2005, Palma *et al.* 2008). Current human activities, however, threaten their populations, and though some species seem to be abundant, others do not, and more importantly, the genetic status for many of them remains unknown. In

fact, a genetic approach would reveal critical information, not attainable by conventional ecological methods, about the viability status of populations, not mentioning genetic diversity. For example, several studies have evaluated the effect of beekeeping upon both genetic variability (Carvalho-Zilse *et al.* 2009) and maintenance of populations (Alves *et al.* 2010), while others have evaluated genetic variability and population structure of stingless bees (Costa *et al.* 2005, Francisco *et al.* 2006, Garcia Tavares *et al.* 2007, Hurtado-Burillo *et al.* 2013). Additionally, population genetics is a powerful tool to estimate colony density for the study of population dynamics (Cameron *et al.* 2004, Moritz *et al.* 2007, Kraus *et al.* 2008), a better alternative to find and count colonies in the field (Zayed 2009). Thus in this paper we investigated the genetic diversity and colony density of two stingless bee species, *Trigona nigerrima* (Cresson 1878) and *T. corvina* (Cockerell 1913) in rural areas in Chiapas, Mexico, by genotyping free foraging individuals. We chose these species because they are the dominant native social species in the study sites and are frequently found foraging in several species of plants, both cultivated and wild.

METHODS AND MATERIALS

Study site. Foragers were collected between February and April 2011 in four localities in Chiapas, Mexico (Fig 1). Shade coffee is the main crop in the region, though other plants like corn and timber are also cultivated. The majority of the farmers possess small pieces of land (< 3 ha), thus a highly patchy landscape is formed. Local agriculture practices are mostly human-labor based, with low input of agrochemicals. All these conditions seemingly support habitat diversity and provide a variety of resources to animal populations.

Sampling scheme. Specimens were collected between 9:00-13:00 hours using standard entomological nets. Species were collected in sufficient number to perform comparative genetic analyses only in some sites: *T. corvina* in Tzajalchen and Chijil, and *T. nigerrima* in Plan Ocosingo and Rancho Naranjo. *Trigona* species are known to recruit nest mates to profitable resources (Lindauer & Kerr 1960, Nieh 2004, Sánchez *et al.* 2004), which could lead to an underestimation of allelic richness if foragers are analyzed. To avoid this, specimens were collected such that: 1) two to four foragers were collected where they were found in groups of more than ten individuals and, 2) they were collected in patches separated from each other by at least 500 m. Foragers were sacrificed with ethyl acetate; one hind leg was removed from each collected specimen and placed in absolute ethanol for further storing at -20°C and DNA extraction. The rest of the insect was kept in an entomological collection in ECOSUR, San Cristobal de las Casas, Chiapas.

Microsatellite analysis. DNA was extracted following the HotSHOT method (Truett *et al.* 2000). All foragers were genotyped at four microsatellite loci (T3-32, Tc3-302, T4-171 and T8-40) following single locus routine PCR protocols (Kraus *et al.* 2008) to a final reaction volume of 5µL. Microsatellite fragments were separated us-

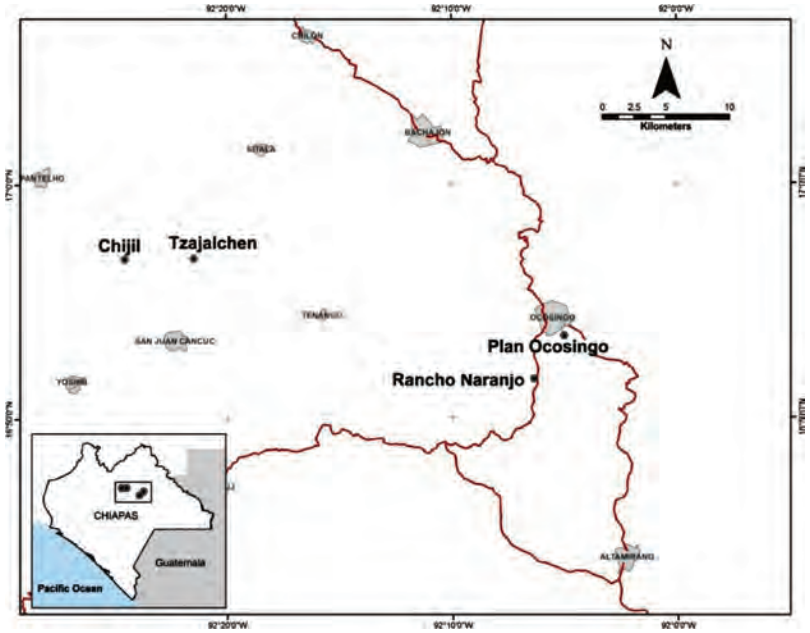

Figure 1. Location of study sites.

ing a semiautomated LI-COR 4200 slab-gel sequencer (6% denaturing polyacrylamide gels); fragment size was determined using the software SAGA MX (LI-COR Inc., US). Forward oligos were tagged with an M13(-29) sequence, which served as a priming site to a M13(-29) primer tagged with IRDYE800 (Schuelke 2000). In order to estimate amplification errors or allele miss-callings 10% of foragers were subjected to reamplification at all loci. All reamplifications gave identical results.

Analysis of genetic data. Screening of data for large allele dropout, null alleles and scoring errors was carried out using MicroChecker (Van Oosterhout *et al.* 2004). Departures from Hardy-Weinberg proportions of each loci, pairwise genotypic linkage disequilibrium, expected and observed heterozygosities, and tests for population differentiation were performed using GENEPOP v4.0 (Rousset 2008). Allelic richness was calculated with FSTAT v2.9.3.2 software (Goudet 2001), which was used to compare the genetic diversity between sites with a paired T-Students' test using S-Plus v6. Number of colonies in each site was estimated with COLONY v1.2 software (Wang 2004).

RESULTS

A total of 194 female individuals from the four sites were genotyped: ninety-eight *T. nigerrima* and ninety-six *T. corvina* individuals. Scoring errors, allele dropout and null alleles were not detected in any loci in any species; however the T8-40 locus in *T. corvina* from the two sites departed significantly from Hardy-Weinberg equilibrium, perhaps because primers were developed for other species (Hurtado-Burillo *et al.* 2013), thus it was removed from allelic richness comparisons. All loci in both species were not in significant linkage ($P > 0.05$). Tables 1 and 2 show the expected and observed heterozygosity and the allelic richness per locus, population and species. Allelic richness was not significantly different between sites for both species (*T. corvina*: $t = 3.05$, $df = 2$, $P = 0.09$; *T. nigerrima*: $t = 2.54$, $df = 3$, $P = 0.09$) though genotypic differentiation between each pair of sites was highly significant in both species (*T.corvina*: $G = 46.48$, $df = 8$, $P < 0.001$; *T nigerrima*: $G = 143$, $df = 8$, $P < 0.001$). Number of colonies estimated for *T. corvina* was 26 and 24 for Rancho Naranjo and Plan Ocosingo sites, respectively. For *T. nigerrima* the numbers were lower, 20 and 22 for the Tzajalchen and the Chijil sites, respectively.

Table 1. Genetic diversity parameters estimated for *Trigona corvina*. N_I: Number of individuals genotyped at the corresponding locus, N_A: Number of alleles, A_r: Allelic richness, H_e: Expected heterozygosity; H_o: Observed heterozygosity *Locus excluded from the analysis of allelic diversity due to deviation from Hardy-Weinberg equilibrium.

Trigona corvina – Plan Ocosingo					
	N_I	N_A	Ar	He	Ho
T3-32	45	16	15.96	0.88	0.74
Tc3-302	44	18	17.20	0.92	0.83
T4-171	44	15	14.86	0.89	0.77
T8-40*	45	12	11.98	0.90	0.65
Average	—	15.2	15	0.89	0.74
Trigona corvina – Rancho Naranjo					
	N_I	N_A	Ar	He	Ho
T3-32	39	12	12.00	0.93	0.89
Tc3-302	41	16	15.95	0.88	0.86
T4-171	44	13	12.75	0.92	0.80
T8-40*	46	15	14.51	0.90	0.67
Average	—	14	13.8	0.90	0.80

Table 2. Genetic diversity parameters estimated for *Trigona nigerrima*. Column names as in Table 1.

Trigona nigerrima – Chijil					
	N_I	N_A	Ar	He	Ho
T3-32	40	10	9.92	0.82	0.83
Tc3-302	44	20	19.60	0.92	0.93
T4-171	45	12	11.58	0.83	0.96
T8-40	43	14	13.71	0.90	0.79
Average	—	14	13.7	0.86	0.86
Trigona nigerrima –Tzajalchen					
	N_I	N_A	Ar	He	Ho
T3-32	39	10	10.00	0.83	0.85
Tc3-302	42	18	17.75	0.91	0.93
T4-171	48	8	7.92	0.82	0.94
T8-40	48	10	10.00	0.89	0.85
Average	—	11.5	11.4	0.86	0.89

DISCUSSION

In spite that some of the sampling sites seemed to be more disturbed due to the presence of grasslands, we did not find any significant difference in the allelic richness in any of the *Trigona* species, thus at a regional level both species seem to be in similar conditions. Moreover, observed heterozygocity was high in all sites. We think that it is the low input of agrochemicals, and the presence of a mosaic of crops and trees that is formed due to rural activities, what helps maintaining populations with such high heterozygosity and allelic diversity, though future research should confirm this. Studies with other stingless bees show diverse results. For example, while Francisco *et al*. (2013) found heterozygosity values from 0.551 to 0.749 in *Plebeia remota* (Holmberg 1903) and Francini

et al. (2009) from zero to 0.75 in *Melipona interrupta manaosensis* (Schwarz 1932); other authors as Borges *et al*. (2010) and Tavares *et al*. (2013) describe very low levels of observed heterozygosity in their study with *Partamona helleri* (Friese 1900) and *M. quadrifasciata* (Lepeletier 1836). In the study by May-Itzá *et al*. (2010) with *M. yucatanica* in Mexico and Guatemala very low levels of heterozygosity were also found. In these studies the low value of the observed heterozygosity can be explained by the swarming behavior of stingless bees, since the daughter colony is commonly founded near the original one, favoring endogamy. A weakness in all these studies is the sampling scheme: they collected directly from colonies and many samples were obtained from neighboring colonies. Studies by Cameron *et al*. (2004) and Kraus *et al*. (2008) involved sampling drones, massively grouped in congregations. They found far higher genetic diversity values, since drones were determined to come from as many as 40 colonies for *Scaptotrigona mexicana* (Guérin 1844) and 137 for *T. collina* (Smith 1857). This indicates that a number of colonies might not be sampled if colony surveying is the main sampling scheme, instead of genotyping free flying foragers or drones. It is important to emphasize how important to our approach of surveying free flying foragers is the fact that stingless bees are mainly single mated (Palmer *et al.* 2002, Paxton *et al.* 2003, Toth *et al.* 2004), since estimation of colonies using microsatellites is greatly far more accurate than for multiple mating species, like *Apis* spp. (Fuchs & Moritz 1999), in which a large number of workers is necessary to estimate a queen's genotype with some accuracy, and thus colony density.

According to our data populations were genotypically different. We think that the short flight range of *Trigona*, in addition to forest fragments surrounded by a matrix of crops and villages and the presence of orographic discontinuities, contribute to explain the genotypic differences between sites; this has to be proven yet. We also were able to estimate colony density with our data, something that had not been done before with free flying workers of stingless bees. Though we did not survey exhaustively our sampling sites, we could estimate colony density as 0.2 to 0.26 nests/ha, since our sites corresponded to approximately 100 ha. Such density is similar to that described for other stingless bees in non disturbed sites (Slaa 2006). These densities also reflect that populations of these species seem not to be compromised. Therefore it is highly likely that the degree of patchiness and the actual quality of the patches in the study sites are not affecting bee populations at detectable levels. Hurtado-Burillo *et al.* (2014) compared the genetic richness of two *Melipona* species occurring in Mexico, *M. colimana* and *M. beecheii*, and found no significant differences in expected heterozygosity; moreover, these authors found no significant differences between the populations from deforested and conserved areas, with results similar to ours. However, as mentioned lines above, many lines of research arise to develop a more detailed view of the situation of stingless bees populations in this part of Mexico, but our results give the baseline for these studies.

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