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RESEARCH ARTICLE

Population genetics and geometric morphometrics of the *Bombus ephippiatus* species complex with implications for its use as a commercial pollinator

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Abstract Mexico and Central America are among the most biodiverse regions on Earth, harboring many species with high levels of interpopulation morphological and genetic diversity. The mountainous topography of this region contains isolated sky island habitats that have the potential to promote speciation. This has been studied in vertebrates, yet few studies have examined the phylogeographic and genetic structure of insect species encompassing this region. Here we investigate geographic patterns of genetic and morphological divergence and speciation among widespread populations of the highly polymorphic bumble bee *Bombus ephippiatus* and its closest relative *B. wilmattae*. We used DNA sequences from a fragment of *cytochrome oxidase I* (COI), genotypes for twelve microsatellite markers, and morphometric data from wings to construct a well-supported inference of the divergences among these taxa. We have found complex patterns of genetic isolation and morphological divergence within *B. ephippiatus* across its geographic range and present evidence that *B. ephippiatus* comprises multiple independent evolutionary lineages. The pattern of their diversification corresponds to geographic and environmental isolating mechanisms, including the Mexican highlands, the lowlands of the Isthmus of Tehuantepec in southern Mexico, the Nicaraguan Depression, the patchily distributed volcanic ranges in Nuclear Central America and Pleistocene glacial cycles. These results have important implications for the development and distribution of *B. ephippiatus* as a commercial pollinator in Mexico and Central America.

Keywords Bumble bees · Microsatellites · Cytochrome oxidase I · STRUCTURE · GENELAND · Species delimitation

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Introduction

Mexico and Central America are well known for their biological complexity (Mittermeier et al. 2000). The great biodiversity in this region is frequently attributed to its location between two large continents, arising from biotic interchange between North and South America. This region is also a transition zone between the northern Nearctic and southern Neotropical biogeographic regions (Heilprin 1887). Its volcanic topography has led to isolation and speciation in birds (Cracraft and Prum 1988; Roy et al. 1997; García-Moreno et al. 2006), mammals (Vrba 1993; Sullivan et al. 2000; León-Paniagua et al. 2007), and herpetofauna (Mulcahy et al. 2006; Castoe et al. 2009; Daza et al. 2010). Many endemic forms have been restricted within the last few million years to particular ecosystems, such as montane pine-oak or cloud forests (Escalante et al. 1993; León-Paniagua et al. 2007; Kerhoulas and Arbogast 2010; Barber and Klicka 2010). For example, many bird species restricted by ecological limits exist as a series of isolated populations in islands of suitable habitat (García-Moreno et al. 2004).

There are two particularly notable lowland regions within Mexico and Central America that have served as geographic barriers between these montane sky island habitats, preventing species movement. One of these barriers is the Isthmus of Tehuantepec (IT) (Fig. 1), a continental strait ending in two plains. This large, narrow area of low elevation with a hot, humid climate separates the Sierra Madre del Sur of southern Mexico from the highland regions of the Maya Block, a fault block encompassing Mexico south of the IT through central Guatemala (Gutiérrez-García and Vázquez-Domínguez 2013). The IT is proposed to be a major barrier to dispersal in toads (Mulcahy et al. 2006), snakes (Castoe et al. 2009; Daza et al. 2010), birds (Barber and Klicka 2010), and rodents (Sullivan et al. 2000). The other significant barrier is the Nicaraguan Depression (Fig. 1), which is a lowland expanse separating the Chortis Block highlands of Honduras and Nicaragua from the highlands of Costa Rica. The Nicaraguan Depression is an important isolating mechanism in snakes and rodents (Castoe et al. 2009; Daza et al. 2010; Gutiérrez-García and Vázquez-Domínguez 2013).

The highlands of Mexico are also important geographic barriers for many plant and animal taxa, especially during more recent Pleistocene glacial cycles (Ornelas et al. 2013; Mastretta-Yanes et al. 2015). North of the IT, there are four distinct mountain ranges that have shaped the genetic structure of the organisms that live within them: the Sierra Madre Occidental, the Sierra Madre Oriental, the Trans-Mexican Volcanic Belt and the Sierra Madre del Sur (Fig. 1). South of the IT, there are four mountain ecoregions: the mid-elevation Central American pine-oak forest that extend into Guatemala and Honduras and the high elevation Sierra Madre de Chiapas moist forest, Chiapas montane forest, and Central American montane forest (Fig. 1). These latter three ranges are partially separated by the Central American pine-oak forest and a lowland region called the Central Depression (CD) (Fig. 1), a barrier to hummingbirds and passerines (Ornelas et al. 2013).

This pattern of high endemism and diversification across Mexico and Central America via geological isolation has likely affected insect diversity in the region. Biogeographic patterns of some groups of beetles, for instance, suggest that the uplift of montane regions in Central America encouraged the southerly movement of Nearctic beetles, while the tropical lowlands of Central America enabled South American beetles to move northward after the Panamanian land bridge connection $\sim$3 mya (Halffter 1987; Liebherr 1994; Lobo and Halffter 2000; Marshall and Liehberr 2000; Morrone 2006). These studies have generated numerous hypotheses to explain the diversification of Mexican and Central American beetles, but the explanations for how geological barriers could have shaped diversification are constrained by reliance on species distribution data alone. Multi-locus phylogenetic and population genetic analyses can clarify how evolutionary divergence is shaped by historical events using gene genealogies. Furthermore, to understand whether whole communities respond similarly to the same historical events and shared barriers, it is important to compare patterns across diverse groups of taxa. To date, a small amount of molecular data is available for insect species in Mexico and Central America (beetles: Morse and Farrell 2005; Anducho-Reyes et al. 2008; Ruiz et al. 2010; Baselga et al. 2011; Sánchez-Sánchez et al. 2012; true bugs: Dorn et al. 2009; stingless bees: May-Itzá et al. 2010).

In both the Old and New World, bumble bees (*Bombus*) have been the focus of intense molecular phylogenetic (Koulianos and Schmid-Hempel 2000; Kawakita et al. 2004; Ellis et al. 2005; Hines et al. 2006; Cameron et al. 2007; Williams et al. 2011, 2012, 2015; Lecocq et al. 2011; Hines and Williams 2012; Carolan et al. 2012; Bossert et al. 2016; Françoso et al. 2016; Sheffield et al. 2016) and population genetic analysis (Estoup et al. 1996; Widmer et al. 1998; Widmer and Schmid-Hempel 1999; Chapman et al. 2003; Shao et al. 2004; Ellis et al. 2006; Darvill et al. 2006; Schmid-Hempel et al. 2007; Herrman et al. 2007; Lozier and Cameron 2009; Kraus et al. 2009; Darvill et al. 2010; Charman et al. 2010; Lye et al. 2011; Cameron et al. 2011; Lozier et al. 2011; Kraus et al. 2011; Goulson et al. 2011; Carvell et al. 2012; Jha and Kremen 2013; Lozier et al. 2013;

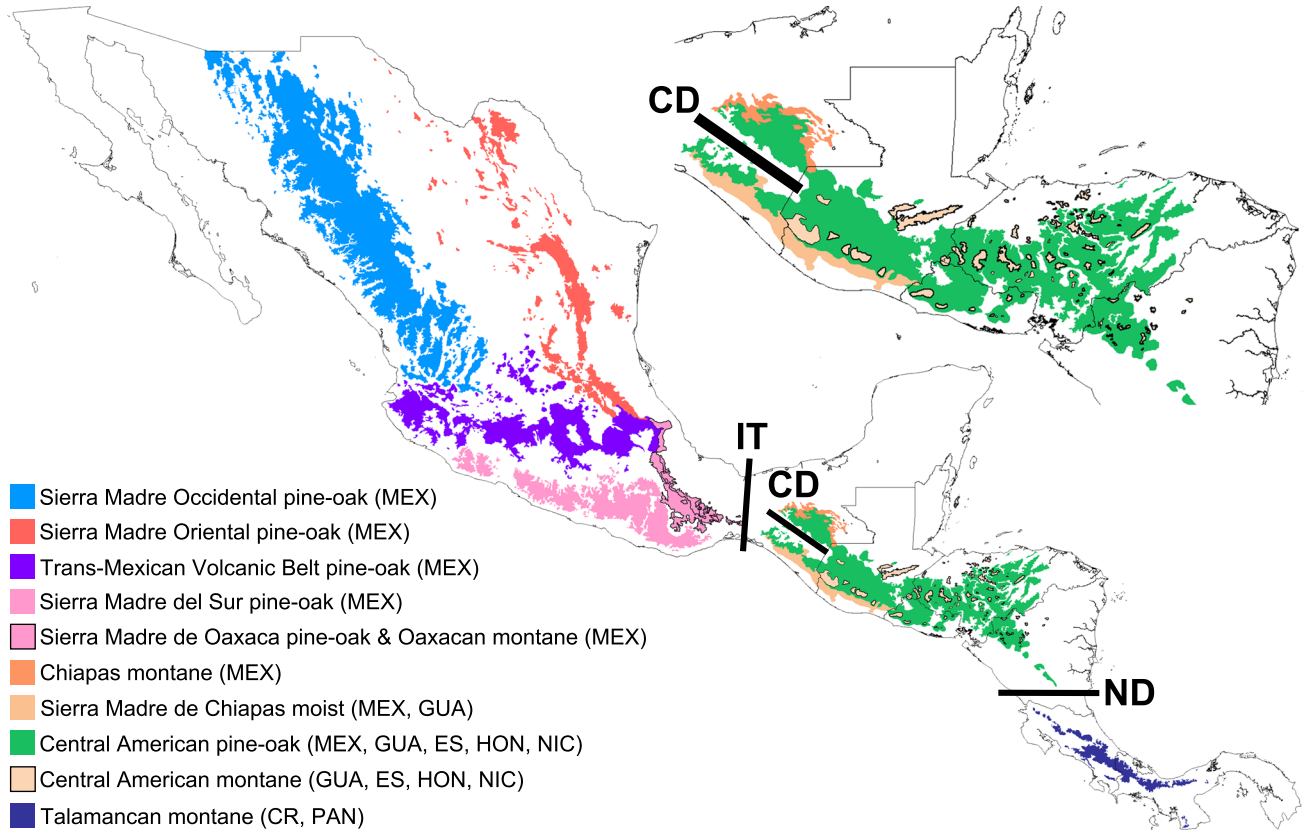

Fig. 1 Map illustrating the distinct mountain regions that *B. ephippiatus* and *B. wilmattae* inhabit. There are four major mountains chains north of the Isthmus of Tehuantepec (IT), three mountain ecoregions south of the IT and north of the Nicaraguan Depression (ND), which are further split in Mexico by the Central Depression (CD), and the Talamancan montane forests south of the ND in Costa Rica and Panama. All regions highlighted are World Wildlife Federation (WWF) recognized ecoregions (Olson et al. 2001). (Color figure online)

Maebe et al. 2013; Lecocq et al. 2013; Dreier et al. 2014; Moreira et al. 2015; Jha 2015; Santos Júnior et al. 2015; Huang et al. 2015; Lecocq et al. 2015a, b, c; Francisco et al. 2016). Despite the presence of nineteen different species (Labougle 1990) of bumble bees in Mexico and Central America, only a single study to date has explored the phylogeography of bumble bees in this region (Duennes et al. 2012). The phylogenetic patterns and known biogeographic distributions of *Bombus* provide a rich background for investigating the structure of inter- and intraspecific genetic diversity in Mexico and Central America and for examining whether *Bombus* diversification correlates temporally with historical events associated with the speciation of vertebrate and other insect taxa.

Bombus ephippiatus and *B. wilmattae* are a species complex especially relevant for the study of Mexican and Central American biodiversity. First, the estimated divergence of these southern species from their North American relative *B. impatiens* (~1 mya, Duennes et al. 2012) fits within the pertinent timescale of recent geological and climatic events in this region. Second, *B. ephippiatus* is distributed widely throughout Mexico and Central America and is found in diverse montane habitats (Duennes and Vandame 2015), while *B. wilmattae*, of uncertain species status (distinguished from *B. ephippiatus* primarily by the presence of a band of yellow or white hairs on the anterior pronotum near the head; Labougle et al. 1985; Labougle 1990; Williams 1998; Duennes et al. 2012), is restricted to a relatively small geographic range in southern Mexico and eastern Guatemala. These distributions allow comparative studies of genetic diversity in widespread and restricted populations. Third, *B. ephippiatus* is highly polymorphic across its large range, exhibiting both a gradation in color pattern and genetic diversity from north to south (Duennes et al. 2012). Preliminary studies of the genetic diversity of this group suggested five main lineages within *B. ephippiatus* and *B. wilmattae*: one lineage of *B. ephippiatus* north of the IT, two sympatric lineages of *B. ephippiatus* south of the IT through Honduras, one lineage of *B. ephippiatus* in Costa Rica and a fifth lineage comprising *B. wilmattae*, which was nested within *B. ephippiatus* (Duennes et al. 2012). Overall, the widespread geographic distribution of the *B. ephippiatus-B. wilmattae* species group over a topologically complex region, its highly polymorphic color pattern, and its genetic diversity suggest the possibility of additional cryptic diversity.

The *B. ephippiatus* complex is of conservation concern as it is currently under development as an alternative commercial pollinator that could replace the non-native *B. impatiens* sold extensively throughout Mexico and Guatemala for greenhouse and field crop pollination. The continued use of *B. impatiens* for commercial pollination in Mexico and Central America poses threats to the population health of native bumble bee species. These threats, which have been indicated in other parts of the world as a result of global intercontinental transport of non-native *Bombus*, include competition for floral resources (Morales et al. 2013) and spread of diseases (Arbetman et al. 2013; Sachman-Ruiz et al. 2015). Because *B. impatiens* is sister species to the *B. ephippiatus-B. wilmattae* complex (Duennes et al. 2012), the threat of reproductive disturbance (Kondo et al. 2009) and inter-specific hybridization (Yoon et al. 2009) is of particular concern. BioBest (http://www.biobestgroup.com/) and Koppert Biological Systems (https://www.koppert.com/), both pioneers in European commercial bumble bee production, have facilities in Mexico that produce and distribute *B. impatiens* throughout Mexico and Central America; both companies are interested in producing *B. ephippiatus* as a native alternative pollinator (Torres-Ruiz and Jones 2012). Moreover, twelve independent breeders of *B. ephippiatus* across five states in Mexico have formed an association with the goal of developing this species as a sustainable crop pollinator (Asociación Mexicana de Criadores de Abejorros Nativos, A.C.). While *B. ephippiatus* appears to be a promising native alternative to *B. impatiens*, its widespread commercial use without understanding the taxonomic status and population genetic structure could result in a homogenization of native population genetic diversity (Bourret et al. 2011; Williams et al. 2012; Lecocq et al. 2016).

Here we describe how both montane and lowland barriers across Mexico through Honduras (Fig. 1) have shaped the genetic structure of the *B. ephippiatus* complex. We add four additional microsatellite loci to the eight used by Duennes et al. (2012) and add nearly 2000 new specimens from across the range of this species complex, especially from northern Mexico. We explore whether morphological variation corresponds to genetic variation and isolation using wing morphometric data from over 600 specimens. We discuss possible geographic barriers and climatic events that could have caused the observed patterns of diversification within this group and provide an in-depth comparison of the efficacy of the Bayesian assignment programs STRUCTURE (Pritchard et al. 2000) and GENELAND (Guillot et al. 2012). We also discuss taxonomic consequences of this study and implications for the trade of *B. ephippiatus* for commercial pollination in Mexico and Central America.

Materials and methods

Taxa examined

Phylogenetic analysis

To resolve the basal relationships among the lineages in the *B. ephippiatus-B. wilmattae* species complex, sequence fragment data were collected from *cytochrome oxidase I* for 254 specimens spanning the group's broad geographic distribution. One hundred and fifty one specimens from Mexico and Guatemala were added to the 103 specimens previously analyzed in Duennes et al. (2012). In total, 76 specimens of *B. wilmattae* and 167 specimens of *B. ephippiatus* were included in the analysis. Nine specimens of *B. impatiens* and one specimen each of *B. huntii* and *B. vosnesenskii* were selected as outgroup taxa. A list of all samples used for phylogenetic analyses can be found in Online Resource 1.

Microsatellite analysis

To delimit distinct genetic groups within the *B. ephippiatus-B. wilmattae* complex, extensive sampling was conducted throughout Mexico and Guatemala. Samples were collected from one to three sites approximately three kilometers apart within a single population. A maximum of 20 samples was collected from each population, and each population sampled was at least 30 km apart. This scheme was used to minimize repeated sampling of individuals from the same colony. Samples previously genotyped in Duennes et al. (2012) were also included and genotyped at an additional 4 loci. In total, 1917 female samples of *B. ephippiatus* and *B. wilmattae* were genotyped at 12 microsatellite loci (Online Resource 1).

Geometric morphometric analysis

To test whether genetic structure has shaped morphology in this group, geometric morphometric data were collected from dorsal left forewings of *B. ephippiatus* and *B. wilmattae*. The wings were removed from 606 worker caste specimens (Online Resource 1) across the distribution of the species complex. Following Aytekin et al. (2007), 20 landmarks on the forewing were mapped and analyzed (Online Resource 2, see details below).

Phylogenetic inference

Cytochrome oxidase I

An 811 base pair fragment of the *cytochrome oxidase I* (COI) gene was amplified from 254 specimens (Online

Resource 1) using the primers RevmtR and FormtR (used in Duennes et al. 2012). These highly specific primers minimized amplification of mitochondrial insertions into the nuclear genome and span a variable region of the gene. Other mitochondrial (*cytochrome oxidase B*; *16S ribosomal RNA*) and nuclear (*Phosphoenolpyruvate carboxykinase,* [PEPCK]; *carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase* [CAD]) genes were explored but lacked informative nucleotide variation (Duennes et al. 2012 for nuclear genes; data not shown for mitochondrial genes).

DNA extraction, PCR, and DNA sequencing

A single foreleg was used for DNA extraction to generate template DNA for both COI amplification and microsatellite genotyping. Tissue was digested and DNA extracted in 150 µL of 5% Chelex® 100 resin (Bio-Rad, Hercules, CA) and 3 µL of Proteinase K (20 mg/µL) for 60 min at 55 °C, 15 min at 99 °C, 1 min at 37 °C, and then 15 min at 99 °C using a thermocyler. Standard conditions for PCR amplification were an initial denaturation step of 95 °C for 3 min; 35 cycles of denaturation for 60 s at 94 °C, annealing for 60 s at 48–57 °C, and elongation for 60 s at 72 °C, and a final extension of 3 min at 72 °C, 25 µL. PCR reactions were conducted in 5 µL of 5X GoTaq® reaction buffer (Promega, Fitchburg, WI), 1.875 mM $MgCl_2$, 0.2 mM each dNTP, 10µL of each primer and 0.4 U of GoTaq® DNA polymerase (Promega) with 2.5 µL of genomic DNA. PCR products were purified using ExoSAP-IT® (Affymetrix, Santa Clara, CA). BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used for sequencing of PCR products with the corresponding primers. Sequencing was performed at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois using an ABI 3730XL (Applied Biosystems) capillary sequencer. Tissue was obtained from the specimens by removing one of the forelegs. All samples are housed either at the University of Illinois (stored in 95–100% ethanol at 4 °C) or at El Colegio de la Frontera Sur (ECOSUR) (pinned and dried).

Alignment and phylogenetic analysis

DNA sequences were edited in Geneious 8.1.7 (Biomatters Ltd) and aligned using MUSCLE (Edgar 2004). The HKY + I model was selected for Bayesian phylogenetic inference based on Bayesian information criteria (BIC) calculated by jModelTest 2.1.7 (Darriba et al. 2012). For phylogenetic inference, the aligned COI sequences were analyzed using MrBayes 3.2.5 (Ronquist et al. 2012) with 10,000,000 generations, four chains, flat priors and sampling trees every 1,000 generations. Log-likelihood plots of the two parallel runs from one MrBayes analysis were compared using Tracer 1.6.0 (Rambaut et al. 2014) and discarded as burnin the first 2,500,000 generations (2500 trees). The two runs were combined and the posterior probability support for each node of the consensus tree was calculated. Pairwise F_{ST} values between clades and average number of nucleotide differences within and between clades from the Bayesian phylogeny were calculated with DnaSPv5.10.1 (Librado and Rozas 2009). In addition to using Bayesian methods to reconstruct relationships, a parsimony haplotype network was generated with TCS1.21 (Clement et al. 2000) using the default parsimony connection limit of the program.

Species delimitation

To establish a threshold for species determination with sequence data, the general mixed Yule-coalescent model (GMYC) was implemented in a Bayesian framework with the program bGMYC (Reid and Carstens 2012). First, unique haplotypes were identified with COLLAPSE 1.2 (accessed 2016: http://www.softpedia.com/get/Science-CAD/Collapse.shtml); individuals with identical haplotypes were subsequently removed so that only a single representative of each haplotype remained in the dataset (N = 48 remaining taxa). This reduced dataset was also run through jModelTest 2.1.7 (Darriba et al. 2012); Bayesian information criteria (BIC) selected HKY + I as the best fit model for the data. To build ultrametric trees for bGMYC, BEAST 1.8.3 (Drummond et al. 2012) was run with the uncorrelated lognormal clock model and constant-size coalescent process tree-speciation prior for 10,000,000 generations, sampling trees every 1000 generations. To account for phylogenetic uncertainty when examining the GMYC model, the last 100 trees generated were used to run bGMYC. The analysis was run for 50,000 generations with a burnin of 40,000 generations and a thinning interval of 100. The MCMC chain was visually inspected for proper mixing and stationarity to determine the number of generations to run and to set the burnin.

Microsatellite analysis

Microsatellite genotyping

To identify areas of restricted gene flow and genetic structure within this widespread species group, 1917 female specimens were genotyped at twelve microsatellite loci using the following published PCR primers: B10, B124, B126 (Estoup et al. 1995); B96, B100, B131, B132 (Estoup et al. 1996); BT10, BL13, BT30, BT28 (Reber-Funk et al. 2006); BTMS0125 (Stolle et al. 2009). A total

of twenty microsatellite loci were tested for use with *B. ephippiatus* and *B. wilmattae*, but these twelve markers were selected for their consistent amplification across multiple *Bombus* species. The amplification of the twelve loci chosen was also tested randomly via re-genotyping a subset of samples to look for genotyping errors, none of which were detected. To examine the possible presence of null alleles in our dataset, GENEPOP 4.2 (Rousset 2008) was used to test for deviations from Hardy–Weinberg equilibrium (HWE) via heterozygote deficiency. Markov chain parameters for all tests were set to 1000 for dememorization at 100 batches with 1000 iterations per batch. We tested all populations with sample sizes greater than ten, as well as all loci. No populations deviated significantly from HWE ($P > 0.01$). Of the twelve loci selected, only locus B10 displayed heterozygote deficiency ($P = 0.0011$); its removal from the dataset did not affect population inference results. These HWE tests as well as the amplification consistency of the selected loci suggest the problem of null allleles is not a strong factor in this dataset. PCR reaction protocols and thermal cycling conditions are described in Lozier and Cameron (2009). Final PCR products with flouresecently-tagged probes were purified and genotyped at the high throughput DNA facility, W.M. Keck Center at the University of Illinois using ABI 3730xl capillary DNA analyzer (Applied Biosystems). Genotypes were scored manually with the Geneious Microsatellite Plugin 1.4 in Geneious 8.1.7 (Biomatters Ltd) using the same allele bin-set for all species. A random subset of samples was genotyped a second time to check accuracy of allele identification; no inconsistencies were observed. For consistency between datasets, all of the microsatellite genotypes for *B. ephippiatus* and *B. wilmattae* taken from Duennes et al. (2012) were re-scored with the Geneious Microsatellite Plugin 1.4 in Geneious 8.1.7 (Biomatters Ltd) for this study (Online Resource 1). The same thermocyclers and sequencing machines were used for amplification and sequencing in this study as in Duennes et al. (2012).

Population differentiation

All microsatellite data were tested for population differentiation using STRUCTURE 2.3.4 (Pritchard et al. 2000). Multiple analyses were run with different groups of samples because it is known that the assignment of individuals to genetic groups by STRUCTURE can be strongly influenced by sample size (Kalinowski 2011). The following groups of samples were assessed for the following K values: all samples ($N = 1917$; $K = 2–7$), only the samples that have also been sequenced for the COI fragment ($N = 225$; $K = 2–7$), equal sample sizes for each region (Sierra Madre Occidental, Sierra Madre Oriental, Trans-Mexican Volcanic Belt, Sierra Madre del Sur, two for the sympatric groups south of the IT through Honduras, Costa Rica; $N = 119$; $K = 2–7$), only the samples from the Mexican state of Chiapas, Guatemala, and Honduras ($N = 664$). All analyses were run with the model parameter defaults (admixture model with allele frequencies correlated among populations and no prior sample information) with a burnin of 150,000 generations followed by an additional 150,000 generations. Between three to six independent runs were conducted for each value of K tested. While the ΔK method (implemented in STUCTURE HARVESTER; Earl and vonHoldt 2012) was used to assess the optimal K value (Evanno et al. 2005) for each dataset, other values of K that corresponded to potential geographic barriers or biologically relevant factors were also considered and are reported below.

The Bayesian assignment program GENELAND 4.0.5 was also used to assess population structure (Guillot et al. 2012). Six analyses (Table 1) designed to test the influence of geographic and morphometric data on the program's assignment of individuals to distinct genetic groups were run with the following parameters for 1,000,000 iterations with every 1,000th iteration saved: maximum rate of Poisson process at 100; uncertainty on spatial coordinates of 1; uncorrelated allele frequency, null allele and spatial models. Ten independent runs were computed for each analysis for K values from one to ten. The iteration with the highest log likelihood after successive burnin values of 100, 200, 300, 400 and 500 was chosen as the best fit to the data and the MCMC plot for that iteration was independently examined to determine stationarity and burnin for the results.

Geometric morphometric analysis

Imaging and landmark mapping

The left forewing was removed and mounted in glycerol on glass slides with glass slipcovers. All slide-mounted wings were imaged at 10X with a Leica microscope camera (DFC425) in Leica Application Suite 3.8.0. After the order of specimen images was randomized using tpsUtil 1.64 (Rohlf 2015b), the coordinates of twenty landmarks on the forewing (Online Resource 2) were mapped on the images using tpsDIG2.22 (Rohlf 2015a). C. Petranek removed, mounted and imaged all wings and mapped all landmark coordinates to ensure that landmarks were placed in the same location and to avoid researcher bias in the mapping of the landmarks.

MorphoJ analyses

The program MorphoJ1.06d (Klingenberg 2011) was used for all post-processing analyses of the landmark coordinate

Table 1 Summary of the six analyses run in GENELAND

	DNA	GPS	MORPH	N MEX	NUC CA	N	burnin	K (logLk)
1	+	+	+	+	+	606	300	8 (81,607.2106)
2	+	+		+	+	606	200	8 (−24,524.6806)
3	+	+	+	+		281	200	3 (40,686.0608)
4	+	+		+		1229	450	5 (−42,727.4268)
5	+	+	+		+	310	200	5 (40,414.5477)
6	+	+			+	624	300	6 (−26,724.7870)

If a "+" is indicated under "GPS," a spatial dataset was included in the analysis that consisted of the GPS coordinates for each specimen in UTM format. If "+" is indicated under "MORPH," a phenotype dataset was included in the analysis that consisted of the regression residuals of the 20 Procrustes superimposed landmark coordinates for each sample. "N" indicates the total sample size for each analysis and "burnin" indicates the number of iterations discarded before the MCMC chain reached stationarity (we assessed this individually for each analysis). Under "K (logLk)" are the K values for the replicates with the highest log likelihood values with their corresponding log likelihood values (after burnin) in parentheses

dataset. A Procrustes fit was performed to scale, rotate and superimpose landmark data across specimens, thereby eliminating all variation due to non-shape differences among individuals. A regression was then conducted with Procrustes coordinates (representing shape) as the dependent variable and log centroid size (an approximation of size) as the independent variable. To control for any size-based shape variation (allometry), the residuals from this regression were used for further analysis. To test whether wing shape variation corresponded to genetic differentiation, a canonical analysis of variance (CVA) was conducted. Each specimen was assigned to a haplotype group based on its genotypic assignment in the STRUCTURE analysis (>50% assignment to a STRUCTURE-determined K group using the K = 6 result from the analysis of the entire population genetic dataset [N = 1917]) and these groups were used as the classifier variables for the CVA. A permutation test for 1000 iterations was run to test the significance of the difference in mean shape between haplotype groups. Leave-one-out cross validation tests were also conducted on all pairwise comparisons of groups via discriminant function analysis (DFA) to assess the reliability of assignment of individuals to a haplotype group based on wing shape. CVA and DFA differ in that the CVA examines the relative separation of groups using the pooled variance -covariance matrix, while DFA examines the separation of each pair of groups using only the covariance matrices of the two groups in question for each comparison. CVA therefore focuses on the degree to which groups differ from one another, while DFA focuses on the degree of difference between groups and the probability of correct group membership for each specimen. Although CVA and DFA are robust to unequal sample size when the number of variables is high (in this case, 20 landmarks), the sample size of the Costa Rican lineage (N = 17) was much smaller than the sample size for all other haplotype groups, so CVA was also run on a randomly reduced dataset in which each haplotype group had an equal sample size of N = 17.

Results

COI phylogenetic relationships and concordance with genotypic divergences

Bayesian analysis of *Bombus ephippiatus* and *B. wilmattae* COI sequence data (Fig. 2a; Online Resource 3) recovers six major polytomous clades. All *B. ephippiatus* from north of the IT and a subset of *B. ephippiatus* from south of the IT to Honduras form one clade (Fig. 2a; clade i), with the Honduran *B. ephippiatus* and Mexican *B. ephippiatus* north of the IT basal to the Mexican samples south of the IT and Guatemala (Fig. 2a; clade ii). Five additional clades are recovered (Fig. 2a; clades iii–vii), each structured by species and geographic distribution.

The parsimony haplotype network of the COI sequence fragment data (Fig. 3) is highly congruent with the Bayesian phylogeny. All strongly supported clades from the phylogeny correspond with distinct haplotype groups in the network (Fig. 3). The most divergent lineage in the network is the group from Costa Rica, with four steps separating it from a haplotype of *B. wilmattae* (Fig. 3). Clade iii, iv and v from the phylogeny are also distinct in the haplotype network (Fig. 3). Differences between groups in the network are also reflected in the pairwise F_{ST} (Online Resource 4) between clades and the average number of nucleotide differences between and among clades in the phylogeny (Online Resource 5). Both distance measures demonstrate high divergence of Costa Rican individuals.

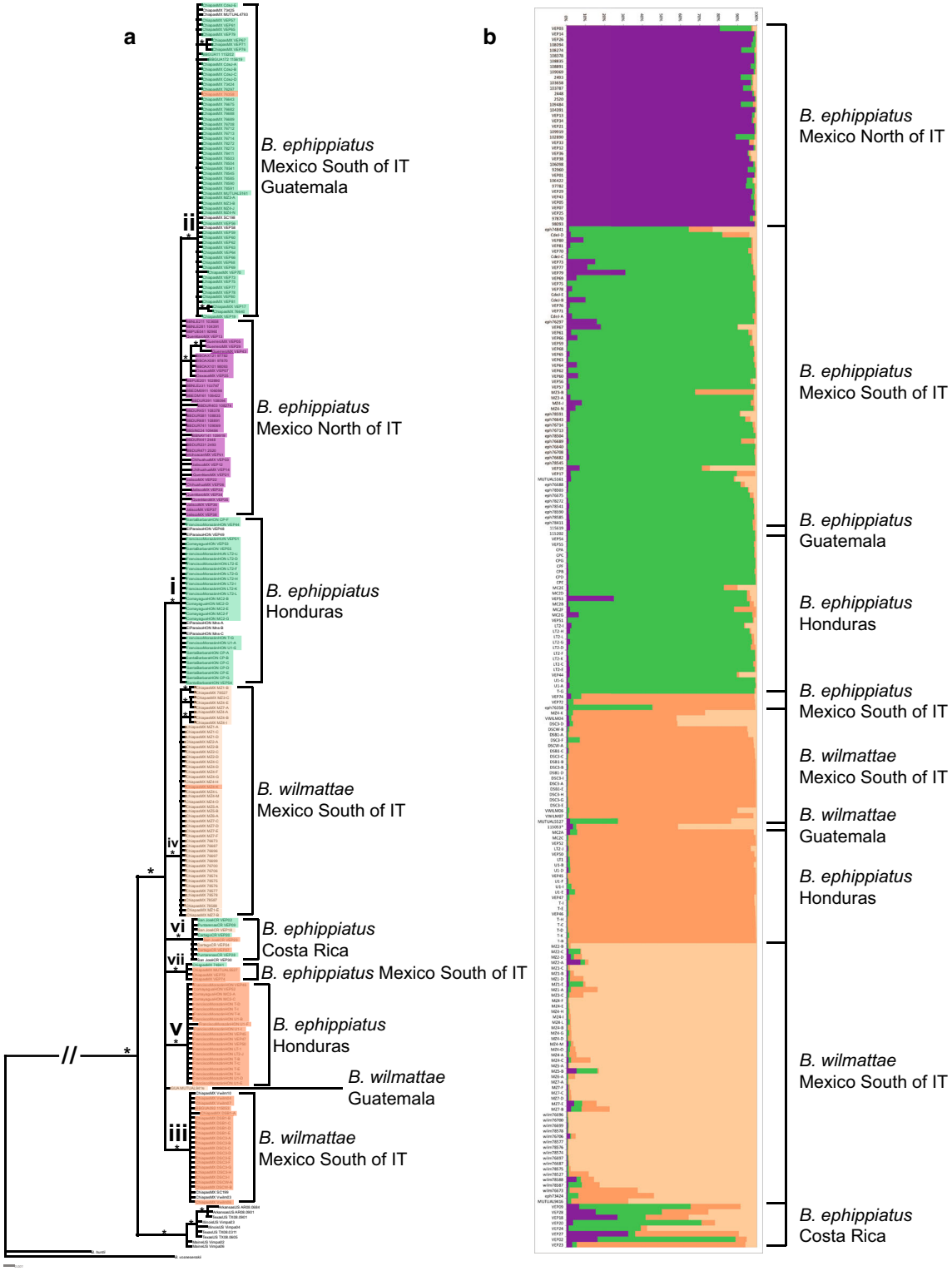

Fig. 2 **a** Bayesian phylogeny of 811 bp of the COI locus for 254 samples of *B. ephippiatus*, *B. wilmattae* and their sister species. All nodes in the phylogeny are colored according to their assignment in **b** STRUCTURE analysis of 12 microsatellite loci from the same samples. Individuals in the STRUCTURE graph are *ordered* and labeled by species designation and geographic location. (Color figure online)

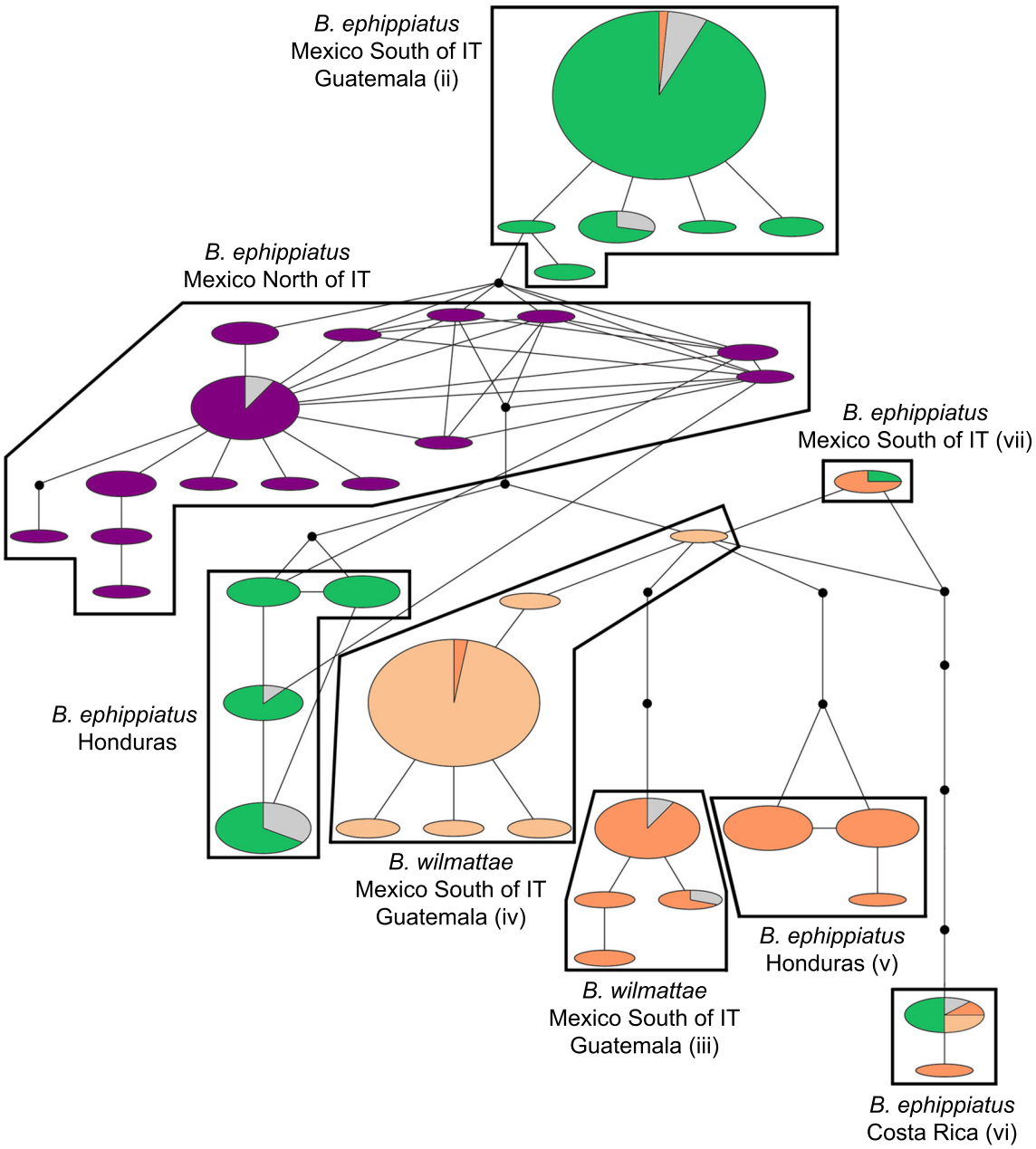

Fig. 3 Parsimony haplotype network of 811 bp of the COI locus for samples of *B. ephippiatus* and *B. wilmattae*. The size of the *circles* is relative to the number of samples possessing each haplotype and *black dots* along connection lines represent steps. Each haplotype is colored to the assignment of each sample in the STRUCTURE analysis of 12 microsatellite loci from the same samples presented in Fig. 2. *Grey-shaded* areas represent samples with COI sequence data that lack microsatellite data. (Color figure online)

High divergences also separate the different sympatric clades from south of the IT to Honduras (Online Resource 4; Online Resource 5).

A STRUCTURE analysis of microsatellite genotype data for samples that were also sequenced for COI (Fig. 2b) was also conducted for a direct comparison of the COI phylogeny (Fig. 2a) and haplotype network (Fig. 3). Using ΔK criteria, $K = 4$ is the best fit to the genotype data (Online Resource 6). The genetic groups recovered from this analysis are largely congruent with the results of the Bayesian phylogeny and the haplotype network, and correspond to geographic subclades (Figs. 2, 3). The only strong incongruence between the datasets is the mixed assignment of the Costa Rican individuals in the STRUCTURE analysis. While STRUCTURE could not assign the Costa Rican samples to a single K cluster, they all belong to the well-supported clade vi in the phylogeny (Fig. 2) and fell out as a distinct group in the haplotype network (Fig. 3).

Species delimitation with bGMYC

Outgroups (*B. huntii*, *B. vosnesenskii*, and *B. impatiens*) are assigned low posterior probabilities of belonging to the same species (PP = 0.00–0.50; Online Resource 7). Phylogenetic clades ii–vii receive low/moderate probability of being conspecific (PP = 0.50–0.90; Online Resource 7) when compared to each other. Within clade i not all taxa are assigned to the same species (Online Resource 7). *B. ephippiatus* from Mexico North of the IT are all assigned to the same species as each other, but eight taxa from that region area also assigned to the same species as clade ii, while the other seven taxa area assigned low/moderate probability of belonging to the same species as clade ii (Online Resource 7). The uncertain species determination of the taxa in clade i coincides with their polytomous placement within the clade in the Bayesian phylogeny (Fig. 2) as well as their multiple connections within the haplotype network (Fig. 3).

Genetic groups inferred from STRUCTURE analyses of the microsatellite genotype data

Using the ΔK criteria, K = 5 was the best fit for the complete genotype dataset (n = 1917) (Online Resource 8). The K groups assigned are highly congruent with geographic location. Cluster A (Fig. 4) predominates throughout the Sierra Madre Occidental, but also occurs in high frequency in samples from the western region of the Trans-Mexican Volcanic Belt where these two mountain ranges overlap (Fig. 4). Cluster B (Fig. 4) is found throughout the eastern side of Mexico in the Sierra Madre Oriental, the Sierra Madre del Sur and the eastern region of the Trans-Mexican Volcanic Belt. While the samples from the Trans-Mexican Volcanic Belt share genotypes with mountain ranges in the far western and eastern portions of the region, a distinct cluster C (Fig. 4) occurs in the middle of the range. The genetic structure presented in this analysis corresponds to the mountains of Mexico, but there are signatures of gene flow in samples where these mountain chains connect and overlap (Fig. 4).

The genotypic composition of *B. ephippiatus* and *B. wilmattae* in Nuclear Central America (Nuc CA) and south of the IT through Honduras was distinct from populations north of the IT (Fig. 4). Two distinct sympatric clusters occur within Nuc CA: one that includes *B. ephippiatus* from south of the IT through Honduras (Fig. 4, cluster D) and another that includes *B. ephippiatus* from Mexico south of the IT and Honduras and all samples of *B. wilmattae* from south of the IT (Fig. 4, cluster E).

Cluster E (Fig. 4) separates into two groups in the smaller STRUCTURE analysis of samples with COI data (Fig. 2b) and is placed in multiple lineages in the COI phylogeny (Fig. 2a); the larger analysis of all samples identifies one panmictic cluster at higher values of K. While ΔK criteria selected K = 5 for this dataset, K = 6 suggests further genetic structure corresponding to the mountain ranges of Mexico (Online Resource 9); a new cluster (cluster F) is revealed that corresponds to the Sierra Madre Oriental and the eastern sides of the Trans-Mexican Volcanic Belt and the Sierra Madre del Sur. The net nucleotide distances between the clusters at K = 6 (Online Resource 10) imply that cluster E (*B. wilmattae* and *B. ephippiatus* from Nuc CA) and cluster D (*B. ephippiatus* from Nuc CA) are most divergent from the populations north of the IT. The northwestern Sierra Madre Oriental (cluster A) and the southern Sierra Madre del Sur (cluster B) also exhibit high differentiation (Online Resource 10).

Both the large analysis and the smaller analysis using samples with COI sequence data (Fig. 2b) did not always assign Costa Rican individuals to a single cluster, so a separate analysis with all sample sizes equal to that for Costa Rica (N = 17 for each of the seven main regions; see Methods; Online Resource 11) was run. At successive K values from 2 to 7, Costa Rica is defined as a distinct lineage from K = 5–7 (Online Resource 11). For K = 2–4, Costa Rica is grouped with the samples from Nuc CA (Online Resource 11).

A separate analysis with the samples from Nuc CA was also run (N = 664; Online Resource 12). Across K values, cluster D from the larger analysis remains the same, but cluster E separates into smaller groups (Online Resource 12). The results from K = 2 mirror those of the analysis containing all (N = 1917) samples; the samples separate into the same two clusters from the larger dataset. At K = 3, cluster E is separated into one group of *B. ephippiatus* and *B. wilmattae* from Chiapas and a second group of *B. wilmattae* from Chiapas and Guatemala and *B. ephippiatus* from Honduras (Online Resource 12). At K = 4, cluster E split into one group of *B. ephippiatus* from Chiapas, a group of *B. wilmattae* from Chiapas and a third group of *B. wilmattae* from Chiapas and Guatemala and *B. ephippiatus* from Honduras (Online Resource 12). At K = 5, cluster E is split into four groups: a group of *B. ephippiatus* from Chiapas, a group of *B. wilmattae* from Chiapas, a group of *B. wilmattae* from Chiapas and Guatemala and a fourth group of *B. ephippiatus* from Honduras (Online Resource 12; Online Resource 13).

Genetic groups inferred from the GENELAND analyses of the microsatellite genotype data

The analysis of all samples with wing morphometric data and genotype data (scenario 1 in Table 1) reveals eight distinct genetic clusters (Fig. 5). Two groups are differentiated north of the IT; the samples from the Sierra Madre

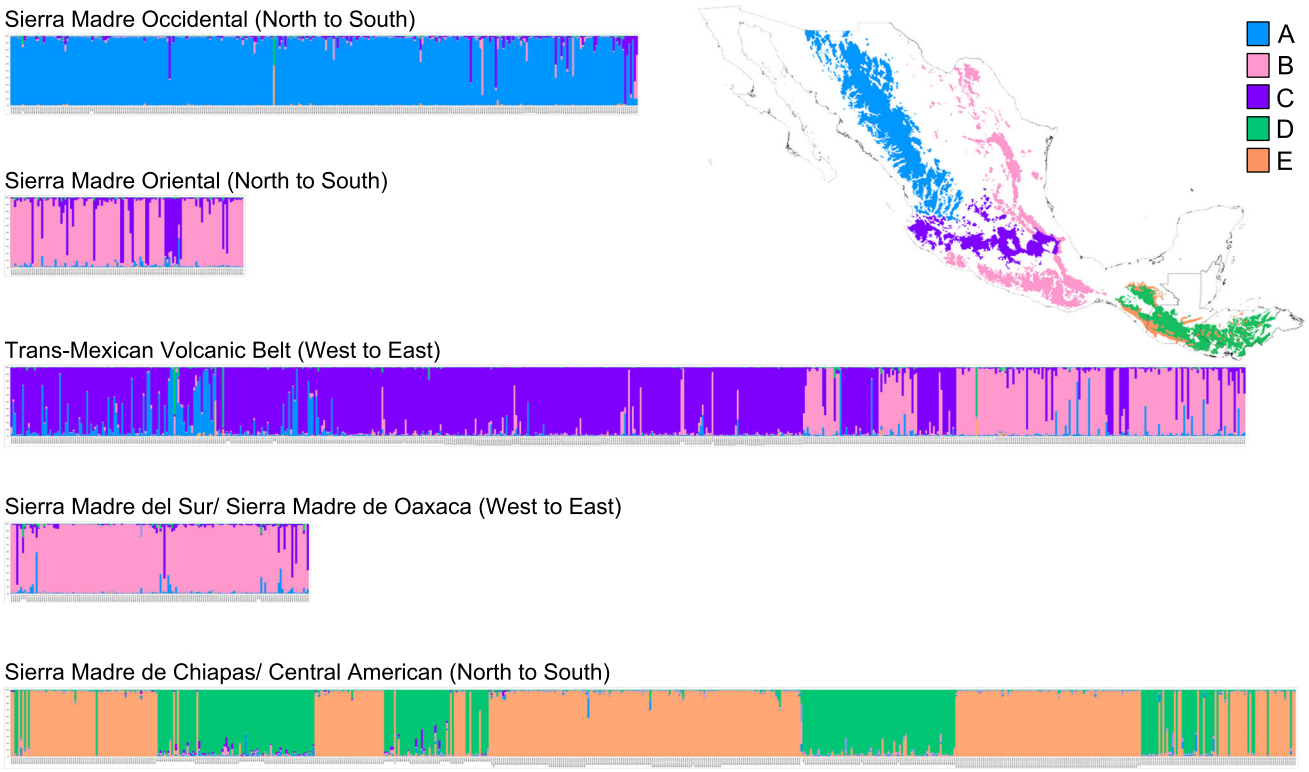

Fig. 4 Results from the K = 5 STRUCTURE analysis of the microsatellite genotype data for all samples. Samples are separated by mountain range and ordered either North to South or East to West depending on the orientation of the mountain range. The *inset* map has WWF (Olson et al. 2001) ecoregions *colored* according to the genetic cluster assignment of the majority of samples present within each region. (Color figure online)

Occidental separate into a cluster (8) distinct from the rest of Mexico north of the IT (4; Fig. 5). A single distinct group is assigned to the individuals from Costa Rica (7; Fig. 5). In Nuc CA, five groups are assigned to the samples (1, 2, 3, 5, 6; Fig. 5). When compared to the STRUCTURE analysis of all samples, GENELAND differentiates less genetic groups north of the IT and more genetic groups south of the IT (Online Resource 14).

When the morphometric data are excluded from the analysis (scenario 2 in Table 1; Online Resource 15), the results are largely congruent with those that include morphometric data, with some minor differences (Online Resource 16). Because the results of scenario 1 and 2 are similar, the pairwise comparison of scenario 2 to the STRUCTURE analysis (Online Resource 17) yield similar results, with STRUCTURE identifying more groups north of the IT and fewer groups south of the IT (Online Resource 17).

GENELAND analyses of the samples from north of the IT with morphometric data (scenario 3) and without morphometric data (scenario 4) reveal three and five groups, respectively (Table 1), which correspond to geography. In scenario 3 (Online Resource 18), Sierra Madre Oriental, the Trans-Mexican Volcanic Belt and the eastern edge of the Sierra Madre del Sur form a group, the Sierra Madre del Sur and southeastern Trans-Mexican Volcanic Belt form a group, and the Sierra Madre Occidental is a distinct group. These groups also correspond to the same regions identified by STRUCTURE (Online Resource 19). When morphometric data are excluded (scenario 4), five genetic groups are identified by GENELAND (Online Resource 20). Group 1 occurs in the Sierra Madre del Sur, group 2 occurs in the Sierra Madre Oriental through to the eastern Sierra Madre del Sur, group 3 occurs in the Sierra Madre Occidental, group 4 occurs in the western Trans-Mexican Volcanic Belt and southern Sierra Madre Occidental and group 5 occurs in the Trans-Mexican Volcanic Belt (Online Resource 20). These groups are also highly congruent with the STRUCTURE clusters assigned to these samples (Online Resource 21).

When the samples from Nuc CA are analyzed separately, five groups are identified with morphometric data (scenario 5; Online Resource 22) and 6 groups are identified without morphometric data (scenario 6; Online Resource 23). With the exception of a few outlier samples, the five groups identified with morphometric data largely correspond to the five STRUCTURE groups identified in the analysis of the Nuc CA samples (Online Resource 24;

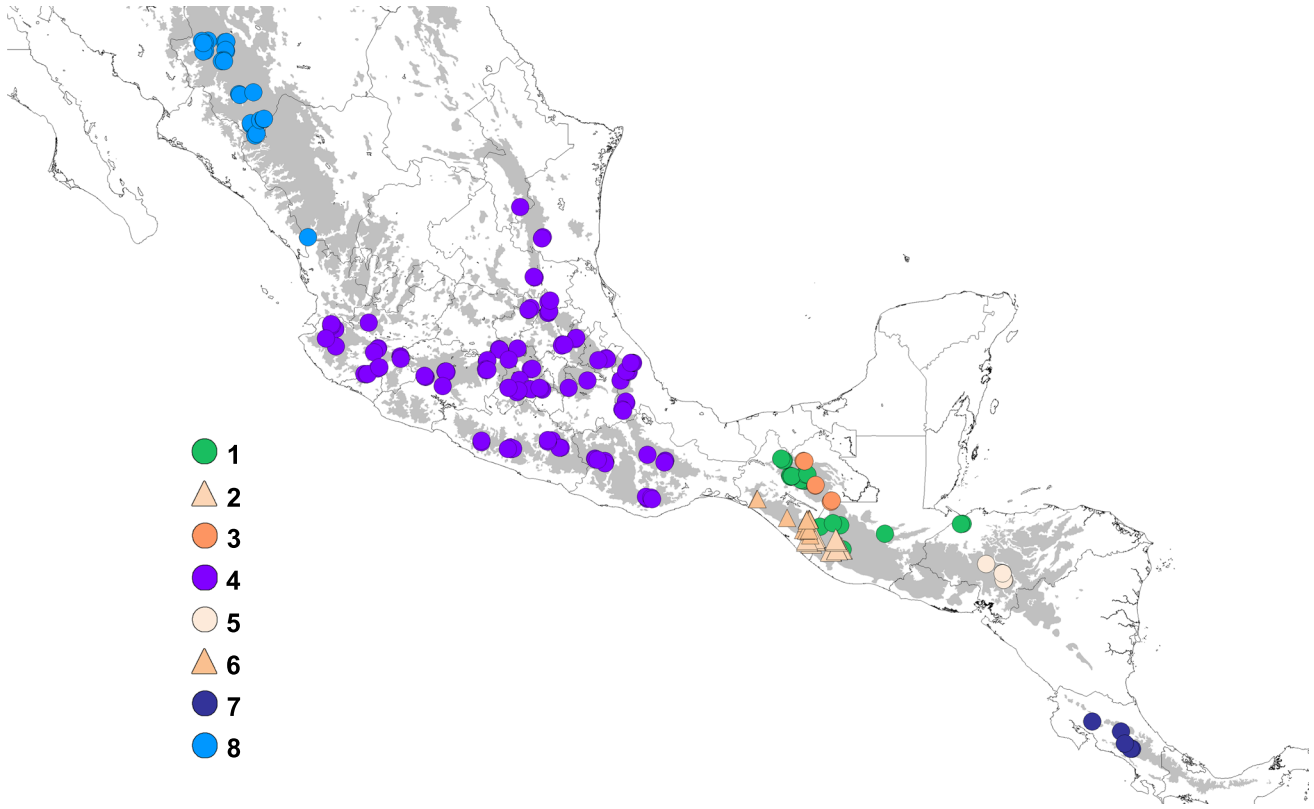

Fig. 5 Map displaying the geographic distribution of each genetic lineage identified by GENELAND for scenario 1 (Table 1). *Grey-shaded* areas of the map represent WWF (Olson et al. 2001) ecoregions in which the species can be found. (Color figure online)

Online Resource 12). When morphometric data are excluded (scenario 6; Online Resource 23), a sixth group is added and the distribution of samples among groups corresponds less so to the STRUCTURE results (Online Resource 25).

Morphological divergence inferred from wing morphometric data

A CVA of all morphometric data (N = 606) with samples classified by their genotype assignment reveals that wing shape difference between each pair of haplotype groups is statistically significant (Online Resource 26) and 67.36% of the variation in the data is explained in the first two canonical variates (Online Resource 27). All samples are correctly identified to their pre-assigned group via pairwise DFA except when cluster B (Sierra Madre del Sur) is compared to cluster F (Sierra Madre Oriental) (Online Resource 26). Costa Rica is most divergent in wing shape from all other haplotype groups (Online Resource 26; Online Resource 27). The Nuc CA lineage with both *B. ephippiatus* and *B. wilmattae* (cluster E) is the second most different from all groups (Online Resource 26; Online Resource 27). Of the three haplotype groups south of the IT, the Nuc CA group (cluster D) containing *B. ephippiatus* is the least different in wing shape from the samples north of the IT (Online Resource 26; Online Resource 27), which also reflects its close relationship to the group north of the IT, as shown in the COI phylogeny (Fig. 2a). The Sierra Madre Oriental and the Sierra Madre del Sur are the least different in wing shape (Online Resource 26). This close relationship is also reflected in the STRUCTURE analyses (Online Resource 10).

The CVA with equal samples sizes (N = 17 for each haplotype group; N = 119 total) demonstrates the same patterns seen in the larger dataset, with 72.55% of the variation in the data explained by the first two canonical variates (Table 2; Fig. 6). The only substantial difference between the results of the analyses is that no pairwise comparison between haplotypes north of the IT has a greater Mahalanobis distance than the comparisons between haplotypes south of the IT (Table 2). In the larger analysis, the Mahalanobis distance between the most northern (Sierra Madre Occidental) and the most southern (Sierra Madre del Sur) haplotypes north of the IT is greater than any comparison of the Nuc CA group (cluster D) containing *B. ephippiatus* to any haplotype group north of the IT (Table 2).

Table 2 Pairwise Mahalanobis distances between haplotypes groups calculated by MorphoJ for equal sample size haplotype groups (N = 17 for each haplotype group, N = 119 total)

Mahalanobis distance	A	B
8.4982[a]	Sierra Madre del Sur	Costa Rica
8.3784[a]	Sierra Madre Oriental	Costa Rica
8.1556[a]	Trans-Mexican Volcanic Belt	Costa Rica
7.2092[a]	Sierra Madre Occidental	Costa Rica
6.3474[a]	Nuc CA (eph/wilm)	Costa Rica
6.1282[a]	Sierra Madre del Sur	Nuc CA (eph/wilm)
6.0663[a]	Nuc CA (eph)	Costa Rica
5.9101[a]	Trans-Mexican Volcanic Belt	Nuc CA (eph/wilm)
5.7076[a]	Sierra Madre Oriental	Nuc CA (eph/wilm)
5.3348[a]	Sierra Madre Occidental	Nuc CA (eph/wilm)
5.165[a]	Nuc CA (eph)	Nuc CA (eph/wilm)
4.9026[a]	Sierra Madre Occidental	Nuc CA (eph)
4.7405[a]	Sierra Madre del Sur	Nuc CA (eph)
4.6114[a]	Trans-Mexican Volcanic Belt	Nuc CA (eph)
4.3754[a]	Sierra Madre Oriental	Nuc CA (eph)
4.3493[a]	Sierra Madre del Sur	Sierra Madre Occidental
3.9552[a]	Trans-Mexican Volcanic Belt	Sierra Madre del Sur
3.8866[a]	Sierra Madre Oriental	Sierra Madre Occidental
3.3786[a]	Trans-Mexican Volcanic Belt	Sierra Madre Occidental
3.2525[a]	Trans-Mexican Volcanic Belt	Sierra Madre Oriental
2.3874[b]	Sierra Madre del Sur	Sierra Madre Oriental

Comparisons are ordered from most to least different

Nuc CA Nuclear Central America, eph, *B. ephippiatus,* wilm, *B. wilmattae*

[a] The permutation test P value was < 0.0001

[b] The permutation test P value was < 0.05

Discussion

Utility of methods for species delimitation

Mitochondrial and nuclear sequence data have been widely used to examine *Bombus* genetic diversity worldwide (Koulianos and Schmid-Hempel 2000; Kawakita et al. 2004; Hines et al. 2006; Cameron et al. 2007; Williams et al. 2012, 2015), and also regionally in Europe (Ellis et al. 2005; Lecocq et al. 2011; Carolan et al. 2012; Lecocq et al. 2013, 2015a, b, c; Bossert et al. 2016), Asia (Williams et al. 2011; Hines and Williams 2012; Huang et al. 2015), South America (Santos Júnior et al. 2015; Françoso et al. 2016),) and North America (Cameron and Williams 2003; Sheffield et al. 2016). Population, landscape, and conservation genetic studies of *Bombus* (Europe: Chapman et al. 2003; Ellis et al. 2006; Darvill et al. 2006, 2010; Herrman et al. 2007; Kraus et al. 2009, 2011; Charman et al. 2010; Goulson et al. 2011; Carvell et al. 2012; Maebe et al. 2013; Dreier et al. 2014; United States: Lozier and Cameron 2009; Cameron et al. 2011; Lozier et al. 2011, 2013; Jha and Kremen 2013; Jha 2015) as well population genetics research on invasive *Bombus* species (New Zealand: Schmid-Hempel et al. 2007; Lye et al. 2011) have utilized microsatellite genotype data extensively to examine patterns of gene flow and isolation. In comparison, few studies have used sequence data and microsatellites in concert to examine species-level and population-level patterns of genetic structure (Europe: Estoup et al. 1996; Widmer et al. 1998; Widmer and Schmid-Hempel 1999; Moreira et al. 2015; Asia: Shao et al. 2004; South America: Francisco et al. 2016); a single study to date has investigated bumble bee genetic diversity in Mexico and Central America (Duennes et al. 2012).

In this study, one of our aims was to balance evidence of older divergence patterns based on sequence data with more recent patterns of gene flow and isolation using faster-evolving microsatellite genotypes to provide a more robust inference of species delineation in the *B. ephippiatus–B. wilmattae* complex. The COI phylogeny and haplotype network presented here are largely congruent with the patterns present in the microsatellite genotype data, but there is evidence of contemporary gene flow in clusters D and E and contemporary genetic isolation in Mexico north of the IT that would not have been discerned from COI sequence data alone. The species delimitation model

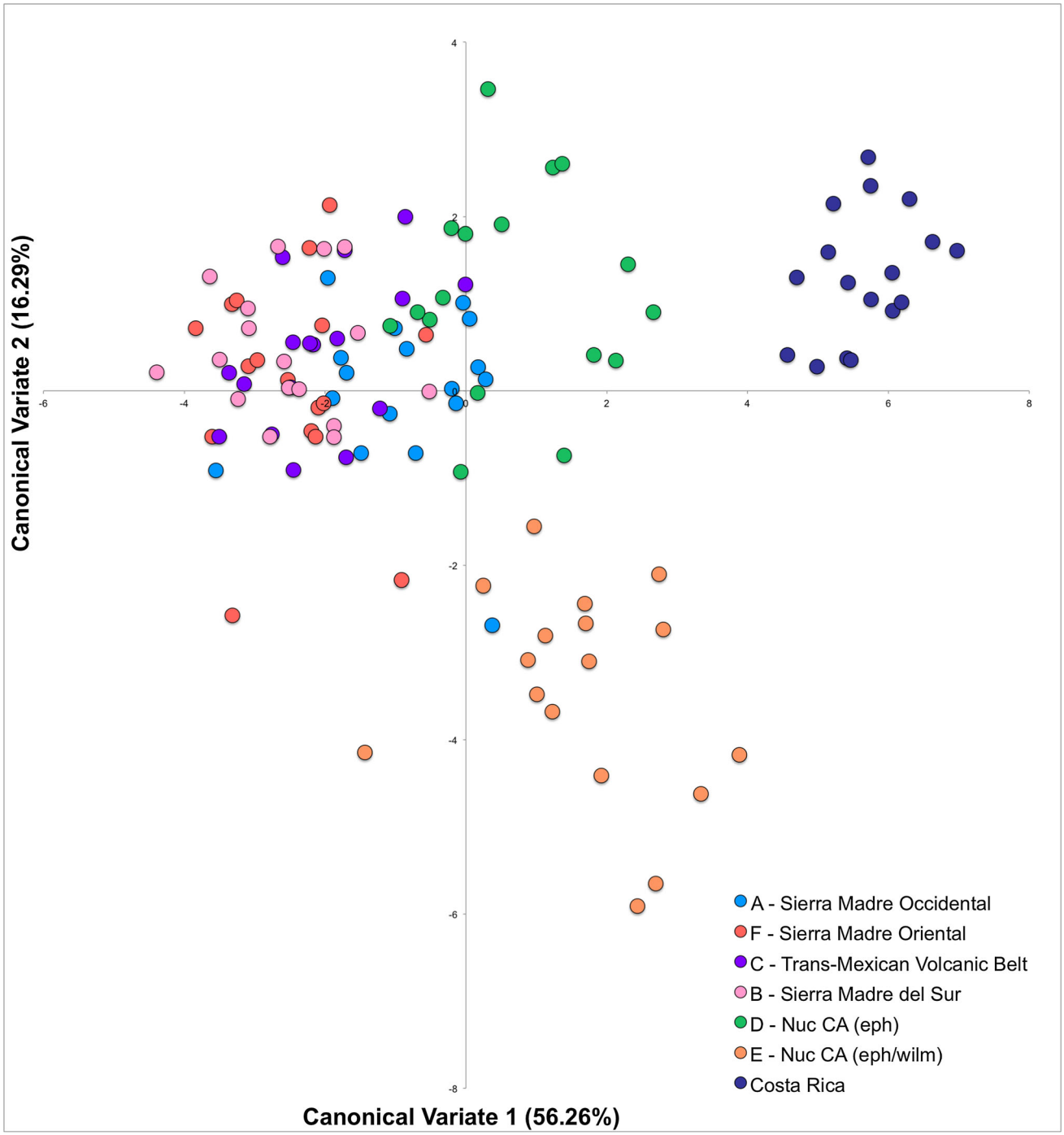

Fig. 6 Graph of the canonical analysis of variance conducted on equal samples sizes from each region (N = 119). All samples are color-coded according to their genotypic assignment by the K = 6 analysis of all samples (N = 1917). *Nuc CA (eph)* Nuclear Central America *B. ephippiatus, Nuc CA (eph/wilm)* Nuclear Central America *B. ephippiatus* and *B. wilmattae*. (Color figure online)

bGMYC assigned low posterior probabilities of conspecificity to the major clades in the COI phylogeny, but could not discern sublineages that, in separate analyses, display a complete lack of gene flow at microsatellite loci. Previous research has reported similar assignment problems with bGMYC and attributed it to introgression and incomplete lineage sorting within sequence data of butterfly taxa (Talavera et al. 2013). The recent diversification of *B. ephippiatus–B. wilmattae* (~1 mya; Duennes et al. 2012) and the conflicting inferences between sequence data and microsatellite data suggest that incomplete lineage sorting and/or introgression are confounding sequence data in this

group as well. While sequence data can be useful for delimiting bumble bee species with older divergences and species delimitation models can aid in choosing a threshold for species identification, future studies of recently diverging groups should consider independent lines of evidence (in addition to sequence data) that can provide a robust examination and estimation of contemporary gene flow between lineages (Schlick-Steiner et al. 2010).

Microsatellite genotype data provide detailed information on genetic diversity and gene flow within this complex, but we have demonstrated that caution should be taken when using genotype assignment programs to assess genetic diversity with microsatellites. The difficulty STRUCTURE had in assigning the Costa Rican samples to a single cluster, except when samples from all regions were reduced to equal those of Costa Rica, illustrates a pitfall in using the STRUCTURE algorithm to assign unequal population samples to genetic groups (Kalinowski 2011). However, GENELAND, which incorporates geospatial data, readily identifies Costa Rica as a unique genetic group in an analysis of all samples with morphometric data included and excluded. The results from GENELAND do not change notably when sample sizes are changed or samples are excluded, but fewer populations are identified by GENELAND when morphometric data are included in the analysis.

The analyses of the Nuc CA region show that STRUCTURE and GENELAND can differ substantially in their assignments. STRUCTURE consistently identifies cluster D as distinct from all other samples from Nuc CA, regardless of the K value being tested; this suggests that there is little to no gene flow between these lineages. In contrast, GENELAND does not identify cluster D as a unique lineage, suggesting admixture throughout Nuc CA. When a map of the groups assigned by GENELAND is compared to a map of the STRUCTURE assignments, it is clear that GENELAND weights geographic proximity much greater than genotype when assigning samples to groups. The ability to include spatial and phenotypic information suggests that GENELAND provides a more holistic picture of evolutionary history, yet here GENELAND appears to prioritize geospatial data over genetic data when multiple cryptic, sympatric lineages are included (i.e. populations deviate from Hardy–Weinberg equilibrium).

This study also aimed to include non-molecular methods as additional evidence towards the delineation of unique groups. Discriminant function analysis of landmark data indicates significant differences in wing shape between all but one pair of genetic lineages and returned high hit ratios for cross-validation tests. While one can use discriminant analyses such as CVA and DFA to reliably differentiate between user-specified groups, these tests can detect significant differences even when the magnitude of morphological shape difference is miniscule, potentially supplying weak information for phylogenetic inference. Lecocq et al. (2015b) implemented a non-discriminant analysis (Schlick-Steiner et al. 2010) of bumble bee wing morphometric data to avoid the bias implicit to designating a priori species hypotheses, but the results suggested that all lineages within the *Bombus lapidarius* species complex were not distinguishable by wing shape. The results of Lecocq et al. (2015b) could therefore be explained by convergent or stabilizing selection on wing shape (Dockx 2007). However, the efficacy of non-discriminant methods should be further explored by examining wing shape divergence in other well-supported groups of bumble bees, as analyzing landmark data without a priori hypotheses may lack the statistical rigor to tell groups apart (Mutanen and Pretorius 2007).

Biogeographic patterns

When the results of our three independent datasets are considered as a whole, the *B. ephippiatus* lineage in Costa Rica is highly divergent from the rest of the complex. Analyses of all datasets identified the Costa Rican samples as a distinct group, with the exception of some STRUCTURE analyses. The strong differentiation of the Costa Rican lineage suggests that the Nicarguan Depression is an important isolating mechanism for this species complex, as has been demonstrated in several vertebrate taxa (Castoe et al. 2009; Daza et al. 2010; Gutiérrez-García and Vázquez-Domínguez 2013).

For such a small region, Nuclear Central America (south of the IT and north of the ND) contains a remarkable amount of genetic diversity. The volcanoes and mountains chains in this region have generated a mosaic of unique habitats across elevations, which in turn led to high levels of diversification for many taxa (Strecker et al. 2004; Crawford and Smith 2005; Gutiérrez-García and Vázquez-Domínguez 2012; Suárez-Atilano et al. 2014; Pérez and Vázquez-Domínguez 2015). The separation of clade ii Chiapas and Guatemala samples from the Honduras samples at the base of clade i, despite being placed into the same K cluster in the STRUCTURE analysis of the genotype data, suggests that this might be a case of secondary contact in which the ancestral distribution arose south of the IT, dispersed into northern Mexico and then dispersed south, back across the IT, introgressing with the ancestral population in the mid-elevation Central American pine-oak forests (Fig. 1). The large amount of COI genetic diversity (also present in the microsatellite data) in the four groups comprising cluster E provides further support for the hypothesis that these bees have been in Nuc CA much longer than the taxa comprising cluster D. These lineages,

which correspond to the high elevation Chiapas montane forest, Sierra Madre de Chiapas, and Central American montane forest ecoregions (Fig. 1), might even represent the ancestral range of the group with retention of ancestral polymorphism. The cluster E subgroups also show that the Central Depression might restrict gene flow between populations, a result that could not have been discerned from the smaller sampling of Duennes et al. (2012). Divergence estimates from Duennes et al. (2012) suggest that both of these possible scenarios could have occurred during Pleistocene glacial cycles; studies across animal and plant taxa inhabiting montane regions of Mexico and Central America suggest multiple dispersal events across the IT have occurred during the late Pliocene and Pleistocene (Barber and Klicka 2010; Ornelas et al. 2013).

Bombus ephippiatus in Mexico north of the IT also contain substantial genetic differentiation that corresponds with the elevation barriers imposed by the mountain ranges of this region. In contrast to Nuclear Central America, pine-oak forests are continuous throughout north Mexican mountains and converge with other ranges via subalpine forests. Gene flow between these distinct populations is greatest where the separate mountain chains meet each other; this pattern has been found in other taxa within the region (Ornelas et al. 2013; Mastretta-Yanes et al. 2015). While geographic distance seems to have a strong impact on differentiation in northern Mexico where mountain chains and habitat are more continuous, habitat and elevation seem to be a more important isolating mechanism in Nuclear Central America.

While some population genetic studies of *Bombus* have found less genetic variation across much larger geographic areas (Lozier et al. 2011; Moreira et al. 2015; Francisco et al. 2016), other research reveals population structure within bumble bee species. Multiple studies suggest that oceanic barriers between island and mainland populations have facilitated genetic differentiation within bumble bees (Widmer et al. 1998; Shao et al. 2004; Darvill et al. 2006, 2010; Kraus et al. 2009; Charman et al. 2010; Lye et al. 2011; Goulson et al. 2011; Lozier et al. 2011; Hines and Williams 2012; Jha 2015; Moreira et al. 2015; Lecocq et al. 2015c; Williams et al. 2015; Francisco et al. 2016). Like the lineages in this complex (Duennes et al. 2012), many bumble bee species also exhibit genetic differentiation corresponding to unique eco-regions across latitudinal/longitudinal and elevational space, implying that mountainous regions in concert with glacial refugia are responsible for generating this diversity (Widmer and Schmid-Hempel 1999; Hines and Williams 2012; Lecocq et al. 2013, 2015; Lozier et al. 2013; Santos Júnior et al. 2015; Françoso et al. 2016; Sheffield et al. 2016).

Conservation implications

Upon the discovery of cryptic diversity, species once thought to have wide distributions and to be of least concern for conservation can actually comprise multiple species with contracted ranges and small population sizes that are in need of conservation (Bickford et al. 2007; Funk et al. 2012; Niemiller et al. 2012, 2013). Currently, *B. ephippiatus* is listed as "least concern" by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (*B. wilmattae* was considered conspecific for this assessment; Duennes and Vandame 2015). In light of the genetic and morphometric patterns we see here, a taxonomic revision of this species complex will be important, as well as a subsequent conservation status assessment: a reassessment for the IUCN Red List could result in different risk categorizations for each species within this complex. We strongly encourage phylogenetic and population genetic studies of the other seventeen species (Labougle 1990) present in Mexico and Central America to develop more accurate and effective conservation assessments for these important native pollinators inhabiting fragile cloud forest habitat (Ornelas et al. 2013).

Within Mexico alone, twelve independent companies across five different states are rearing *B. ephippiatus* for commercial pollination of greenhouse crops (Asociación Mexicana de Criadores de Abejorros Nativos, A.C.). Although raising *B. ephippiatus* as a native species is a more sustainable alternative to the non-native North American *B. impatiens* currently used for commercial pollination in Mexico and Central America, the inter-regional movement of *B. ephippiatus* colonies with unique population structure could be detrimental to the other three un-described species in this complex as well as to native population diversity within northern Mexico. Not only does their movement pose the threat of facilitating the spread of potential diseases (the same *B. ephippiatus* specimens used for this study demonstrated relatively high pathogen infection compared to other Mexican *Bombus spp.;* Gallot-Lavallée et al. 2016), but commercial *B. ephippiatus* could outcompete native populations for resources and even cause genetic pollution of the native populations through interbreeding (Goulson 2010; Kraus et al. 2011; Williams et al. 2012). The results presented here demonstrate that *B. ephippiatus* exhibits substantial population structure across its range and we suggest that bombiculture companies restrict the movement of colonies to within the distinct mountain ranges of Mexico and not move colonies between ranges, thereby preserving the native diversity across the species' range.

Consequences for species delimitation

The genetic and morphometric results presented here demonstrate that a taxonomic revision of the *B. ephippiatus–B. wilmattae* complex is needed. Mexico north of the IT is clearly a distinct taxonomic unit with genetically unique populations in the Sierra Madre Occidental, Sierra Madre Oriental, Sierra Madre del Sur and the Trans-Mexican Volcanic Belt. There are at least two sympatric taxonomic units from Mexico south of the IT to Honduras, with one of these lineages containing the previously recognized *B. wilmattae* as well as substantial population structure corresponding to the Central Depression. A fourth distinct taxonomic unit inhabits Costa Rica, and presumably also Panama based on the consistent color pattern phenotype present through these regions. The results presented here highlight the importance of thorough investigations of cryptic species diversity for the conservation of native species.

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