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Geographical patterns of genetic divergence in the widespread Mesoamerican bumble bee *Bombus ephippiatus* (Hymenoptera: Apidae)

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ABSTRACT

Bumble bees (*Bombus* Latreille) are an important group of social insects, well recognized throughout northern temperate regions as important pollinators of wild and agricultural plants. Little is known about the biology of this group in southern portions of the Americas, especially in Mesoamerica, a region of geological and ecological complexity from Mexico through Central America. One ubiquitous Mesoamerican species, *Bombus ephippiatus*, is enigmatic. Like many other *Bombus*, this species is homogeneous in body structure yet exhibits striking intraspecific color pattern polymorphism across its range, leading to uncertainty about its genealogical boundaries. It has been grouped taxonomically with *B. wilmattae*, a species narrowly restricted to southern Mexico and northern Guatamala. Furthermore, the relationships between these two taxa and a third species, *B. impatiens*, found only in America north of Mexico, have been controversial. Our phylogenetic analysis of DNA sequences from mitochondrial COI and nuclear PEPCK and CAD resolves the phylogeny of these three taxa as (*B. impatiens*, (*B. ephippiatus*, *B. wilmattae*)). Additional data from eight nuclear microsatellite markers reveal complex patterns of genetic divergence and isolation among populations of *B. ephippiatus* across its extensive geographic range, providing evidence for multiple independent evolutionary lineages. These lineages correspond not only to geographic and habitat variation across their range, but also to distinct color pattern groups present in the species. Knowledge of the phylogeny and genetic divergence of the *B. ephippiatus* group will provide a framework for understanding evolutionary and ecological origins of color pattern polymorphism in bumble bees, as well as providing insight into geographical factors enhancing speciation in Mesoamerica.

1. Introduction

Bumble bees (*Bombus*) have long held the attention of naturalists in Europe and North America who were fascinated by their social behavior, cold-temperature tolerance, and charismatic color patterning (Darwin, 1859; Sladen, 1912; Plath, 1934; Heinrich, 2004). They have also received recognition as important wild pollinators (Kremen et al., 2002; Morandin and Winston, 2005; Greenleaf and Kremen, 2006; Julier and Roulston, 2009) throughout much of the temperate world, but current attention has turned to the apparent population decline of many species worldwide (reviewed in Cameron et al., 2011; Williams and Osborne, 2009). Much less is known, however, about bumble bee biology and species status in the southern regions of the Americas, in spite of the fact that numerous exotic European species including *B. ruderatus* and *B. terrestris* in Chile and Argentina (Montalva et al., 2011) have been introduced for agricultural crop pollination, and have established viable populations. These introductions have the potential to threaten the genetic and ecological integrity of native populations. Even less is known about bumble bees in Mesoamerica, the region from central Mexico through Central America that is remarkable for its geological and ecological complexity. An exotic North American species, *B. impatiens*, which is routinely exported to Mexico for greenhouse pollination, has reportedly been found in the wild (Vergara, 2008) and is known to mate in the laboratory with a native species and close relative, *B. ephippiatus* (JI Cuadriello, unpublished data).

To better understand and manage threats to bumble bee populations, including potential transmission pathways of important pathogens (Meeus et al., 2011) and genetic dilution from hybridization between native and commercially transported domesticated species (Yoon et al., 2009; Ryan et al., 2009), a thorough understanding of native species boundaries and evolutionary history will be critically important.

Historically, species determination has been difficult in *Bombus*, in part because they exhibit exceptional color pattern diversity

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within species across often wide geographic distributions as well as remarkable mimetic color pattern convergence among species within a region (Williams, 2007). Otherwise they appear morphologically similar. A recently published comprehensive molecular phylogeny of bumble bees (Cameron et al., 2007) helped to resolve the pattern of species relationships but also highlighted the need to improve our understanding of species boundaries in some lineages.

One closely related New World *Bombus* complex, with both divergent and convergent color patterns across a complicated landscape, has the potential to reveal new insights into the association between color pattern, species divergence, and biogeography. The phylogenetic relationships of the three lineages comprising this group, *B. impatiens*, *B. ephippiatus* and *B. wilmattae*, are unresolved (Hines et al., 2006; Cameron et al., 2007). Phylogenetic analysis of a five-gene dataset (Cameron et al., 2007) of the genus *Bombus* suggested that in this complex the North American *B. impatiens* was most closely related (with low support) to the Mexican–Guatemalan *B. wilmattae*, with *B. ephippiatus*, which is distributed throughout Mexico and Central America, positioned as sister group. Because support was low for these relationships we consider the complex as an unresolved polytomy.

Bombus impatiens is common throughout the Eastern half of the United States (Cameron et al., 2011), separated from *B. ephippiatus* by a narrow region in southwestern Texas (Vergara, 2008). *B. ephippiatus* is commonly found from Northwest Mexico (Chihuahua) to Panama (Labougle, 1990), and possibly as far south as the northwestern region of South America (Ecuador and Colombia, Franklin, 1913) (Fig. 1), while *B. wilmattae* is found only in higher elevation volcanic areas of southern Mexico (Chiapas) and northern Guatemala (Labougle, 1990). Given the distribution of these species one might predict that *B. ephippiatus* and *B wilmattae* would likely be sister lineages, with *B. impatiens* as sister group to them.

Unlike *B. impatiens*, *B. ephippiatus* is unique in displaying a remarkable degree of polymorphic color pattern across its range (Fig. 1). This variation historically has caused a great deal of taxonomic confusion, contributing to 11 names assigned to the lineage (Williams, 1998). While widely distributed throughout Mesoamerica, like most bumble bees, *B. ephippiatus* is restricted mostly to montane habitat. Lowland areas, particularly the Isthmus of Tehuantepec and the Nicaraguan Depression (Fig. 1), impart probable breaks in the widespread geographic distribution of this species (Franklin, 1913; Labougle, 1990). Together with the topographic complexity of the region, such breaks may result in considerable barriers to dispersal in *B. ephippiatus*. We hypothesize that the remarkable color pattern variation in this species (Fig. 1) is the result of a long history of isolation associated with barriers to dispersal, and that such patterns of isolation will also be reflected by considerable genetic diversity and structure.

The color pattern of *B. wilmattae* is very similar to that of the darker forms of *B. ephippiatus* (Fig. 1) (Williams, 2007) and its taxonomic status as a separate species has been debated over the years (Cockerell, 1912; Labougle et al., 1985). It is still questionable whether or not these species are conspecific as a result of gene topology incongruence in phylogeny (Hines et al., 2006). In light of the color pattern similarities between *B. ephippiatus* and

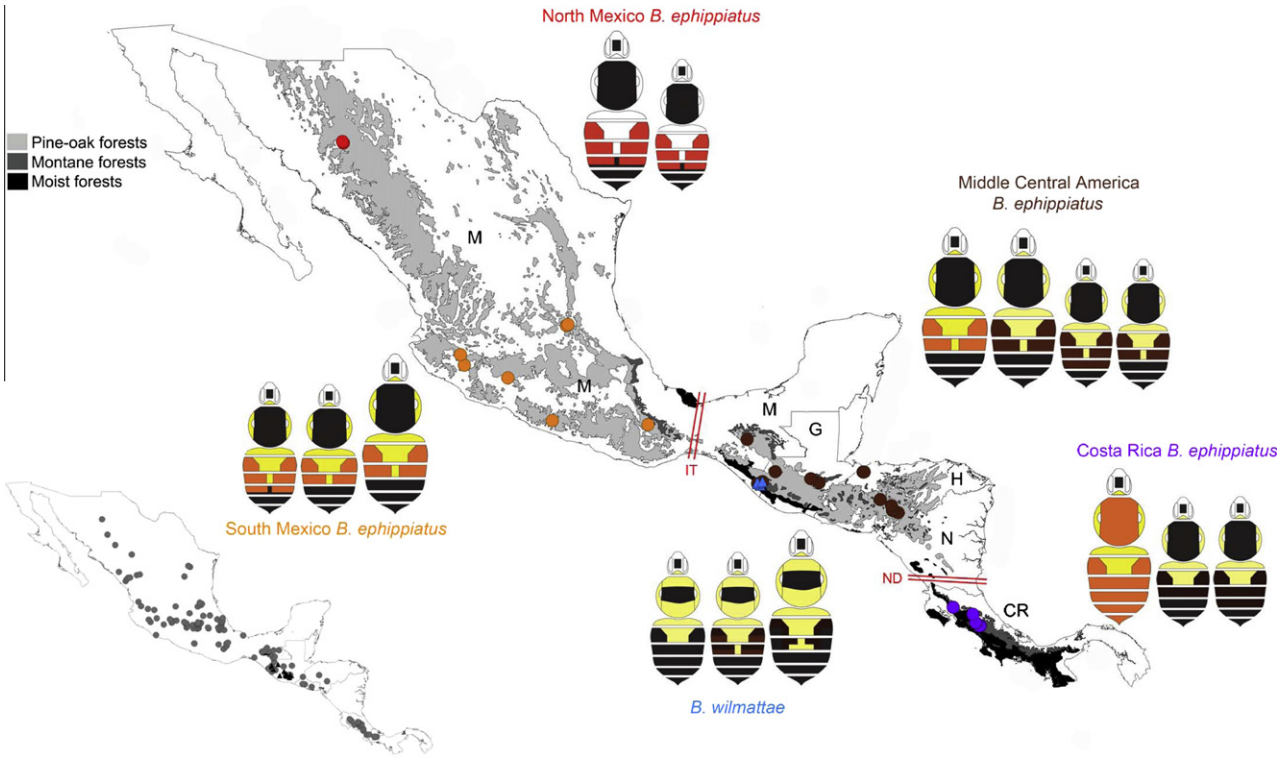

Fig. 1. Map of the collection localities and distribution of *B. ephippiatus* and *B. wilmattae* color patterns throughout the species' ranges in Mesoamerica. Countries are outlined and labeled: M = Mexico, G = Guatemala, H = Honduras, N = Nicaragua, CR = Costa Rica. The lowland areas that have caused potential isolation within *B. ephippiatus* and *B. wilmattae* are in red and indicated with double red lines labeled: IT = Isthmus of Tehuantepec, ND = Nicaraguan Depression. World Wildlife Federation (WWF) ecoregions that include the distribution of *B. ephippiatus* and *B. wilmattae* (Olson et al., 2001) are highlighted in shades of grey. Collection localities of *B. ephippiatus* and *B. wilmattae* are represented by circles and triangles; colors correspond to the color patterns in that region: red circles = North Mexico *B. ephippiatus* with white, red, black color pattern; orange circles = South Mexico *B. ephippiatus* with yellow, orange, black color pattern; blue triangles = *B. wilmattae* with yellow, brown, black color pattern; brown circles = Middle Central America *B. ephippiatus* with yellow, orange, black and yellow, brown, black color pattern; purple circles = Costa Rica *B. ephippiatus* with orange, yellow and yellow, black color pattern. The larger bees represent the color patterns of queens and are adjacent to their respective distributions on the map; the smaller bees represent the color patterns of workers. The inset map in the lower left-hand corner represents the full distribution of *B. ephippiatus* (grey circles) and *B. wilmattae* (black triangles) as reported by Labougle (1990).

B. wilmattae, integrating color pattern information, geographical range, and genetics can be a powerful approach for understanding species and their boundaries (Ross et al., 2010).

In addition to their uncertain phylogenetic history, *B. ephippiatus* has a broad biogeographic distribution across complex habitats. The phylogenetic patterns and known biogeographic distributions of these putative species provide the background for investigating the structuring of inter- and intraspecific genetic diversity and for examining how bumble bee diversification correlates with the same historical events that have shaped the speciation of vertebrate taxa. The intricate history of this species complex makes this an excellent system in which to explore the evolutionary phenomena that can confound the process of species delimitation.

Here, we present an analysis of multiple loci (mitochondrial COI and nuclear PEPCK and CAD sequences, as well as microsatellite genotype data) to test the hypothesis that *B. ephippiatus* comprises multiple independent evolutionary lineages across its geographic range. We also infer the phylogeny among the major taxa, estimate divergence times, and discuss possible geographic barriers and climatic events that could have caused the observed genetic and morphological patterns of diversification within this group and other taxa.

2. Materials and methods

2.1. Taxa examined

2.1.1. Interspecific phylogenetic analysis

We included a total of 102 specimens for the three species examined (Table 1). To represent the widespread range of *B. ephippiatus*, we included 86 individuals. We sequenced six individuals of *B. wilmattae* and ten individuals of *B. impatiens*. Exemplars of *B. huntii* and *B. vosnesenskii* were selected as outgroup taxa based on the *Bombus* phylogeny of Cameron et al. (2007). Ingroup taxa, including both males and females, were sampled from regions throughout their range (Table 1 and Fig. 1).

2.1.2. Microsatellite analysis

Because male bumble bees are haploid, only female bees (either workers or queens) were used for genotyping. We used a more extensive set of 120 specimens of *B. ephippiatus*, four specimens of *B. wilmattae*, and 30 specimens of *B. impatiens* (Table 1). Most of the same samples were also used for phylogenetic estimation with the exception of 44 *B. ephippiatus* and 25 *B. impatiens*. Specimen vouchers are housed in the Cameron lab facilities at the University of Illinois, Urbana.

2.2. Phylogenetic inference and divergence time estimation

2.2.1. Genes

All taxa are represented by sequences from three independently evolving genes, although not every individual was sequenced for each gene (Table 1). We obtained sequence fragments from the following genes: mitochondrial cytochrome oxidase I (COI), nuclear-encoding carbamoyl-phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD), and phosphoenolpyruvate carboxykinase (PEPCK). We amplified 811 bp of COI using primers RevmtR (5′-AACCAGTAATTATTGGATATCATGA) and FormtR (5′-GG TTGAACTGTATATCCTCCA) (developed by H. Hines). Most of the Costa Rican *B. ephippiatus* individuals are represented only by COI because these specimens were at least 15 years old and nuclear loci could not be amplified by PCR. To amplify CAD, we designed two forward primers, CDBF1 (5′-TGAATCCTGATTGATTCTACAGAG-CAGTC) and CDBF2 (5′-TGGTCTCAGTATCGGTCAAGCG), and used the reverse primer CD688R (Wild and Maddison, 2008) to amplify 840 bp, including two intron fragments totaling 357 bp. We used PEPCK primers from Cameron et al. (2007) to amplify 878 bp, including two intron fragments totaling 515 bp. We obtained five PEPCK sequences from GenBank (Cameron et al., 2007), one for each of the three ingroup taxa and the two outgroups. Sequences and collection localities are listed in Table 1. New sequences have been submitted to GenBank (GenBank ID: JF798930–JF799088, Table 1).

2.2.2. PCR and DNA sequencing

We obtained tissue from specimens (preserved in 95–100% ethanol at 4 °C) by either removing one of the forelegs or removing thoracic muscle through a small puncture made in the side of the thorax. We also included some pinned specimens from which a foreleg was used for DNA extraction. We re-associated legs with specimens following DNA extraction. Tissue was digested for six or more hours in proteinase K at 56 °C on a shaking platform. DNA was extracted using either a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) or a modified Chelex® (BIO-RAD, Hercules, CA) reaction (Lozier and Cameron, 2009).

Standard conditions for PCR amplification included 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 44–57 °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 5 × GoTaq® reaction buffer (Promega, Fitchburg, WI), 1.875 mM $MgCl_2$, 0.2 mM each dNTP, 10 μM of each primer and 0.4 U of GoTaq® DNA polymerase (Promega) with 2.5 μL of genomic DNA. Annealing temperatures were 44–52 °C for COI, 48–57 °C for CAD, and 45–52 °C for PEPCK. We carried out a nested PCR reaction for CAD, initially using primers CDBF2 and CD688R to amplify a longer fragment, then using primers CDBF1 and CD688R to amplify an internal fragment 840 bp in length. We purified PCR products using ExoSAP-IT® (Affymetrix, Santa Clara, CA). BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used for sequencing sense and anti-sense strands 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.

2.2.3. Alignment

DNA sequences were edited and aligned manually in BioEdit v7.0.5 (Hall, 1999). The PEPCK dataset contained a five bp indel removed from the sequence alignment and coded as either a single binary (presence–absence) character (weight equal to a base substitution) in Bayesian analyses (Simmons and Ochoterena, 2000), or as a fifth character in maximum parsimony analyses. Individuals heterozygous for the CAD locus were identified by a frameshift in the chromatogram caused by a nine bp indel in one of the alleles. Composite sequences of the heterozygotes were pieced together by using the readable chromatogram regions before and after the indel, or were re-sequenced using the following primer combinations to sequence the allele without the indel: CDBF1 with CAD1insert (5′-CATCCGAATTAATCGCGAGACTTGCG), CAD2insert (5′-CGCA AGTCTCGCGATTAATTCGGATG) with CD688R. Relatively few SNPs (represented by double peaks in the chromatogram) were detected in the heterozygotes; their allele assignment was determined through re-sequencing the allele without the nine bp indel (see above) or they were coded as ambiguous positions based on the peaks present in the chromatogram. For all analyses we excluded the nine bp indel plus three associated downstream adjacent sites.

2.2.4. Phylogenetic analysis

For interspecific phylogenetic inference, we analyzed genes individually and combined using Bayesian and maximum

Table 1
Collection information, sequence and genotype sources of taxa examined. Unless otherwise indicated with a ♂, all specimens are female. A plus sign + indicates that the full set of eight microsatellite loci were successfully genotyped for that individual. All spaces left blank under the loci categories indicate that sequencing of that gene or genotyping was unsuccessful for that individual, or was not attempted in the case of some of the *B. impatiens* specimens. No microsatellite data were collected for males. Accession numbers are given for all gene sequences obtained and submitted to GenBank. An asterisk * indicates that the sequence was taken from GenBank from a previous study.

Bombus species	Collection locality	Latitude	Longitude	Collector	Year	Voucher #	GenBank ID			MSAT
							COI	CAD	PEPCK	
ephippiatus	CR: San José: Villa Mills	9.564651	−83.707901	A. Picado	1996	VEP23	JF798945	JF799053	JF799073	+
ephippiatus	CR: San José: Cerro de la Muerte	9.566583333	−83.75018333	L. Masner	1998	VEP02	JF798931			+
ephippiatus	CR: Cartago: Madre Selva: L. Lagos	9.676111111	−83.87722222	P. Hanson	1993	Cart-A				+
ephippiatus	CR: Cartago: Madre Selva: L. Lagos	9.676111111	−83.87722222	P. Hanson	1993	Cart-B				+
ephippiatus	CR: Cartago: Madre Selva: L. Lagos	9.676111111	−83.87722222	P. Hanson	1993	Cart-C				+
ephippiatus	CR: Cartago: Madre Selva: L. Lagos	9.676111111	−83.87722222	P. Hanson	1993	Cart-D				+
ephippiatus	CR: Cartago: Madre Selva: L. Lagos	9.676111111	−83.87722222	P. Hanson	1993	VEP27	JF798949			+
ephippiatus	CR: Cartago: Cañon	9.7	−83.9	P. Hanson	1995	VEP20	JF798942	JF799031		+
ephippiatus	CR: Cartago: Cañon	9.7	−83.9	P. Hanson	1995	VEP24	JF798946			+
ephippiatus	CR: San José: Zurquí de Moravia	10.02	−84	P. Hanson	1991	SnJ1-C				+
ephippiatus	CR: San José: Zurquí de Moravia	10.02	−84	P. Hanson	1991	SnJ2-A				+
ephippiatus	CR: San José: Zurquí de Moravia	10.02	−84	P. Hanson	1991	SnJ2-B				+
ephippiatus	CR: San José: Zurquí de Moravia	10.02	−84	P. Hanson	1991	SnJ2-C				+
ephippiatus	CR: San José: Zurquí de Moravia	10.02	−84	P. Hanson	1991	SnJ2-D				+
ephippiatus	CR: San José: Zurquí de Moravia	10.02	−84	P. Hanson	1991	VEP18	JF798940			+
ephippiatus	CR: San José: Zurquí de Moravia ♂	10.02	−84	P. Hanson	1991	VEP30	JF798952			
ephippiatus	CR: Puntarenas: RB Monteverde	10.28396	−84.75961	K. Martines	1994	VEP09	JF798935	JF799052	JF799068	+
ephippiatus	CR: Puntarenas: RB Monteverde	10.28396	−84.75961	K. Martines	1994	VEP28	JF798950			+
ephippiatus	HON: El Paraiso: Montserrat ♂	13.93166667	−86.87666667	M. Duennes	2009	VEP48	JF798984		JF799079	
ephippiatus	HON: El Paraiso: Montserrat ♂	13.93166667	−86.87666667	M. Duennes	2009	VEP49	JF799007	JF799062	JF799080	
ephippiatus	HON: El Paraiso: Montserrat ♂	13.93166667	−86.87666667	M. Duennes	2009	MONA	JF798985			
ephippiatus	HON: El Paraiso: Montserrat ♂	13.93166667	−86.87666667	M. Duennes	2009	MONB	JF798986			
ephippiatus	HON: El Paraiso: Montserrat ♂	13.93166667	−86.87666667	M. Duennes	2009	MONC	JF798987			
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-A				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-B	JF798974			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-C	JF798975			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-D	JF798964			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-E	JF798976			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-G	JF799008			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-H	JF798977			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-I	JF798965			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-J				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-K	JF798966			+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-L				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-M				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-N				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-O				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-P				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-Q				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-R				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	T-S				+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	VEP46	JF798963	JF799060	JF799077	+
ephippiatus	HON: Francisco Morazán: Tatumbla	14.018834	−87.096436	M. Duennes	2009	VEP47	JF798973	JF799061	JF799078	+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-A	JF798982			+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-B	JF798960			+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-C				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-D	JF798971			+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-E	JF798972			+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-F	JF798961			+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-G	JF798983			+

ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-H				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-I	JF798962			+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-K				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-L				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-M				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-N				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-O				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-P				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-Q				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-R				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-S				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	U1-U				+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	VEP44	JF798981		JF799075	+
ephippiatus	HON: Francisco Morazán: Uyuca	14.03055556	−87.08	M. Duennes	2009	VEP45	JF798970	JF799059	JF799076	+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19444444	−87.12972222	M. Duennes	2009	LT1	JF798979			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19444444	−87.12972222	M. Duennes	2009	VEP50	JF798978			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-A				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-B				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-C	JF798988			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-D	JF799003			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-E	JF799010			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-F	JF799004			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-G	JF798989			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-H	JF799011			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-I	JF799012			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-J	JF798980			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-K	JF799005			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-L	JF798990			+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-M				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-N				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-O				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-P				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-Q				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	LT2-R				+
ephippiatus	HON: Francisco Morazán: La Tigra	14.19722222	−87.12583333	M. Duennes	2009	VEP51	JF799009			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.43611111	−87.56083333	M. Duennes	2009	VEP52	JF798967		JF799081	+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2A	JF798968			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2B	JF799014			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2C	JF798969			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2D	JF798991			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2E	JF798992			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2F	JF799006			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	MD2G	JF798993			+
ephippiatus	HON: Comayagua: Mnt. Comayagua	14.4375	−87.56083333	M. Duennes	2009	VEP53	JF799013		JF799082	+
ephippiatus	MX: Chiapas	15.05	−92.283333	M. Guzman	2003	SC198	JF799015		EF050844*	
ephippiatus	GTM: Zacapa	15.083336	−89.916665	M. Metz	1997	VEP10				+
ephippiatus	MX: Chiapas: Volcán Tacaná	15.117761	−92.107067	D. Sanchez	2007	VEP17	JF798939	JF799055	JF799070	+
ephippiatus	MX: Chiapas: Volcán Tacaná	15.120847	−92.096547	D. Sanchez	2007	VEP19	JF798941	JF799051	JF799071	+
ephippiatus	GTM: Baja Verapaz: Purulhá	15.239095	−90.235607	M. Sharkey	1987	VEP06				+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-A	JF798995			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-B	JF798996			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-C	JF798997			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-D	JF798998			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-E	JF798999			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-F	JF799002			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	CP-G	JF799000			+
ephippiatus	HON: Santa Barbara: PN Cusuco	15.49222222	−88.22666667	M. Duennes	2009	VEP55	JF799001			+

(*continued on next page*)

Table 1 (*continued*)

Bombus species	Collection locality	Latitude	Longitude	Collector	Year	Voucher #	GenBank ID			MSAT
							COI	CAD	PEPCK	
ephippiatus	HON: Santa Barbara: Buenos Aires	15.49638889	−88.18083333	M. Duennes	2009	VEP54	JF798994		JF799083	+
ephippiatus	GTM: Huehuetenango: Cuchumatán	15.511595	−91.60564	M. Sharkey	1987	VEP08				+
ephippiatus	MX: Chiapas: Huitepec	16.744444	−92.685	P. Sagot	2010	VEP56				+
ephippiatus	MX: Chiapas: Huitepec	16.744444	−92.685	P. Sagot	2010	VEP57				+
ephippiatus	MX: Oaxaca: San Pablo Guelatao	17.31736	−96.495108	H. Hines, Ayala	2004	VEP07	JF798934	JF799046	JF799067	+
ephippiatus	MX: Oaxaca: San Pablo Guelatao	17.31736	−96.495108	H. Hines, Ayala	2004	VEP25	JF798947	JF799047	JF799074	+
ephippiatus	MX: Guerrero: Puerto del Gallo	17.474758	−100.178778	H. Hines, Ayala	2004	VEP05	JF798933	JF799043	JF799066	+
ephippiatus	MX: Guerrero: Puerto del Gallo	17.474758	−100.178778	H. Hines, Ayala	2004	VEP29	JF798951	JF799045		+
ephippiatus	MX: Guerrero: Puerto del Gallo	17.474758	−100.178778	H. Hines, Ayala	2004	VEP43	JF798959	JF799044		+
ephippiatus	MX: Michoacan: Cheranastico	19.118402	−101.893329	H. Hines, Ayala	2004	VEP01	JF798930	JF799054	JF799064	+
ephippiatus	MX: Jalisco: Volcan Colima ♂	19.6143	−103.56765	R. Ayala	2007	VEP22	JF798944	JF799036		
ephippiatus	MX: Jalisco: Volcan Colima	19.6143	−103.56765	R. Ayala	2007	VEP36	JF798956	JF799038		+
ephippiatus	MX: Jalisco: Volcan Colima ♂	19.6143	−103.56765	R. Ayala	2007	VEP37	JF798957	JF799037	JF799084	
ephippiatus	MX: Jalisco: Volcan Colima	19.6143	−103.56765	R. Ayala	2007	VEP38	JF798958			+
ephippiatus	MX: Jalisco: Tapalpa	20.0085	−103.70833	R. Ayala	2007	VEP12	JF798936	JF799035	JF799069	+
ephippiatus	MX: Jalisco: Tapalpa	20.0085	−103.70833	R. Ayala	2007	VEP33	JF798953			+
ephippiatus	MX: Querétaro: Pinal de Amoles	21.13517	−99.63268	R. Ayala	2007	VEP21	JF798943	JF799040	JF799072	+
ephippiatus	MX: Querétaro: Pinal de Amoles	21.13517	−99.63268	R. Ayala	2007	VEP34	JF798954	JF799041	JF799085	+
ephippiatus	MX: Querétaro: Jalpan	21.17631	−99.57348	R. Ayala	2007	VEP13	JF798937	JF799039		+
ephippiatus	MX: Querétaro: Jalpan ♂	21.17631	−99.57348	R. Ayala	2007	VEP35	JF798955	JF799042	JF799086	
ephippiatus	MX: Chihuahua: Candemeña	28.168724	−108.2131	K. Shaw	1991	VEP14	JF798938	JF799033		+
ephippiatus	MX: Chihuahua: Candemeña	28.168724	−108.2131	K. Shaw	1991	VEP26	JF798948	JF799034		+
ephippiatus	MX: Chihuahua: Basaseachic River	28.221098	−108.251971	K. Shaw	1991	VEP03	JF798932	JF799032	JF799065	+
wilmattae	MX: Chiapas: Cacahuatan ♂	14.98333	−92.16667	S. Cameron	2005	Vwilm03	JF799016	JF799056		
wilmattae	MX: Chiapas: Volcán Tacaná	15.05	−92.283333	R. Vandame	2003	SC199	JF799021		EF050843*	
wilmattae	MX: Chiapas: Tapachula	15.05	−92.283333	R. Vandame	2003	Vwilm06	JF799018	JF799057	JF799087	+
wilmattae	MX: Chiapas: Tapachula	15.05	−92.283333	R. Vandame	2003	Vwilm07	JF799019	JF799058		+
wilmattae	MX: Chiapas: Volcán Tacaná	15.09036667	−92.28638333	R. Vandame	2003	Vwilm10	JF799020			+
wilmattae	MX: Chiapas: Volcán Tacaná	15.120847	−92.096547	D. Sanchez	2007	Vwilm04	JF799017	JF799048		+
impatiens	USA: Texas	30.67105	−94.71843	S. Cameron et al.	2008	TX08.0311	JF799030			+
impatiens	USA: Texas	30.67105	−94.71843	S. Cameron et al.	2008	TX08.0605	JF799029			+
impatiens	USA: Texas	30.67105	−94.71843	S. Cameron et al.	2008	TX08.0901	JF799028			+
impatiens	USA: Alabama	31.85426	−86.64129	S. Cameron et al.	2009	AL09.0029				+
impatiens	USA: Alabama	31.85426	−86.64129	S. Cameron et al.	2009	AL09.0513				+
impatiens	USA: Georgia	34.26571	−84.27237	S. Cameron et al.	2009	GA09.0058				+
impatiens	USA: North Carolina	35.043611	−80.025	S. Cameron et al.	2009	NC09.0074				+
impatiens	USA: Arkansas	35.8207	−94.15895	S. Cameron et al.	2008	AR08.0684	JF799026			+
impatiens	USA: Arkansas	35.8207	−94.15895	S. Cameron et al.	2008	AR08.0901	JF799027			+
impatiens	USA: Virginia	37.25568	−78.68094	S. Cameron et al.	2009	VA09.0641				+
impatiens	USA: Illinois	37.37934	−88.59228	S. Cameron et al.	2008	IL08.1033				+
impatiens	USA: Illinois	37.77345	−89.41856	S. Cameron et al.	2008	IL08.0871				+
impatiens	USA: Indiana	39.06533	−85.43764	S. Cameron et al.	2008	IN08.0115				+
impatiens	USA: Missouri	39.89866	−92.47369	S. Cameron et al.	2008	MO08.0455				+
impatiens	USA: Indiana	39.9602	−87.0686	S. Cameron et al.	2008	IN08.0008				+
impatiens	USA: Ohio	40.09789	−84.11588	S. Cameron et al.	2009	OH09.0277				+
impatiens	USA: Illinois: Urbana	40.11058889	−88.20726944	H. Hines	2002	SC060			EF050842*	
impatiens	USA: Illinois: Urbana	40.11058889	−88.20726944	H. Hines	2004	Vimpa03	JF799023		JF799088	
impatiens	USA: Illinois: Urbana ♂	40.11058889	−88.20726944	H. Hines	2002	Vimpa04	JF799024	JF799050		
impatiens	USA: Illinois	40.916516	−89.803093	S. Cameron et al.	2008	IL08.0642				+
impatiens	USA: New York	40.923923	−73.12382	S. Cameron et al.	2009	NY09.0333				+
impatiens	USA: Nebraska	40.95999	−96.86378	S. Cameron et al.	2008	NE08.0151				+
impatiens	USA: Nebraska	40.99976	−95.87263	S. Cameron et al.	2008	NE08.0431				+

impatiens	USA: Iowa	41.32979	−92.64732	S. Cameron et al.	2008	IA08.0051					+
impatiens	USA: Pennsylvania	41.40279	−78.02639	S. Cameron et al.	2009	PA09.0299					+
impatiens	USA: Illinois	42.01375	−88.25199	S. Cameron et al.	2008	IL08.0406					+
impatiens	USA: Massachusetts	42.29117	−71.31905	S. Cameron et al.	2009	MA09.0001					+
impatiens	USA: Wisconsin	43.04103	−89.43149	S. Cameron et al.	2008	WI08.0843					+
impatiens	USA: Iowa	43.06555	−92.6489	S. Cameron et al.	2008	IA08.0140					+
impatiens	USA: Wisconsin	43.62648	−89.80605	S. Cameron et al.	2008	WI08.1051					+
impatiens	USA: Wisconsin	43.94267	−90.74896	S. Cameron et al.	2008	WI08.0369					+
impatiens	USA: Minnesota	43.97524	−91.4277	S. Cameron et al.	2008	MN08.0902					+
impatiens	USA: Maine: Isle Au Haut	44.07536111	−68.63335833	S. Cameron	2003	Vimpa02	JF799022	JF799049			
impatiens	USA: Maine: Isle Au Haut	44.07536111	−68.63335833	S. Cameron	2003	Vimpa06	JF799025	JF799063			
impatiens	USA: Minnesota	44.21959	−93.2283	S. Cameron et al.	2008	MN08.0064					+
huntii	USA: Washington: Umtanum Recreation Area	46.85	−120.483333	J. Whitfield	2003	SC151	JN400357	JN400355	EF050840*		
vosnesenskii	USA: Washington: Seattle, U of W	47.659494	−122.309647	J. Whitfield	1994	SC112	JN400358	JN400356	EF050841*		

likelihood (ML) analyses. Because we had a much larger sampling of individuals for COI than for either nuclear gene, we used a reduced COI taxon dataset for concatenation with the nuclear genes. We did not sequence more taxa for the nuclear genes to match the larger number sequenced for COI because of the low variation and limited utility of the nuclear data at the interspecific level of analysis. Bayesian inference of species relationships was implemented in a parallel (MPI) version of MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Altekar et al., 2004) with evolutionary models based on corrected Akaike information criteria calculated in jMODELTEST v0.1.1 (Posada, 2008). We partitioned nuclear DNA sequences into exon and intron regions and partitioned the PEPCK indel. The models calculated for each partition are as follows: COI (GTR + I), CAD intron (F81), CAD exon (HKY + I), PEPCK intron (GTR + I), PEPCK exon (HKY), PEPCK indel character (standard). For each gene and the concatenated data, we conducted six independent runs (three sets of parallel runs) in MrBayes to ensure efficient sampling of tree space (8,000,000 generations, four chains, mixed models, flat priors, sampling trees every 1000 generations). We compared log likelihood plots of the two parallel runs from one MrBayes implementation using Tracer v1.5 (Rambaut and Drummond, 2007) to determine proper mixing of the runs. All trees estimated prior to stationarity were discarded (burnin). The six runs of each analysis were summarized and clade support was determined by calculating posterior probabilities of nodes on retained trees in MrBayes. To test whether the Bayesian topology and posterior probability (pp) values were supported across different inference methods, we also implemented maximum likelihood approximate likelihood-ratio tests (Anisimova and Gascuel, 2006) on individual genes in PhyML 3.0 (Guidon et al., 2010) (the concatenated data set was not analyzed using this method because it does not allow one to partition the data and implement multiple substitution models). We present results only from the Bayesian analysis in the text, but see Supplementary material Figs. S1–S5 for results from individual gene datasets with Bayesian and maximum likelihood analyses.

2.2.5. Divergence time estimation

In order to roughly estimate the time to most recent common ancestor (TMRCA) for these three species, as well as to confirm the timing proposed by Hines (2008), we analyzed the 103 taxon COI dataset in BEAST v1.6.1 (Drummond and Rambaut, 2007). Using the GTR + I model selected by jModelTest, we ran Markov chain Monte Carlo simulations with the coalescent constant population size tree model and the strict clock model. The relaxed uncorrelated lognormal molecular clock model (Drummond et al., 2006) was implemented first to assess the clock-like nature of the data. The sampled marginal posterior probability (PP) distribution of both standard deviation and coefficient of variation of substitution rates among tree branches included 0 in the 95% HPD (data not shown), indicating that the there was no strong evidence for substantial rate heterogeneity among lineages (Drummond and Rambaut, 2007). We specified a range of possible substitution rates for insect mitochondrial genes taken from the literature using a flat prior ranging from 1×10^{-9} to 1×10^{-7} substitutions site^{-1}/year^{-1}, which encompasses rates including those from Farrell (2001) and Brower (1994). Simulations were run for 20 million generations, sampling every 2000 generations. Four independent runs were assessed in Tracer v1.4.1 (Drummond and Rambaut, 2007) to confirm convergence, determine burnin, and examine the effective sample size of all posterior parameters. Log files from each run were combined in LogCombiner v1.6.1 (Drummond and Rambaut, 2007) for final parameter estimates.

2.3. Microsatellite analysis

2.3.1. Microsatellite genotyping

To provide additional information about patterns of species and population divergence from nuclear genomic markers, including possible admixture, we amplified microsatellite genotypes for a total of 154 females. These specimens were genotyped at eight microsatellite loci using published PCR primers: B10, B124, B126 (Estoup et al., 1995); B96 (Estoup et al., 1996); BT10, BL13, BT30, BT28 (Reber-Funk et al., 2006). The markers were selected for their consistent amplification across multiple *Bombus* species and the lack of evidence for null alleles or other scoring errors that could complicate analyses (Cameron et al., 2011). Data for *B. impatiens* were taken from Cameron et al. (2011). See Lozier and Cameron (2009) for PCR reaction protocols and thermal cycling conditions. Final PCR products were genotyped at the high throughput DNA facility at the W.M. Keck Center at the University of Illinois using ABI 3730xl capillary DNA sequencers (Applied Biosystems). Genotypes were scored manually with GeneMapper® 4.3 (Applied Biosystems) using the same allele bin-set for all species. A random subset of samples was genotyped a second time to check the accuracy of allele identification and no inconsistencies were observed.

2.3.2. Microsatellite analyses

We tested the five well-sampled Honduras *B. ephippiatus* populations for deviations from Hardy–Weinberg and linkage equilibria using the Markov chain method implemented in GENEPOP v4.0.10 (Raymond and Rousset, 1995; http://genepop.curtin.edu.au/). We used default parameters except for an increase to 800 iterations for the Hardy–Weinberg and 1600 iterations for linkage disequilibrium tests. Populations collected from areas other than Honduras had too few samples to test for these deviations.

To test whether patterns of genetic structure in the microsatellite data support the same clades resolved from the phylogenetic estimation procedures, and also to examine any possible admixture among the clades, we used the Bayesian genotype clustering method implemented in STRUCTURE v2.3 (Pritchard et al., 2000). All model parameters were set to the STRUCTURE defaults (admixture model with allele frequencies correlated among populations and no prior sample information) and run for 1,000,000 generations following a burn-in of 300,000 generations using a range of K (designated number of genetic clusters) values between two and eight; four independent runs were conducted for each value of K. The Evanno method of evaluating the optimal number of clusters (Evanno et al., 2005) was implemented in STRUCTURE HARVESTER v0.6.7 (Earl and vonHoldt, 2011). The highest ΔK (185.58) was found for $K = 4$, however, this pattern of clustering makes little biological sense because it groups Mexican and Costa Rican *B. ephippiatus* samples into the same cluster. Increasing K to 5 and 6 resulted in consistent and informative clustering, rather than over-splitting individuals into clusters for which no individuals had high assignment probability, as we began to observe for $K > 6$. As $K = 6$ received the next greatest ΔK value (114.93) and the highest log likelihood values, we elect to report results for this K value.

3. Results

The Bayesian phylogenetic analysis of the concatenated gene sequences (Fig. 2) resolved *B. impatiens* as sister group to *B. ephippiatus* + *B. wilmattae* with strong support (PP = 1.00). Based on the COI data, the estimated divergence of the *B. impatiens* + *B. ephippiatus* + *B. wilmattae* complex (IEW) was within the range of 0.07–3.7 mya, with a mean of 985,720 years and median of 404,110 years. Correspondingly, simple pairwise divergence between *B. impatiens* and EW clades ranged from 1.96% to 2.22% (Tables S6–S7) or ~1 mya based on the 2% divergence per million years clock commonly applied to insect mitochondrial DNA (Brower, 1994). Together, these data suggest divergence of the major clades in the IEW complex corresponds to the period between the late Pliocene and the early to late Pleistocene. CAD and PEPCK provide little resolution within the *B. ephippiatus* + *B. wilmattae* species complex (hereafter referred to as EW). We therefore focus on the results from COI for further discussion of relationships within the EW clade (Fig. 3A).

Within EW, there is strong support (COI PP = 0.99) for a monophyletic clade comprising *B. wilmattae* (**a**) and some, although weak, support (COI PP = 0.78) for *B. wilmattae* as sister group to a strongly supported (COI PP = 0.97) Costa Rican clade of *B. ephippiatus* (**b**). Two additional *B. ephippiatus* clades (**d** and **e**, Fig. 3A) are resolved with strong support: **d** comprises all the Mexican specimens plus a large subset of the Honduran samples (PP = 0.98); **e** comprises all of the remaining Honduras samples. The relationships among the three major *B. ephippiatus* clades (**c**, **d**, **e**) are unresolved, however. The Honduras individuals are not geographically divided, but specimens from the same locality are apportioned between the two clades (**d**, **e**). In contrast, the Mexican *B. ephippiatus* do appear somewhat geographically structured, with all individuals from Guerrero in the Southwest (VEP05, VEP29, VEP43) comprising a well-supported clade with the individuals from the adjacent southern state Oaxaca (VEP07, VEP25), and individuals from the southernmost state of Chiapas (VEP17, VEP19, SC198) forming a distinct clade. Divergence of clades within EW was lower than divergence from *B. impatiens*, but was not insubstantial, ranging from 0.1% to 1.3% (Table S6).

The microsatellite data further support the moderate levels of divergence in the IEW complex observed for COI. The STRUCTURE analysis reveals six major clusters within IEW (highest log likelihood for our data was $\ln \Pr[X|6] = -3834.1$ for the run presented). The six clusters (Fig. 3B) are largely congruent with those obtained from the phylogenetic analysis (Fig. 3A), with *B. impatiens* individuals belonging to a distinct genetic group (K6) and substantial geographic structure apparent within the EW complex.

Congruent with the COI sequence data, Costa Rican *B. ephippiatus* and *B. wilmattae* individuals were assigned in large part to a single cluster K1. Similarly, *B. ephippiatus* from Honduras were split into multiple distinct groups (K2, K3, K4), none of which are strictly concordant with geography. The clustering of the Honduras individuals also corresponds to the clustering of individuals in the Bayesian analysis of the COI data (Fig. 3A). Clusters K2 and K3 correspond to the Honduras individuals that are sister to the Mexican *B. ephippiatus* in the Bayesian tree, and the K4 cluster corresponds to the Honduras individuals in the unresolved clade (**e**). K4 also includes two weakly assigned *B. wilmattae* specimens (each with ~50% probability). The mean F_{ST} values for each STRUCTURE cluster (Table 2) demonstrate the high degree of differentiation within this species complex. The K4 Honduras population was the most differentiated population ($F_{ST} = 0.301$), while the Honduras K2 population was least divergent from the ancestral population ($F_{ST} = 0.1114$). All F_{ST} estimates for each cluster are large, highlighting the strong differentiation within this species group.

The spatial co-occurrence of Honduras individuals with different genetic compositions is also supported by deviations from Hardy–Weinberg equilibrium (Fig. 3B); that is, in the La Tigra ($P = 0.0017$), Tatumbla ($P = 0.0042$), and Uyuca ($P < 0.001$) populations, but not in the Comayagua ($P = 0.3159$) population, which may be an effect of small sample size, or Cusuco ($P = 0.6977$), where no mix of clusters was found. No other deviations from Hardy–Weinberg or linkage equilibria were detected. Mexican *B. ephippiatus* largely separate into a distinct genetic cluster (K5) with the exception of individuals from Chiapas, which group with K2 Honduras samples (Fig. 3).

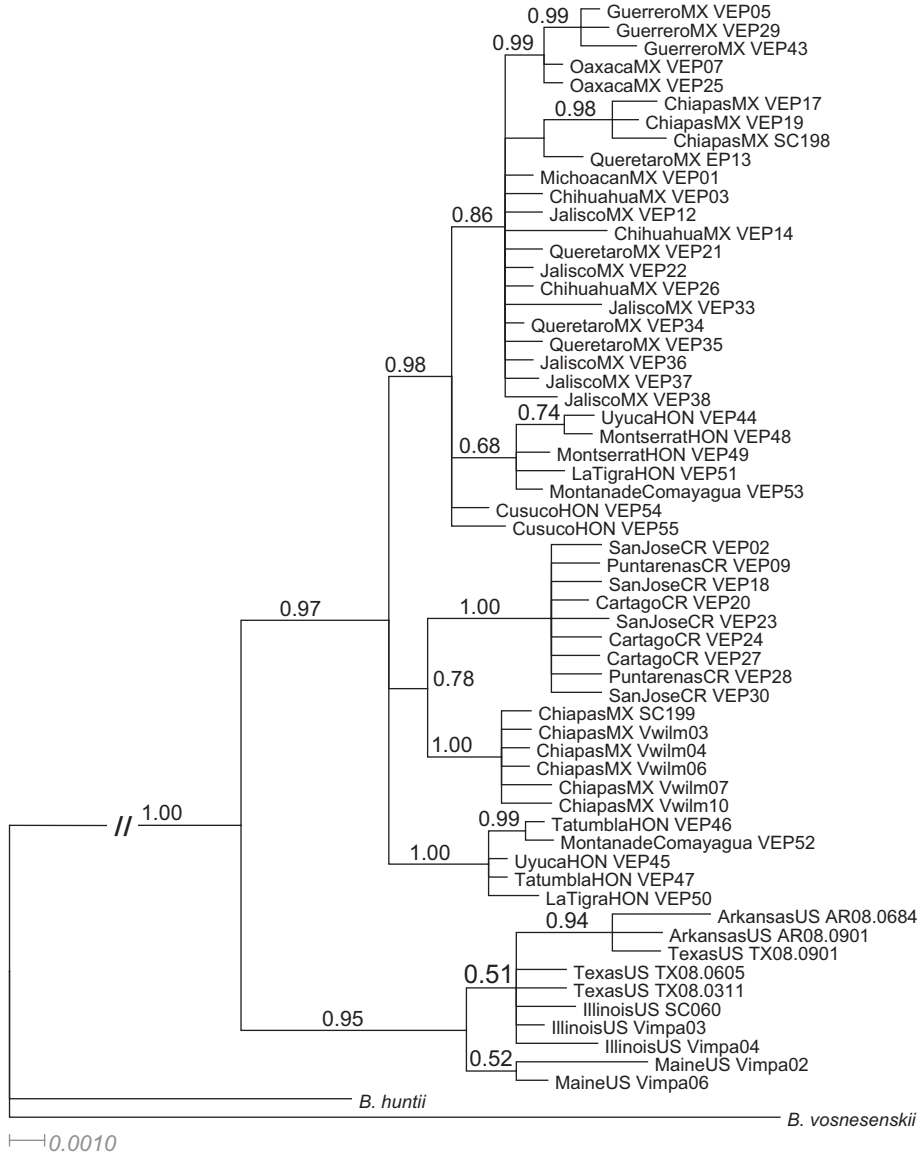

Fig. 2. Bayesian phylogeny based of the concatenated gene dataset (CAD + PEPCK + COI). Phylogeny estimated from 237,000 trees (2 runs: 12,000,000 generations, four chains, sampling every 1000 trees, burnin = 1500 trees per run) using flat priors, mixed models, and partitioning by gene, exon/intron, and gap characters when suitable. Clade support values are Bayesian posterior probabilities. The scale bar indicates branch lengths in expected substitutions per site.

4. Discussion

4.1. Phylogenetic resolution of species relationships

Our analyses strongly support the hypothesis that *B. impatiens* is the sister species to the EW assemblage and that *B. ephippiatus* is paraphyletic with respect to *B. wilmattae*. The overall topology is strongly associated with the spatial distributions of the three taxa; *B. impatiens* isolated from EW in the Americas north of Mexico, while *B. wilmattae* is localized to Chiapas nested within the range of multiple *B. ephippiatus* lineages. Levels of COI divergence (Tables S6 and S7) of *B. impatiens* from EW indicate divergence between the late Pliocene and Pleistocene, a timing more recent than that proposed by Hines (2008; ~ 6 mya for lEW). Our phylogeny also implies a different directionality of dispersal in the lEW clade relative to that proposed by Hines (2008). Based on a weakly supported (*B. wilmattae* + *B. impatiens*) + *B. ephippiatus* relationship, Hines postulated a northward dispersal of *B. impatiens* into North America. Our well supported phylogeny indicating *B. impatiens* as sister to the Mesoamerican taxa instead suggests dispersal from North America into Mesoamerica.

Our analyses resolve EW as a complex assemblage of lineages, with *B. wilmattae* nested as a genetically distinct monophyletic lineage within multiple lineages of *B. ephippiatus*, most closely related to the Costa Rican *B. ephippiatus* lineage. While the species status of *B. wilmattae* has been controversial, our data support the hypothesis that *B. wilmattae* arose as a population isolate of a more southerly clade of *B. ephippiatus*, exhibiting relatively distinct genetic divergence at COI. This distinction is also evident in the color pattern of *B. wilmattae*, which is similar to that of *B. ephippiatus* but with a consistent band of yellow hairs on the anterior of the thorax (Labougle et al., 1985) (Fig. 1). The STRUCTURE analysis, however, failed to identify a distinct *B. wilmattae* cluster, and even suggested the possibility of admixture of the *B. wilmattae* individuals with *B. ephippiatus* (Fig. 3B). The small *B. wilmattae* sample size may limit the power to accurately assign individuals to a distinct cluster with STRUCTURE. Although small sample sizes would not affect the COI polymorphisms unique to *B. wilmattae* in the

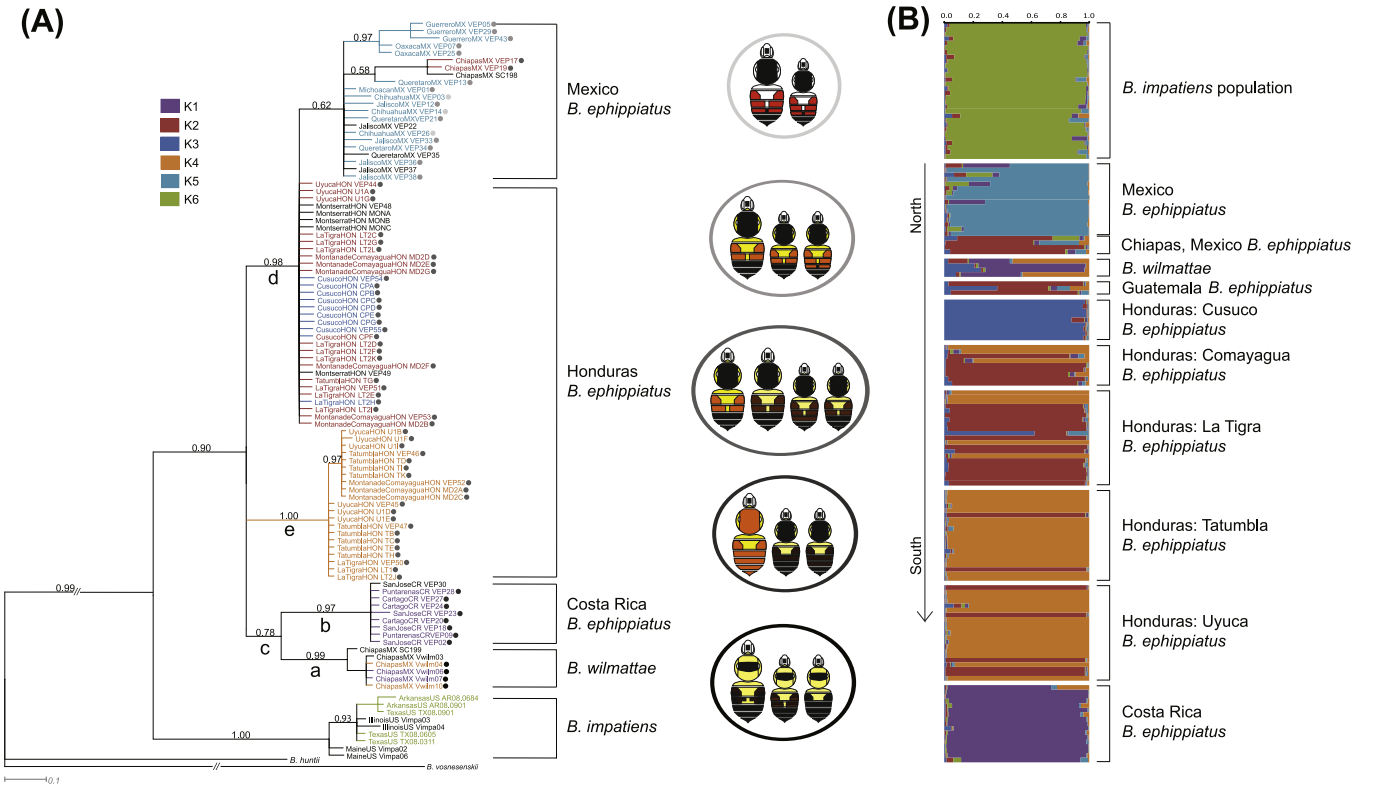

Fig. 3. (A) Bayesian phylogeny based on the expanded COI sequence dataset and STRUCTURE analysis of microsatellite genoptype data. Phylogeny estimated from 15,880 trees (two runs: 8,000,000 generations, four chains, sampling every 1000 trees, burnin = 60 trees per run) using flat priors and mixed models. Values above branches are Bayesian posterior probabilities. The scale bar indicates branch lengths in expected substitutions per site. The color pattern group that each specimen belongs to is mapped out along the tree with grey-shaded dots that correspond to their respective color pattern. Colors on the branches correspond to microsatellite STRUCTURE clusters (B) for $K = 6$ (ln Pr[X|6] = −3834.1). Each horizontal bar (B) represents a single specimen's microsatellite genotype, with the fraction of each color representing the posterior probability of ancestry in each genetic cluster (see inset legend). Samples are sorted by locality from North to South and white spaces separate localities (labeled to the right).

phylogenetic analysis, it is possible that increased sampling of *B. wilmattae* from across its range would reveal individuals with COI haplotypes shared by *B. ephippiatus*. A greater number of *B. wilmattae* samples are needed to more rigorously assess the possibility of admixture with *B. ephippiatus* and to clarify its species status. Furthermore, the genetic diversity and color pattern that distinguish *B. wilmattae* are comparable to the diversity present across the multiple *B. ephippiatus* lineages. Therefore, if *B. wilmattae* is considered a separate species then some of the other *B. ephippiatus* clades should be treated as similarly separate.

4.2. Divergence within B. ephippiatus and B. wilmattae in Mesoamerica

The levels of differentiation of *B. ephippiatus* and *B. wilmattae* lineages for both the COI and microsatellite data indicate that lineages within this complex have remained isolated for a substantial period of time. The exact relationships among all the lineages could not be completely resolved with the current data, however there is evidence for a split between northern and southern lineages and for the maintenance of distinct genetic structure over time. In particular, the data support three major lineages: a Mexican (K5) and Honduran (K2, K3) lineage (Fig. 3A; clade **d**), a distinct Honduran (K4) lineage (Fig. 3A; clade **e**), and a Costa Rican + *B. wilmattae* lineage (K1, K4) (Fig 3A; clade **c**).

Table 2
Mean F_{ST} values for each STUCTURE cluster from Fig. 3 in the main text. Cluster numbers correspond to those used in Fig. 3.

Cluster	F-model (F_{ST})
K1	0.1487
K2	0.1114
K3	0.2589
K4	0.301
K5	0.1813
K6	0.1219

Several aspects of Mesoamerican biogeography have likely contributed to diversification within the EW complex. First, two major low-lying, hot and dry regions in southern Mexico (Isthmus of Tehuantepec, IT) and southern Nicaragua (Nicaraguan Depression, ND) cross the distribution of *B. ephippiatus* + *B. wilmattae*. *Bombus ephippiatus* and *B. wilmattae* occur primarily at high elevations (>1000 m) in temperate forest habitats throughout Mesoamerica (Fig. 1). The marine transgressions of the ND during the Tertiary (Howell, 1969) and the IT in the late Pliocene-early Pleistocene (Campbell, 1999; Mulcahy et al., 2006) would have constituted major vicariant barriers for many organisms, including birds (Barber and Klicka, 2010; Arbeláez-Cortéz et al., 2010; Miller et al., 2007; García-Moreno et al., 2006), rodents (León-Paniagua et al., 2007), reptiles (Castoe et al., 2009; Greenbaum et al., 2011), and plants (Gutiérrez-Rodríguez et al., 2011). Although it is difficult to precisely determine lineage divergence dates using TMRCAs from a single gene (COI), these marine transgressions alone appear too ancient to explain the Pleistocene divergence estimate for the EW obtained here. Nonetheless, the ND and the IT likely play an important role in isolating these lineages during interglacial periods because of their distinctly unsuitable environmental conditions relative to high elevation temperate habitats favored by these bumble bees. Currently, at least, the IT and the

ND are hot and low regions (~200 m above sea level) likely preventing contemporary dispersal among lineages.

Climatic fluctuations throughout the Pleistocene would have altered the distribution of suitable habitat for bumble bees throughout Mesoamerica. Species that occur in topographically complex regions will frequently move between high and low elevation habitats in response to climatic fluctuations during glacial cycles (Rand, 1948). Distributional shifts can lead to variation in population continuity and isolation, possibly explaining why *B. ephippiatus* lineages from different geographic regions are not entirely monophyletic, despite the high degree of genetic differentiation among lineages. For example, in Honduras, the mountainous habitats suitable for *B. ephippiatus* are sparse and separated by large expanses of less favorable mid-elevation pine-oak, bridged by hot and dry lowland habitat that is generally depauperate of floral resources utilized by bumble bees (Fig. 1). Northern Mexico and Costa Rica, in contrast, possess continuous mountain ranges that would allow greater dispersal of *B. ephippiatus* within the respective chains of montane habitat (Fig. 1). Isolated mountains often act as islands that can promote rapid population divergence (Peterson et al., 1992; García-Moreno et al., 1999; Navarro et al., 2001; García-Moreno et al., 2004), which may explain the pattern we see in the *B. ephippiatus* complex. Climatic changes following such isolation events could have contributed to mixture without gene flow if the initial isolation period was sufficient (Phillips et al., 2004). The likelihood of long-term isolation within Central American *B. ephippiatus* is exemplified in Honduras, where three well-differentiated genetic groups coexist (K2–K4) in two well-defined COI clades (**d** and e, ~1% divergence) without substantial admixture, even when collected at the same localities. We can only speculate about the timing or nature of these biological events, but it seems clear from the multilocus analysis that multiple distinct *B. ephippiatus* lineages coexist on mountains in Honduras, apparently with limited gene flow, suggesting they may be in an early stage of species formation.

The only major incongruence observed between the mitochondrial and microsatellite data sets involves *B. ephippiatus* from Chiapas, Mexico (Fig. 3). In the phylogeny the Chiapas individuals are placed within the Mexico *B. ephippiatus*, but the STRUCTURE analysis clusters them with Honduras K2 (Fig. 3). Chiapas is located south of the IT, and one plausible explanation for this incongruence is secondary dispersal from more northern Mexican *B. ephippiatus* lineages across the IT. Subsequent hybridization with the Honduras K2 group would explain the signature shown in the STRUCTURE analysis. This kind of secondary dispersal following the emergence of the IT barrier has been hypothesized for pitvipers (Castoe et al., 2003) and toads (Mulcahy et al., 2006). The discordance may alternatively be explained by incomplete lineage sorting between the Mexican *B. ephippiatus* clade and the most closely related Honduras clade. It is possible that the Mexico lineage would still retain some of the alleles it shared with the Honduras K2–K3 lineage. It is also possible that *B. ephippiatus* from Chiapas in fact form their own distinct genetic cluster that STRUCTURE is unable to identify due to small sample size. Additional sampling in Chiapas should help to determine which of these explanations is most likely.

4.3. Association of genetic differentiation and color pattern variation

By examining color pattern evolution at the intraspecific level we can improve our understanding of the evolutionary history of color pattern change and corresponding phylogeographic patterns. The color patterns of *B. ephippiatus* lineages in different geographic regions correspond well with their genetic structure (Fig. 3A). Color pattern does match well with inferred structure from nuclear microsatellites, but color pattern does not map perfectly to mitochondrial lineages (Fig. 3A) due to the position of the Chiapas lineage among the Mexican populations. This provides support for the potential for mitochondrial introgression to lead to the discrepancies between the datasets.

The color patterns of *B. ephippiatus* queens and workers found in Middle Central America (Figs. 1 and 3) suggest that there might be morphological differentiation considering the queens of the two distinct genetic groups in Honduras. No queen samples were obtained from Honduras, but observations of museum collections have shown that an orange, yellow and black queen form and a yellow and black queen form both exist in Middle Central America (Fig. 1). Further sampling of queens might reveal that the red queen morph corresponds to one of the Honduras clusters, and the black queen morph corresponds to the other Honduras cluster. Although we refrain here from making formal taxonomic revisions of this species complex until further data are obtained, the correlation between trends in color pattern and genetic data suggest that *B. ephippiatus* comprises multiple distinct species. The extensive genetic diversity demonstrated within this complex may also serve as an indication that numerous other known polymorphic *Bombus* species may contain geographic and genetic structure, especially for other montane species in Mesoamerica and other geographic regions.

4.4. Conservation implications

The research presented here demonstrates that EW comprises at least four independently evolving lineages, if not species; therefore, treating *B. ephippiatus* as a single species will not be appropriate for their development as a commercial pollinator. Efforts are now being made to rear *B. ephippiatus* from Mexico as a commercial pollinator for Mexico and Central America (J. Vermeulen, personal comm.). If *B. ephippiatus* colonies from Mexico are imported throughout Central America for pollination services, the threats of hybridization and reproductive disturbance are likely (Kondo et al., 2009; Yoon et al., 2009). We recommend that bombiculture companies take the results of this study into consideration when exploring new species for pollination services in Mesoamerica.

4.5. Perspectives and future directions

This research represents a major step towards understanding how the intricate geographic history of Mesoamerica has enhanced the divergence of bumble bees. Our data support five putative species within the *B. ephippiatus-B. wilmattae* complex. From our extensive multilocus analyses, as well as general trends in the distribution of color patterns over the distinct genetic clades, it is most likely that the *B. ephippiatus* present in Mexico north of the Isthmus of Tehuantepec are a distinct species. Due to conflicts between the microsatellite and sequence data it is unclear to which putative species the *B. ephippiatus* from Chiapas belong, and additional sampling is needed to resolve this question. Our data also suggest that two sympatric species of *B. ephippiatus* exist in Mexico south of the Isthmus of Tehuantepec and in the highlands of Guatemala and Honduras, in addition to a distinct *B. wilmattae* species. Finally, there appears to be a distinct species of *B. ephippiatus* in Costa Rica evident both from genetic data and a distinct queen phenotype (Fig. 3).

These lineages attest to the potential complexity underlying processes of speciation, particularly with regard to the complex events of the late Pliocene and Pleistocene witnessed by the temperate adapted bumble bees. While it is clear that EW comprises several independently evolving lineages, additional population genetic tests, including model-based coalescent methods (e.g., Knowles and Maddison, 2002; Hey and Nielsen, 2004; Hickerson et al., 2006), as well as larger taxon sampling and additional genetic data will be required to resolve species hypotheses in the EW species

complex. Documenting the evolutionary divisions within extant faunas provides insights into how communities have responded to and been shaped by historical events. This study of bumble bees provides a powerful basis for comparative analyses of evolutionary processes leading to the diversification of wide ranging Mesoamerican taxa.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.03.018.

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