

Thoracobombus from Mexico: a description of the male species-specific cephalic labial gland secretions

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Abstract – The male cephalic labial gland secretions of bumblebees are known to be species-specific semiochemicals. These secretions that are involved in bumblebee pre-mating recognition provide efficient diagnostic characters for species delimitation. The subgenus *Thoracobombus* is the largest group of bumblebees and is found in the Palearctic, Nearctic, and Neotropical regions. Here, the cephalic labial gland secretion (CLGS) composition of six Mexican *Thoracobombus* bumblebee species are analyzed: *Bombus diligens*, *B. medius*, *B. mexicanus*, *B. pensylvanicus*, *B. trinominatus*, and *B. weisi*. Our results suggest the presence of two new potential species into the formerly recognized *B. weisi* as well as one new potential species in the taxon presently identified as *B. pensylvanicus*. Moreover, the male of *B. pensylvanicus*, known to congregate at nest sites awaiting the emergence of virgin queens, is characterized by low concentrations of the C16 component. This observation raises the possibility that courtship behavior as well as environmental constraints could affect the role of the male bumblebees' CLGS.

Thoracobombus / Mexico / cephalic labial glands / courtship behavior / species-specific

1. INTRODUCTION

The family Apidae (Hymenoptera) contains 18 tribes including Bombini. This tribe is composed of social bees of the genus *Bombus* (Michener 2007) and currently includes about 260 species in 15 subgenera (Williams 1998; Williams et al. 2008). The majority of these

species are much more common and diverse in temperate areas of Eurasia and North America than in Neotropical region, where six subgenera and 42 species have been recorded (Abrahamovich and Diaz 2002). Indeed, compared to the Palearctic region, the Neotropical region appears to have a less investigated bumblebee fauna (Williams 1998). This difference in number of recorded species could be explained by (i) a low sampling efforts in tropical regions compared to temperate ones, complicated by the difficulties in collecting specimens in these regions and therefore the presence of a large number of undiscovered species and (ii) the low number of researchers sampling and determining bumblebees.

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After the studies on western hemisphere bumblebees carried out by Franklin (1913) and Frison (1925), the most recent revisions are those of Milliron (1971, 1973a, b). Other regional studies were carried out in Brazil (Eustáquio Santos Júnior et al. 2015; Françoso et al. 2016; Moure and Sakagami 1962), Mexico and Central America (Labougle 1990), Colombia (Lievano-Leon et al. 1994), Argentina (Abrahamovich and Diaz 2001), and Peru (Rasmussen 2003). In Mexico, 19 species belonging to 5 subgenera were recorded (Abrahamovich and Diaz 2002; Franklin 1913; Frison 1925; Labougle 1990; Michener 2007; Milliron 1971, 1973a).

The *Thoracobombus* subgenus is the largest group of bumblebees and is found in the Palearctic, Nearctic, and Neotropical regions (Williams 1998). However, despite its large distribution, it remains quite rare and localized in the New World (Cameron et al. 2011; Milliron 1973a). Within this subgenus, nine species have been registered in Mexico. Compared to most bumblebees living in temperate regions (Lavery and Plowright 1985), some additional social characteristics such as the development of large colonies (Michener and LaBerge 1954; Taylor and Cameron 2003; Zucchi 1973), or well-developed defense systems (Cameron et al. 1999; Dias 1954) have been highlighted in some Neotropical *Thoracobombus* species.

Mexico is a highly diverse country with large deserts (Chihuahuan Desert), mountain ranges (Sierra Madre Occidental, Sierra Madre Oriental, Trans-Mexican volcanic belt), and highlands (Altiplano) and is biogeographically split by the Tehuantepec Isthmus in the south. These characteristics are favorable to the diversification of animals such as birds, insects, and lizards (Bryson et al. 2012; Cognato et al. 2008; Rodríguez-Gómez et al. 2013). However, in the majority of the case, while geographic isolation combined with an interruption of gene flow between populations is recognized as one of the main speciation drivers (Coyne and Orr 2004; Sobel et al. 2010), other process of divergence involving the choice of the sexual partner also play an important role (Panhuis et al. 2001; Vereecken et al. 2007).

The male cephalic labial gland secretions (CLGS) are complex mixtures composed of

mainly aliphatic compounds and are synthesized de novo (Luxová et al. 2003; Žáček et al. 2013). They are known to be species-specific semiochemicals (Ayasse and Jarau 2014; Calam 1969; Terzo et al. 2003). These secretions are involved in bumblebee pre-mating recognition by attracting conspecific virgin females (Ayasse and Jarau 2014) but additional analyses are needed to determine which compounds are directly involved in sexual recognition. Moreover, they provide efficient diagnostic characters for species delimitation (Bergström et al. 1981; Brasero et al. 2015, 2017; Lecocq et al. 2015; Rasmont et al. 2005; Svensson and Bergström 1979; Terzo et al. 2003). Within bumblebees, cephalic labial gland secretions of 88 species are currently known (Brasero 2018). In *Thoracobombus*, as well as in other subgenera, the majority of compounds are shared by a large number of species. Moreover, as already shown in other insect groups such as beetles (Symonds and Elgar 2004), it does not seem to be a phylogenetic pattern in the pheromones of bumblebees.

In this paper, we aim to characterize the CLGS composition of six Mexican *Thoracobombus* bumblebee species in order to highlight divergences in their sexual recognition system: *B. diligens*, *B. medius*, *B. mexicanus*, *B. pennsylvanicus*, *B. trinominatus*, and *B. weisi*.

2. MATERIAL AND METHODS

2.1. Sampling

Sixty-nine male *Thoracobombus* specimens were collected in Mexico between 2015 and 2016. They belong to 6 species: *Bombus diligens* ($n = 1$), *B. medius* ($n = 9$), *B. mexicanus* ($n = 2$), *B. pennsylvanicus* ($n = 8$), *B. trinominatus* ($n = 30$), and *B. weisi* ($n = 19$) (Supplementary file 1, Figure 1). All specimens were identified using references keys (Labougle et al. 1985; Milliron 1973a, b; Williams et al. 2008) and compared to the reference collection of El Colegio de la Frontera Sur (ECOSUR) in Mexico. Following the protocol found in De Meulemeester et al. 2011, males were killed by freezing at $-20\text{ }^{\circ}\text{C}$ and their CLGS were extracted in n -hexane (400 μl). Samples were stored at $-40\text{ }^{\circ}\text{C}$ prior to the analyses.

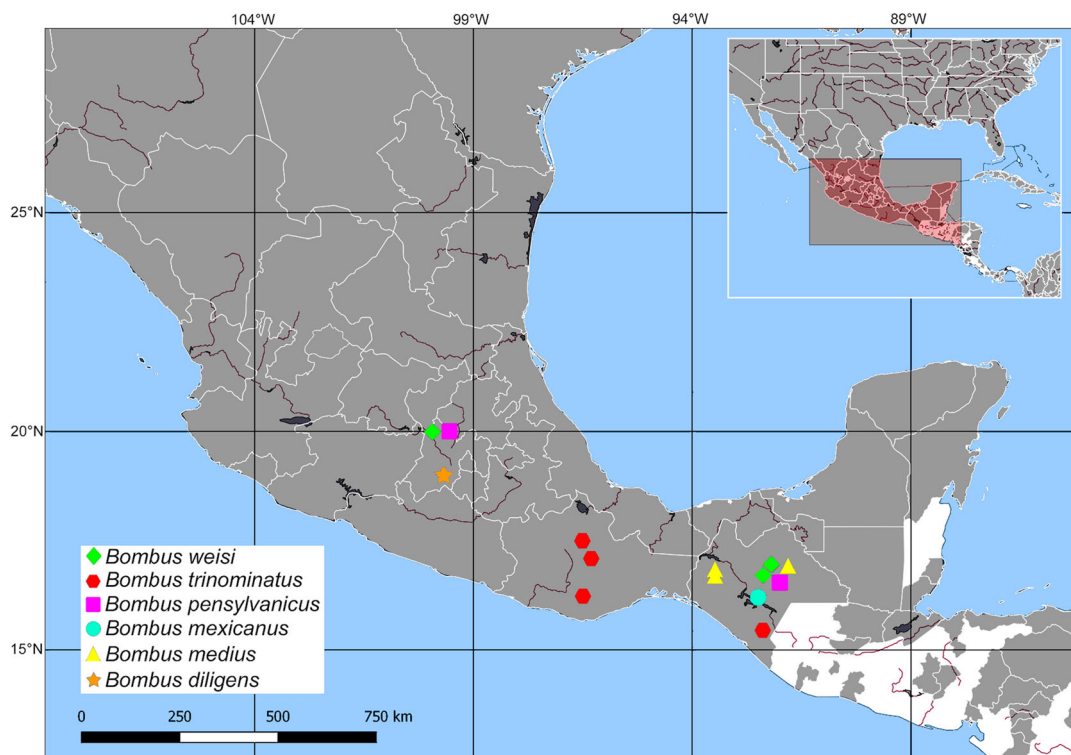


Figure 1 Maps of sampling sites of *B. medius*, *B. diligens*, *B. pensylvanicus*, *B. mexicanus*, *B. trinominatus*, and *B. weisi*.

2.2. Chemical trait differentiation

CLGS compositions were determined by gas chromatography-mass spectrometry (GC/MS) using a Focus GC (Thermo Scientific) with a non-polar DB-5 ms capillary column (5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm) coupled to a DSQ II quadrupole mass analyzer (Thermo Scientific) with 70 eV electron impact ionization. We used a splitless injection mode (220 $^{\circ}\text{C}$) and helium as a carrier gas (1 ml/min). The temperature of the oven was set at 70 $^{\circ}\text{C}$ for 2 min and then heated up at a rate of 10 $^{\circ}\text{C}/\text{min}$ to 320 $^{\circ}\text{C}$. The temperature was then held at 320 $^{\circ}\text{C}$ for 5 min. Compounds were identified in XcaliburTM using the retention times (t_r) and mass spectra of each peak, compared with those at the National Institute of Standards and Technology Library (NIST, U.S.A.). Double bond positions (C=C) were determined by dimethyl disulfide (DMDS) derivatization (Cvacka et al. 2008).

CLGS compounds were quantified using a gas chromatography-flame ionization detector Shimadzu GC-2010 equipped with an SLB-5ms non-polar capillary column (5% phenyl (methyl) polysiloxane stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25- μm film thickness) with the same chromatographic conditions as in GC/MS. Peak areas of compounds were quantified in GC solution Postrun (Shimadzu Corporation) with automatic peak detection and noise measurement. The relative amounts (RA in %) of compounds in each sample were calculated by dividing the peak areas of compounds by the total area of all compounds. We left out all compounds for which RA were lower than 0.1% for all specimens (De Meulemeester et al. 2011). All compounds having an RA equal to or greater than 1% were considered to be abundant compounds. Main components were defined as those with the highest relative amount in at least one individual of the taxon. We used GCaligner 1.0 (Dellicour and Lecocq

2013) to define Kovats indices and the data matrix as the alignment of each compound between all samples (see Supplementary file 2).

R environment was used to perform CLGSs statistical comparative analyses (R Development Core Team 2017) in order to detect differentiations between Mexican taxa. Data consisting of the relative contents of all compounds were transformed ($\log(x + 1)$) to reduce the large content differences between highly and slightly concentrated compounds (Brasero et al. 2015). A clustering method based on a Pearson correlation matrix based and computed with an unweighted pair group method with arithmetic mean (UPGMA) was used (R package ape, Suzuki and Shimodaira 2011). The uncertainty in hierarchical cluster analysis was assessed using p values calculated via multiscale bootstrap resampling with 100,000 bootstrap replications (significant branch supports > 0.85) (R package pvclust, Suzuki and Shimodaira 2011). Moreover, we calculated the CLGS differentiations of taxa by performing a permutation multivariate analysis of variance using distance matrix (PerMANOVA) (R package vegan, Oksanen et al. 2011). A pairwise multiple comparison with an adjustment of p values (the Bonferroni correction) to avoid type I errors was performed when a significant difference was detected.

For each taxon, we determined specific compounds (indicator compounds, IC > 0.70) using the indicator value (IndVal) method (Clausen et al. 2006; Dufrière and Legendre 1997). The given value is the product of relative abundance and relative frequency of occurrence of a compound within a group. The statistical significance of an indicator compound (threshold of 0.01) was evaluated with a randomization procedure.

Photographs have been performed with an Olympus OMDEM1 camera with a Zwiko 60-mm 1:2.8 macro-lens and with three Godox TT355o flash lights. Processing stack has been performed with Zerene Stacker software.

3. RESULTS

Within the CLGS, our chemical analyses revealed 97 compounds (39 from *B. diligens*, 41 from *B. medius*, 30 from *B. mexicanus*, 53 from

B. pensylvanicus, 38 from *B. trinominatus*, and 53 from *B. weisi*) (Supplementary file 2).

The cluster analyses (69 specimens \times 97 compounds) highlighted 9 well-supported groups (bootstrap values > 0.85) all characterized by main compounds (Figure 2): (i) *B. diligens* with an inseparable mixture of isomers eicos-11-en-1-ol/eicos-13-en-1-ol (46.41%) as main compounds; (ii) *B. medius* with the octadec-11-en-1-ol (48.44–70.85%); (iii) *B. mexicanus* with the octadec-9-en-1-ol (40.21–62.85%); (iv) *B. pensylvanicus* (part A: ‘sonorus’) with the octadec-11-en-1-ol (55.55–61.56%); (v) *B. pensylvanicus* (part B: ‘Chiapas’) with the octadec-11-en-1-ol (37.00–51.71%); (vi) *B. trinominatus* with the dodecyl octanoate (2.84–47.03%), eicos-11-en-1-ol/eicos-13-en-1-ol (3.04–32.28%) and the dodecyl icos-11-enoate (1.48–44.24%); (vii) *B. weisi* (part A: ‘nigrodorsalis’) with the eicos-11-en-1-ol/eicos-13-en-1-ol (31.80–37.43%); (viii) *B. weisi* (part B) with the eicos-11-en-1-ol/eicos-13-en-1-ol (19.16–33.21%); (ix) *B. weisi* (part C) with the octadec-11-enyl acetate (13.40–31.28%), eicos-11-en-1-ol/eicos-13-en-1-ol (0.12–17.88%) (Table I). Qualitative differentiations are shown by our analyses between the nine groups, including main compounds for some of them. For the other ones, abundant and minor compounds allowed us to differentiate groups. All identified compounds are listed in Supplementary file 2.

The IndVal method highlighted several significant and unique indicator compounds for each groups (IndVal > 0.70): (i) *B. diligens* (IC = 6); (ii) *B. medius* (IC = 3); (iii) *B. mexicanus* (IC = 8); (iv) *B. pensylvanicus* (part A: ‘sonorus’) (IC = 2); (v) *B. pensylvanicus* (part B: ‘Chiapas’) (IC = 5); (vi) *B. trinominatus* (IC = 8). Indicator compounds were found for (vii) *B. weisi* (part A: ‘nigrodorsalis’) (IC = 6) whereas none were detected for (viii) *B. weisi* (part B) or (ix) *B. weisi* (part C) (Figure 2; Supplementary file 2). A permutation multivariate analysis of variance (PerMANOVA) confirmed differentiations observed between all these taxa (all p values < 0.05) (Supplementary file 3).

4. DISCUSSION

Our analyses based on CLGS of male *Thoracobombus* collected in Mexico permitted us to split the specimen into nine different groups.

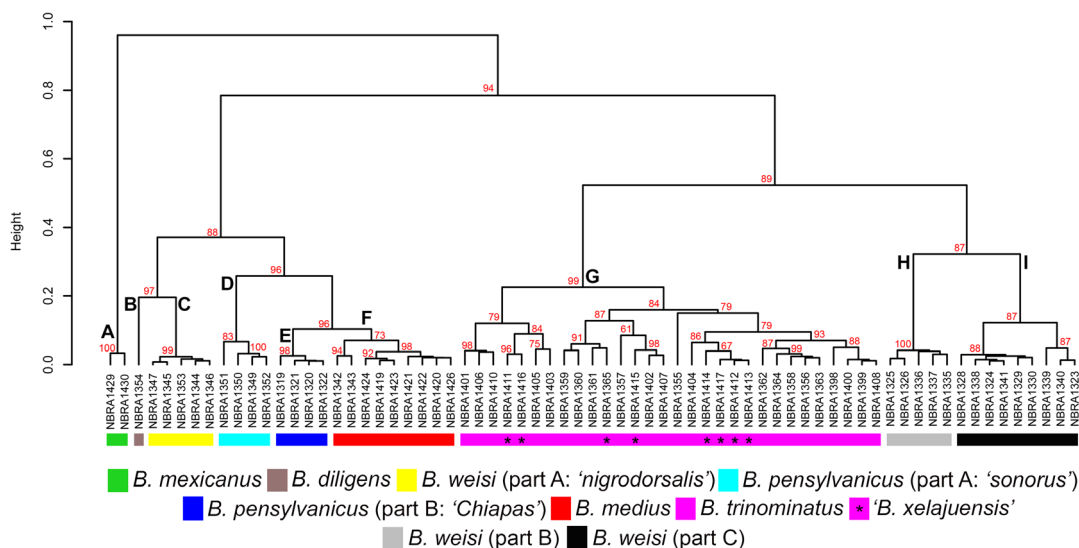


Figure 2 Unweighted pair group method with arithmetic mean cluster based on a correlation matrix calculated from matrix of cephalic labial gland secretions of Mexican *Bombus mexicanus*, *B. diligens*, *B. weisi* (part A: 'nigrodorsalis'), *B. pensylvanicus* (part A: 'sonorus'), *B. pensylvanicus* (part B: 'Chiapas'), *B. medius*, *B. trinominatus*, '*B. xelajuensis*,' *B. weisi* (part B), and *B. weisi* (part C). Values above branches represent multiscale bootstrap resampling. Indval compounds = A: octadec-9-enal, octadecanal, octadec-9-en-1-ol, octadec-9-enyl acetate, octadecenyl butyrate, tetracos-9-ene, hexacos-9-ene and octadecadienyl octadecenoate; B: octadecanol, ethyl octadec-11-enoate, icosenyl acetate, decyl octadec-11-enoate, icosenyl tetradecanoate, and octadecenyl octadecanoate; C: dodecyl butyrate, propyl tetracosane, heptacosene, nonocos-5-ene, hentriacosadiene, and icosenyl dodecanoate; D: farnesene, geranylgeraniol, geranylgeranial, propyl docosane, and dodecyl tetradec-5-enoate; E: dotriacontane and tritriacontene; F: dodecyl acetate, U3, and U4; G: icos-11-enal, icos-11-enyl acetate, icosyl octanoate, icosenyl tetradec-7-enoate, icosyl hexadecenoate, icosenyl octadecenoate, icosenyl octadecatrienoate, and icosenyl icosenoate; H: /; I: /.

This first characterization allowed us to highlight some significant differentiations in the CLGSs composition within the *Thoracobombus* subgenus. However, most of the species studied in this paper have a widespread occurrence and are not limited to Mexico (Abrahamovich and Diaz 2002; Williams 1998), and specimens from the whole distribution area should be analyzed and confirmed by other methods as genetic and morphological tools. Here, we will discuss here the taxonomic knowledge within the species studied and hypothesized on the basis of the results obtained with CLGS.

4.1. *B. pensylvanicus* group

Bombus pensylvanicus is a widespread species and is very variable in color pattern (Williams et al. 2014). The taxonomy of this group remains

unclear and only one species is generally considered: *B. pensylvanicus* Cresson 1863 (Cameron et al. 2007; Labougle 1990; Labougle et al. 1985; Milliron 1973a; Poole and Gentili 1996; Williams 1998). However, two forms exist in the USA, a lighter subspecies named *B. sonorus* Say 1837 distributed in the southwest and the nominal subspecies in the east. These two subspecies are geographically intermixed in the northern part of Mexico and southwestern Texas, where intermediate specimens are recorded (Labougle 1990; Williams et al. 2014). Several other authors considered *B. pensylvanicus* and *B. sonorus* as separated species (Franklin 1913; Stephen 1957; Thorp 1983). In this paper, we analyzed two forms which correspond to the subspecies *B. sonorus* (*B. pensylvanicus* part A) (Figure 3 I, J) and another unnamed taxon (*B. pensylvanicus* part B) (Figure 3 G, H) endemic to Chiapas

Table 1. List of main compounds (black background) identified for *B. diligens*, *B. medius*, *B. mexicanus*, *B. pensylvanicus* (part B), *B. pensylvanicus* (part A), *B. trinominatus*, *B. weisi* (part A), *B. weisi* (part B), and *B. weisi* (part C). MW molecular weight, M median of compound relative proportion (%). Abundant compounds are on gray background. All identified compounds in Supplementary file 1

Compounds	<i>B. diligens</i> (n = 1)			<i>B. medius</i> (n = 9)			<i>B. mexicanus</i> (n = 2)			<i>B. pensylvanicus</i> (part B): 'Chiapas' (n = 4)			<i>B. pensylvanicus</i> (part A): 'sonorus' (n = 4)			
	MW	Min	Max	Min	M	Max	Min	M	Max	Min	M	Max	Min	M	Max	
Octadec-9-en-1-ol	268	0.00	0.00	0.00	0.00	0.00	40.21	51.53	62.85	0.00	0.00	0.00	0.00	0.00	0.00	
Octadec-11-en-1-ol	268	10.56	10.56	48.44	63.49	70.85	0.00	0.00	0.00	0.00	55.55	59.09	61.56	31.45	38.81	
Eicos-11-en-1-ol/ Eicos-13-en-1-ol	296	46.41	46.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
Octadec-11-enyl acetate	310	0.00	0.00	0.00	0.27	0.56	0.07	0.08	0.08	0.08	0.70	0.84	1.89	0.00	0.00	
Dodecyl octanoate	312	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.95	3.96	
Dodecyl icos-11-enoate	478	0.88	0.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
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<i>B. pensylvanicus</i> (part A: 'sonorus') (n = 4)																
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<i>B. trinominatus</i> (n = 22)																
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<i>B. weisi</i> (part A: 'nigrodorsalis') (n = 5)																
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<i>B. weisi</i> (part B) (n = 5)																
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<i>B. weisi</i> (part C) (n = 9)																
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<i>B. xelajúensis</i> ' (n = 8)																
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Compounds	Max	Min	M	Max	Min	M	Max	Min	M	Max	Min	M	Max	Min	M	Max
Octadec-9-en-1-ol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Octadec-11-en-1-ol	51.71	0.00	0.00	0.00	0.00	19.55	19.87	24.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Eicos-11-en-1-ol/ Eicos-13-en-1-ol	0.00	3.04	8.40	32.28	31.80	36.52	37.43	37.43	19.16	28.02	33.21	0.12	1.34	17.88		
Octadec-11-enyl acetate	0.00	0.00	0.00	0.00	0.28	0.37	0.54	3.72	6.38	7.21	13.40	25.79	31.28			
Dodecyl octanoate	6.02	2.84	15.15	47.03	2.68	3.78	5.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dodecyl icos-11-enoate	0.00	1.48	20.54	44.24	0.00	0.00	0.00	0.00	4.00	6.33	11.75	1.85	4.20	15.23		



Figure 3 A Male of *B. diligens*, B female of *B. diligens*, C female of *B. medius*, D male of *B. weisi* (part A: ‘nigrodorsalis’), E female of *B. weisi* (part A: ‘nigrodorsalis’), F female of *B. pensylvanicus* (part B: ‘Chiapas’), G male of *B. pensylvanicus* (part B: ‘Chiapas’), H male of *B. pensylvanicus* (part A: ‘sonorus’), I female of *B. pensylvanicus* (part A: ‘sonorus’), J male of *B. trinominatus*, K female of *B. trinominatus*, L male of *B. weisi*, M female of *B. weisi*, N male of ‘*B. xelajuensis*,’ and O female of ‘*B. xelajuensis*.’ All white lines correspond to a scale of 1 cm. All photographs are by P. Rasmont.

(Figure 1; Supplementary file 1) known to be morphologically (scutellum and first tergite totally black) and genetically different (Hatfield et al. 2015). Our analyses clearly show a differentiation between these two populations suggesting that the taxonomic status of this group should be reconsidered. While these two populations share the same main components (Table I), the subspecies *B. sonorus* (*B. pensylvanicus* part A) is characterized by the presence of geranylgeraniol (9.93–18.30%) which is totally absent in the population in Chiapas (Figure 2, Supplementary file 2). This differentiation is supported by our IndVal analyses, which highlighted specific indicator compounds for the *sonorus* subspecies (*B. pensylvanicus* part A) (IC = 5) as well as for the unnamed taxon (*B. pensylvanicus* part B) from Chiapas (IC = 2) (Figure 2; Supplementary file 2) but also by the PerMANOVA (p values < 0.05; $F = 13.597$).

Bergström et al. 1996 already characterized the CLGS composition of the *B. sonorus* subspecies and our specimens have shown the same composition. However, even if this population is very similar to the population of Chiapas, major differentiations are found in abundant and minor compounds (Figure 2, Supplementary file 2). Such cases have already been described by comparing different species such as *B. filchnerae* and *B. muscorum* (Brasero et al. 2017), as well as within the *Cullumanobombus* subgenus (Brasero et al. 2015).

At the moment, no research has analyzed the CLGS composition of the nominal form. Considering the morphological differences, the specific CLGS composition, and the first genetic approach (Hatfield et al. 2015), we consider to be in presence of a new unnamed species endemic to the state of Chiapas.

Ecologically speaking, males of *B. pensylvanicus* are known to congregate at nest sites, awaiting the emergence of virgin queens (Villalobos and Shelly 1987) as found in *B. muscorum* by Darvill et al. 2007. As mentioned in Brasero et al. 2017 about *B. muscorum* and *B. filchnerae*, the CLGS composition of *B. pensylvanicus* also includes very low concentrations of compounds below 18 carbons. This observation therefore strengthens our hypothesis, suggesting that the long pre-mating recognition distance could be mediated by cues other than the

CLGS such nest site choice (i.e., the habitat) or nest scent (Brasero et al. 2017).

4.2. *B. trinominatus* group

Since Asperen De Boer 1992 described *Bombus xelajuensis* from one locality in Guatemala, the taxonomy of this group remains unclear. This taxon also seems to occur as well in Mexico (south of Chiapas state) with the same pattern (especially the fairly narrow black interalar band intermixed with yellow hairs) (Abrahamovich and Diaz 2002; Asperen De Boer 1992; Martinez Lopez and Vandame 2015). However, only one species is currently considered valid: *B. trinominatus* Dalla Torre 1890 (Williams 1998). Here, we analyzed specimens identified as *B. trinominatus* (Figure 3 K, L) from Oaxaca state as well as specimens identified as *B. xelajuensis* (Figure 3 O) from Chiapas state (Figure 1; Supplementary file 1). These two populations share the same main compounds and have no differences in other compounds that would significantly separate them in our clustering, PCA (Figure 2), and PerMANOVA (p value > 0.05, $F = 1.749$) analysis. In the light of these results, it seems that *B. xelajuensis* and *B. trinominatus* are conspecific as already considered by Williams 1998.

4.3. *B. weisi* group

In this group, *Bombus weisi* and *B. nigrodorsalis* have been respectively described by Friese 1903; Franklin 1907 based on morphological characters. However, only one species is currently considered as valid: *B. weisi* Friese 1903 (Labougle 1990; Williams 1998). While *B. weisi* has a distribution including Mexico, Guatemala, Honduras, and Costa-Rica (Abrahamovich and Diaz 2002; Vandame and Martinez Lopez 2016), *B. nigrodorsalis* occurs in sympatry with *B. weisi* to the north of the isthmus of Tehuantepec and is endemic to Mexico. Here, we analyzed specimens determined as *B. weisi* (Figure 3 M, N) and *B. nigrodorsalis* (Figure 3 E, F) respectively from the south and the north of the isthmus of Tehuantepec (Figure 1; Supplementary file 1). Three well-supported groups are highlighted by our analyses (Figure 2): *B. weisi* (part A: *B. nigrodorsalis*); (ii) *B. weisi* (part B); and (iii) *B. weisi* (part C). While all

groups possessed the eicos-11-en-1-ol/eicos-13-en-1-ol as main compounds, the octadec-11-en-1-ol (19.55–24.61%) has been highlighted as an abundant compound in *B. weisi* (part A: *B. nigrodorsalis*) (Supplementary file 2). Moreover, the octadec-11-enyl acetate (13.40–31.28%) has been highlighted as another main component in *B. weisi* (part C) (Table 1; Supplementary file 2). Indicator compounds were found for *B. weisi* (part A: *B. nigrodorsalis*) (IC = 6) whereas none were detected for *B. weisi* (part B and C) (Supplementary file 2). PerMANOVA analyses confirmed the differentiation between these groups (all p values < 0.05; *B. weisi* (part A: *B. nigrodorsalis*) vs *B. weisi* (part B), $F = 54.76$; *B. weisi* (part A: *B. nigrodorsalis*) vs *B. weisi* (part C), $F = 79.084$, and *B. weisi* (part B) vs *B. weisi* (part C), $F = 34.486$) (Supplementary file 3).

Considering the CLGS composition as an essential criterion used in the pre-mating recognition system (Paterson 1993) and the morphological differences, we support the specific status of *B. nigrodorsalis* Franklin 1907 and hypothesized the presence of two species inside *B. weisi*: *B. weisi* Friese 1903 and a new unnamed species.

4.4. *B. diligens*, *B. medius*, and *B. mexicanus*

Our clustering and PCA analysis enabled us to highlight the species specificity of their CLGS composition (Figure 2). Indeed, PerMANOVA analyses confirmed the CLGS composition differentiations (all p values < 0.05): *B. diligens* vs *B. medius* ($F = 33.953$); *B. diligens* vs *B. mexicanus* ($F = 100.6058$); and *B. medius* vs *B. mexicanus* ($F = 89.968$) (Figure 3 A, B, and C).

5. CONCLUSION

The cephalic labial glands secretions are considered as a practical and useful diagnostic trait to delineate bumblebee species. However, many aspects related to the ecology of these secretions are still unknown. Preliminary studies made (Brasero et al. 2017; Brasero 2018) have shown that males prenuptial behavior influenced the composition of these secretions. Indeed, in males performing patrolling behavior, the average molecular mass of abundant compounds at the beginning of chromatograms is significantly smaller than that of

other males performing nest waiting or perching behaviors. Further studies are needed to confirm these hypotheses.

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AUTHORS CONTRIBUTIONS

NB, RM, and PR conceived this research and designed experiments; NB, BM, and PR participate in the design and interpretation of the data; NB and BM performed experiments and analysis; NB, RM, PS, BM, IV, and PR wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript. Funding information

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Thoracobombus du Mexique: description des sécrétions glandulaires labiales céphaliques spécifiques aux mâles

Thoracobombus / Mexique / glandes labiales céphaliques / comportement de parade nuptiale / spécifique à une espèce

***Thoracobombus* aus Mexiko: Eine Beschreibung der männchenspezifischen Kopflabialdrüsensekrete**

***Thoracobombus* / Mexiko / Kopflabialdrüsen / Paarungsverhalten / Artspezifität**

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