Variability in Sexual Pheromones Questions their Role in Bumblebee Pre-Mating Recognition System

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Abstract

Sex-specific chemical secretions have been widely used as diagnostic characters in chemotaxonomy. The taxonomically confused group of bumblebees has reaped the benefit of this approach through the analyses of cephalic labial gland secretions (CLGS). Most of currently available CLGS descriptions concern species from the West-Palearctic region but few from the New World. Here, the CLGS of four East-Palearctic species *Bombus deuteronymus*, *B. filchnerae*, *B. humilis*, and *B. exil* (subgenus *Thoracobombus*) are analysed. Our results show high levels of variability in the major compounds in *B. exil*. In contrast, we describe a low differentiation in CLGS compounds between *B. filchnerae* and its phylogenetically closely related taxon *B. muscorum*. Moreover, the chemical profiles of *B. filchnerae* and *B. muscorum* are characterized by low concentrations of the C16 component, which is found in higher concentrations in the other *Thoracobombus* species. This raises the possibility that courtship behavior as well as environmental constraints could affect the role of the bumblebee males' CLGS.

Keywords Courtship behavior · Pheromones · Cephalic glands · Social insect · Pollinator

Introduction

In many animal species, sex-specific chemical secretions are involved in pre-mating communication (Hay 2011; Lecocq et al. 2015a; Patricelli et al. 2003). This key role in reproductive behavior has led to the development of chemotaxonomy in which such chemical traits are used as diagnostic characters to assess taxonomic status (Bagnères et al. 2003; Pokorny et al. 2014; Quicke 1993). The systematics of insects has especially reaped the benefits of this approach (Lockey 1991). Bumblebees (Hymenoptera, Apidae) give a stunning example of the improvements conferred by chemotaxonomy (Bertsch and Schweer 2011; Lecocq et al. 2015b). In this taxonomically confused group, morphological traits are often inefficient diagnostic characters because different species can be morphologically highly similar while conspecific populations can be very divergent in their coat color pattern (Lecocq et al. 2015b; Michener 1990; Williams 1998). In contrast, the male cephalic labial gland secretions (CLGS) are known to be species-specific semiochemicals (Ayasse and Jarau 2014; Calam 1969). These secretions are produced *de novo* (Luxová et al. 2003; Žáček et al. 2013) and attract conspecific virgin females (Ayasse and Jarau 2014). They appear to provide efficient diagnostic characters for species delimitation (Bergström et al. 1981; Lecocq et al. 2015c; Rasmont et al. 2005; Svensson and Bergström 1979; Terzo et al. 2003).

During recent decades, an increasing number of studies have based their taxonomic conclusions on inter-taxon comparisons of CLGS composition (e.g. Bertsch and Schweer 2011; Lecocq et al. 2015b, d). However, global taxonomic assessment of bumblebee species through chemotaxonomy

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requires an extensive overview of CLGS composition. Currently, CLGS composition has been described for approximately 65 species (Ayasse and Jarau 2014; Brasero et al. 2015; Lecocq et al. 2015c, d; Rasmont et al. 2005; Terzo et al. 2003; Terzo et al. 2007) out of a total of 250 bumblebee species around the world (Williams 1998).

Most of currently available CLGS descriptions concern species from the West-Palearctic region (Calam 1969; Lecocq et al. 2015b; Rasmont et al. 2005) and some from the New World (Bertsch et al. 2004, 2008; Brasero et al. 2015). In contrast, bumblebee fauna from the East-Palearctic region has received far less attention to date (Bergström et al. 1981), even though this area hosts the main bumblebee diversity hotspot (Williams 1998).

Beyond taxonomic matters, more and more ecological questions about the role of compounds emitted by bumblebees have emerged within the scientific community (e.g. Brasero et al. 2017; Lhomme et al. 2015). However, compared to several neural coding studies focused on sex pheromones in honey bees (e.g. Carcaud et al. 2015; Galizia and Menzel 2001; Sandoz 2012), only a few studies have begun to describe the neurophysiology of olfaction in bumblebees (Fonta and Masson 1984; Strube-Bloss et al. 2015). The description of CLGS contents in bumblebees could help achieve a better understanding of these insects' sexual communication behavior and its neurobiological basis.

In this study, the variability in CLGS of four East-Palearctic taxa was investigated to question their role in the pre-mating recognition system: *Bombus deuteronymus superequester* (Skorikov 1914) (Fig. 1a), *B. filchnerae* Vogt 1908 (Fig. 1b), *B. humilis subbaicalensis* Vogt 1911 (Fig. 1c), and *B. exil* (Skorikov 1923) (Fig. 1d) all belonging to the *Thoracobombus* Dalla Torre 1880 subgenus. Moreover, we compared the CLGS composition of *B. filchnerae* with that of its nearest Palearctic taxon: *B. muscorum* (L. 1758) (Fig. 1e, f).

Fig. 1 **a** *Bombus deuteronymus superequester* from Mongolia. **b** *B. filchnerae* from Mongolia. **c** *B. humilis subbaicalensis* from Mongolia. **d** *B. exil* from Mongolia. **e** *B. muscorum muscorum* from The Netherlands. **f** *B. muscorum liepetterseni* from Norway. All photographed by P. Rasmont

Methods and Materials

Sampling One hundred and forty seven queens of *B. deuteronymus superequester* (n = 2), *B. exil* (n = 65), *B. filchnerae* (n = 77) and *B. humilis subbaicalensis* (n = 3) were collected in Mongolia in 2014. One hundred and twenty two queens were killed during collection and 25 were transported alive and reared in Belgium (Table 1). In order to produce males, we maintained queens in a wooden box in a controlled climate, dark room at 26/30 °C and 50–60% relative humidity, and fed them ad libitum on *Salix sp.* pollen and sugar syrup (Lhomme et al. 2013). Ten different queens produced 51 males (see Table 1): *B. deuteronymus superequester* (n = 10), *B. exil* (n = 13), *B. filchnerae* (n = 15), and *B. humilis subbaicalensis* (n = 13). All breeding males used in the analysis were between 7 and 10 days old, which corresponds to the age of active males (Tasei et al. 1998). In order to compare the CLGS compositions of *B. filchnerae*, we used its phylogenetic sister taxon *B. muscorum* (n = 24) whose composition has been described by Lecocq et al. (2015d) (Table 1). We assessed the taxonomic identification of males using the taxonomical revision of North China bumblebees published by An et al. (2014). Indeed, a taxonomical revision can merge different lines of evidence such as morphological approaches, DNA-based methods, and semiochemical signatures in order to build a consensus and clarify taxonomic status. Prior to chemical analyses, all males were killed by freezing at −20 °C and their CLGS were extracted in 400 µl hexane (De Meulemeester et al. 2011). Samples were stored at −40 °C.

Chemical Analyses We determined the chemical composition of CLGS 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 quadrupol mass analyzer (Thermo Scientific) with 70 eV electron ionization. We used a splitless injection mode (220 °C) and helium as the carrier gas (1 ml/min). The temperature of the oven was set to 70 °C for 2 mins and then heated up at a rate of 10 °C/min to 320 °C. The temperature was then held at 320 °C for 5 mins. Compounds were identified in Xcalibur™ using the retention times (t_r) and

Table 1 East-palearctic *Bombus* (*Thoracobombus*) species

Taxa	N	Collecting sites of founder queens		Coordinates
Thoracobombus subgenus				
B. deuteronymus superequester (Skorikov 1914)	10	Mongolia, Tov, Terelj		47°59′28.0″N 107°22′25.7″E
	/	Mongolia, Tov, Terelj	†	47°59′28.0″N 107°22′25.7″E
B. filchnerae Vogt 1908	8	Mongolia, Tov, Terelj		47°59′45.7″N 107°19′46.1″E
	2	Mongolia, Tov, Terelj		47°59′45.7″N 107°19′46.1″E
	3	Mongolia, Tov, Terelj		47°59′45.7″N 107°19′46.1″E
	/	Mongolia, Tov, Terelj	†	47°59′45.7″N 107°19′46.1″E
	/	Mongolia, Tov, Terelj	†(2)	47°59′28″N 107°22′25.7″E
	/	Mongolia, Tov, Terelj	†(2)	47°59′48.1″N 107°20′11.5″E
	2	Mongolia, Arkhangai, Tsenker		47°00′11.4″N 101°43′50″E
B. muscorum pereziellus (Skorikov 1922)	10	France, Corse, Palneca [2012]		42°42′42″N 09°11′27″E
B. humilis subbaicalensis Vogt 1911	4	Mongolia, Tov, Terelj		47°59′45.7″N 107°19′46.1″E
	1	Mongolia, Arkhangai, Tsenker		47°07′40.4″N 101°57′04.5″E
	8	Mongolia, Arkhangai, Kharkhorin		47°03′50.3″N 102°12′23.6″E
B. exil (Skorikov 1923)	8	Mongolia, Tov, Terelj		47°59′28.0″N 107°22′25.7″E
	5	Mongolia, Tov, Terelj		47°59′28.0″N 107°22′25.7″E
	/	Mongolia, Tov, Terelj	†	47°59′28.0″N 107°22′25.7″E
	/	Mongolia, Arkhangai, Bat-Olziit	†(3)	46°47′50.5″N 101°45′18.2″E
	/	Mongolia, Tov, Terelj	†(5)	47°59′45.7″N 107°19′46.1″E
	n	Collecting sites of males		
B. muscorum muscorum (L. 1758)	1	Sweden, Ystad, Mossbystrand [2008]		55°25′10″N 13°38′55″E
	1	Poland, Olsztynski, Wójtowo [2010]		53°47′11″N 20°35′33″E
	1	Russia, Irkutsk Obl., Ust'-Ordinskiy [2010]		52°54′51.98″N 105°04′29.96″E
	1	Ireland, Clare, Ennis [2009]		52°51′37″N 09°02′12″W
	1	France, Pyrenées -Orientales, Millas [2004]		42°42′44″N 02°42′36″E
	1	The Netherlands, Zuid-Beijerland, Tiengemeten [2015]		51°44′37.1″N 04°19′04.8″E
	2	Denmark, Viborg, Moldrup [2010]		56°37′01.37″N 09°30′01.34″E
B. muscorum liepetterseni Löken 1973	5	Norway, Flatanger [2008]		64°28′11″N 10°43′16″E
B. muscorum allenellus Stelfox 1933	1	Ireland, Galway, Inishmore [2009]		53°07′42″N 09°43′54″W

Collecting sites, number (N) of males produced by reared specimens and number (n) of males collected of *Bombus deuteronymus superequester*, *B. filchnerae*, *B. humilis subbaicalensis*, *B. exil* and *B. muscorum*

Coordinates are given with the reference to the WGS84. All specimens from Mongolia were bred in 2014

† Corresponds to the dead queens who have not founded colonies, numbers in brackets corresponding to the number of queens

mass spectra of each peak, compared with those in 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).

We quantified all samples using a gas chromatograph-flame ionization detector Shimadzu GC-2010 with a SLB-5 ms 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 used for the GC/MS. Peak areas of compounds were detected in GCsolution Postrun (Shimadzu Corporation) with automatic peak detection and noise measurement. We calculated relative amounts (RA in %) of compounds in each sample by dividing the peak areas of compounds by the total area of compounds in each sample. We discarded all compounds for which RA were lower than 0.1% for all specimens (De Meulemeester et al. 2011). Moreover, all compounds having an RA equal to or greater than 1% were considered to be abundant compounds. We defined the data matrix as the alignment of each compound between all samples performed with GCAligner 1.0 (Dellicour and Lecocq 2013a, b) (see supplementary file 1).

Statistical Analyses Statistical analyses were performed using R (R Development Core Team 2017) to detect differences in CLGS between taxa. Data consisting of the relative contents of all compounds were standardized (mean = 0, standard deviation = 1), to reduce the sample-concentration effect (Brasero et al. 2015).

A clustering method was used to assess potential differentiation between taxa. A Pearson correlation matrix based on CLGS data matrix was computed. An unweighted pair group method with arithmetic mean (UPGMA) was used as a clustering method (R-package ape, Suzuki and Shimodaira 2011). We assessed the uncertainty in hierarchical cluster analysis using *P*-values calculated via multiscale bootstrap resampling with 50,000 bootstrap replications (R-package pvclust; Suzuki and Shimodaira 2011). We investigated differentiation among CLGS by performing a multiple response permutation procedure (MRPP) (R package vegan, Oksanen et al. 2017).

For each taxon, we used the indicator value (IndVal) method to identify specific compounds (indicator compounds, IC) (Claudet et al. 2006; Dufrêne and Legendre 1997). The given value is the product of the 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 (Dufrêne and Legendre 1997).

In order to visualize the indicator compounds, we compared *B. filchnerae* and its sister species *B. muscorum* by principal component analyses (PCA; R-package MASS; Venables and Ripley 2002).

Results

One hundred and twenty nine compounds were detected in the CLGS across all species: 36 in *B. deuteronymus superequester*, 70 in *B. exil*, 33 in *B. filchnerae*, 35 in *B. muscorum* and 42 in *B. humilis* (Supplementary file 1). The main compounds detected were (i) for *B. deuteronymus superequester*: octadec-11-en-1-ol (44.40% - 57.05%); (ii) for *B. exil*: hexadec-9-enal (0.04% - 44.35%), octadec-9-enoic acid (1.36% - 15.20%), icos-11-en-1-ol (0.20% - 8.88%) and hexadecenyl hexadecenoate (0.69% - 10.53%); (iii) for *B. filchnerae* and *B. muscorum*: octadec-9-enyl acetate (40.49% - 75.96%) and (iv) for *B. humilis subbaicalensis*: octadec-9-en-1-ol (40.12% - 56.64%) (Table 2; Supplementary file 1). Our chemical analyses showed qualitative differentiation among all taxa including specific major compounds (i.e. compounds that have the highest relative abundance among all CLGS compounds in at least one individual of the taxon), except between *B. filchnerae* and *B. muscorum* (Table 2) where only minor compounds diverged, allowing us to differentiate the two taxa (Table 3).

Our statistical analyses confirmed the differentiations of our samples in five groups supported by high multiscale bootstrap resampling values: (i) *B. exil*, (ii) *B. humilis subbaicalensis*, (iii) *B. deuteronymus superequester*, (iv) *B. filchnerae*, and (v)

Table 2 List of main compounds from cephalic labial gland secretions of *Bombus* (*Thoracobombus*) species

Compounds	C	MW	B. deuteronymus superequester			B. exil			B. filchnerae			B. muscorum			B. humilis subbaicalensis		
			Q1	M	Q3	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3	Q1	M	Q3
Hexadec-9-enal	C16	238	0.00	0.00	0.00	0.14	2.05	3.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Octadec-11-en-1-ol	C18	268	45.51	47.09	49.46	0.00	0.00	0.00	4.17	5.00	5.57	0.00	0.00	0.00	8.13	8.84	9.82
Octadec-9-en-1-ol	C18	268	0.00	0.00	0.00	0.83	1.39	1.92	15.40	16.71	19.00	8.63	20.46	43.31	43.31	44.71	44.91
Octadec-9-enoic acid	C18	282	0.30	0.32	0.53	4.84	8.67	11.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Icos-11-en-1-ol	C20	296	0.00	0.00	0.00	1.87	4.21	6.09	0.04	0.05	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Octadec-9-enyl acetate	C20	310	0.00	0.00	0.00	0.00	0.00	0.00	60.27	66.17	70.60	63.25	65.38	71.92	0.19	0.29	0.38
Hexadecenyl hexadecenoate1	C32	476	0.35	0.40	0.48	4.21	6.81	7.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Main compounds (grey background) identified in cephalic labial gland secretions of *Bombus deuteronymus superequester*, *B. exil*, *B. filchnerae*, *B. muscorum* and *B. humilis subbaicalensis*

Abundant compounds are on a black background. All identified compounds are listed in Supplementary file 1

C, the number of carbon atoms; *MW*, the molecular weight; *Q1*, quartile 1; *M*, median of relative concentration of compound (%); *Q3*, quartile 3

Table 3 List of compounds determined from the indicator value (IndVal) method from *Bombus muscorum* and *B. filchnerae*

Compounds			MW	Indval results		M (%)	
Name	carbons	Code		B. filchnerae	B. muscorum	B. filchnerae	B. muscorum
Hexadecan-1-ol	C16	A	242	0.89	0.08	1.08	0.15
Octadec-9-enal	C18	B	266	0	0.92	0	0.06
Hexadecyl acetate	C20	C	284	1	0	0.58	0
Octadec-11-en-1-ol	C18	D	268	0.93	0	5	0
Octadecanol	C18	E	270	0.87	0	0.02	0
Octadecadienoic acid	C18	F	280	0	0.85	0	0.11
U3	/	G	/	0.73	0	0.10	0
Icos-11-en-1-ol	C20	H	296	1	0	0.05	0
Tricos-7-ene	C23	I	322	0	0.79	0	0.20
Icos-11-enyl-acetate	C22	J	338	1	0	0.07	0
Icos-13-enyl-acetate	C22	K	338	1	0	0.07	0
Tetracosane	C24	L	338	0	1	0	0.15
Pentacos-9-ene	C25	M	350	0.28	0.72	0.39	0.71
U7	/	N	/	0.73	0	0.14	0
Heptacos-11-ene	C27	O	378	1	0	0.86	0
Heptacos-7-ene	C27	P	378	0	1	0	0.14
Nonacosene	C29	Q	406	1	0	0.14	0
Nonacos-9-ene	C29	R	406	0.71	0.27	0.56	0.11
Nonacos-7-ene	C29	S	406	0.71	0.06	0.18	0
Nonacosane	C29	T	408	0.78	0.11	0.50	0.08
Hentriacont-9-ene	C31	U	434	1	0	0.17	0
Hentriacontane	C31	V	436	0.8	0.06	0.08	0
Hexadecenyl octadecenoate3	C34	W	504	1	0	0.10	0
Octadec-9-enyl hexadecanoate2	C34	X	506	1	0	0.05	0
Octadecenyl octadecenoate2	C36	Y	532	0	0.96	0	0.37
Octadecenyl octadecenoate	C36	Z	534	0	1	0	0.18

List of compounds identified from cephalic labial gland secretions of *B. muscorum* and *B. filchnerae* after the identification of characteristic compounds (grey background) calculated using the Indicator Value (IndVal) method (> 0.70)

The compounds we were not able to determine are indicated as *Ux*. The full table with minimum and maximum data (min and max [%]) are in the Supplementary file 1

Code, letters used in PCA; *MW*, the molecular weight; *M*, median of relative concentration of compound (%)

B. muscorum (Fig. 2a, b). Moreover, MRPP confirmed these divisions (all *P*-values <0.001): *B. deuteronymus vs B. exil* (A = 0.2993); *B. deuteronymus vs B. filchnerae* (A = 0.3326); *B. deuteronymus vs B. humilis* (A = 0.3868); *B. deuteronymus vs B. muscorum* (A = 0.2551); *B. exil vs B. filchnerae* (A = 0.2876); *B. exil vs B. humilis* (A = 0.3059); *B. exil vs B. muscorum* (A = 0.2501); *B. filchnerae vs B. humilis* (A = 0.3547); *B. filchnerae vs B. muscorum* (A = 0.186), and *B. humilis vs B. muscorum* (A = 0.2648). Several significant indicator compounds were revealed by the IndVal method (IndVal >0.70) (Supplementary file 1).

Discussion

The chemical characterization of CLGS from four East-Palearctic species (*B. deuteronymus*, *B. filchnerae*, *B. humilis*, and B. exil) was performed. Our results highlight the high levels of variability in the main compounds of CLGS from *B. exil*. In contrast, we found low levels of discrimination in CLGS compounds between *B. filchnerae* and its closely related taxon *B. muscorum*. Moreover, the chemical profiles of *B. filchnerae* and *B. muscorum* are characterized by a lack of highly concentrated C16 components, whereas these components are abundant in the other *Thoracobombus* species.

The analyses of CLGS from *B. humilis subbaicalensis* and *B. deuteronymus superequester* allowed us to highlight the species-specificity of their contents.

The intraspecific variability of main compounds in Bombus exil. Bombus exil is characterized by high levels of intraspecific variability in its CLGS composition (Supplementary file 1). It displays four main components (Table 2) and 45 indicator compounds (Supplementary file 1). We therefore hypothesize that pre-mating communication in *B. exil* involves other cues, leading to a relaxation of selective pressure on CLGS chemistry, and thus to an increase of intraspecific variability (Lecocq et al. 2013). Indeed, males of *B. exil* are the only bumblebees to have the last three articles of their antennae broadened and ventrally concave (Tkalců 1974) (Fig. 1d). During copulation they press this clubbed part of the antennae against the lower part of the face of female bees (P. Williams: com. pers. based on unpublished personal observations from Moerdaga, Neimenggu, 18/08/2009). A relatively similar behavior has been reported in grasshoppers bearing clubbed antennae where they are used to reinforce the visual signal associated with the hind leg strokes (Dumas et al. 2010), and in other species where movement of the antennae is used as a visual display even if their antennae are not clubbed (Dumas et al. 2010), suggesting that the apical thickening of the antennae has another key role. In some other Apoids, this kind of
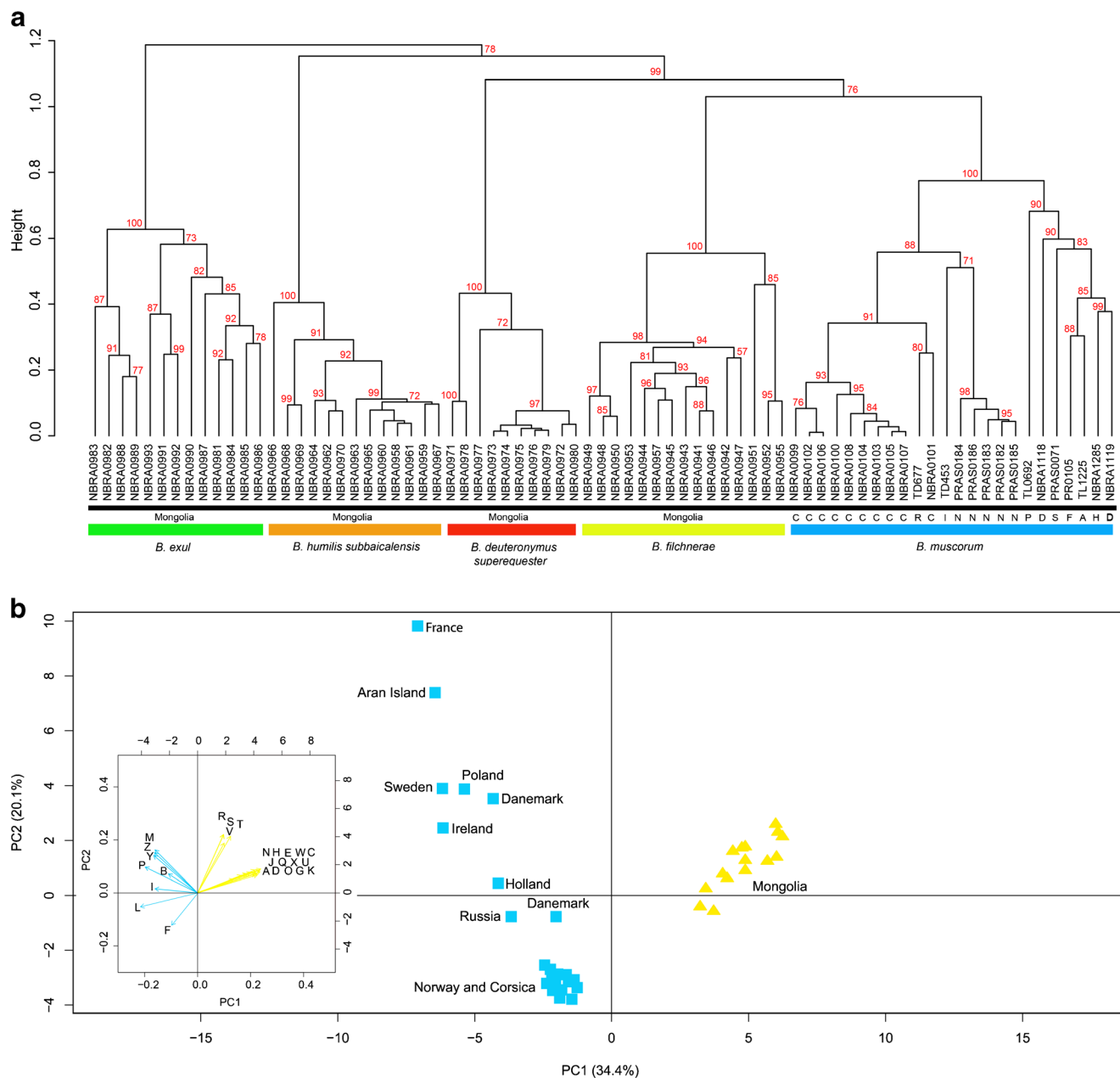
Fig. 2 **a** Unweighted pair group method with arithmetic mean cluster based on a correlation matrix calculated from matrix of cephalic labial gland secretions of *Bombus deuteronymus superequester*, *B. filchnerae*, *B. humilis subbaicalensis*, *B. exil* and *B. muscorum*. Values above branches represent multiscale bootstrap resampling. The letters indicate the geographic origins (C: Corsica; R: Russia; I: Ireland; N: Norway, P:

Poland, D: Denmark; S: Sweden, F: France, A: Aran Island and H: The Netherlands). **b** CLGS Principle components analyses (PCA) of *B. muscorum* (blue squares) and *B. filchnerae* (yellow triangles). PC1 and PC2 are the first and second axes of the PCA. In the left corner is the variable factor map. Each letter (code) corresponds to characteristic compounds revealed by the IndVal method (Table 3)

behavior has also been highlighted both with and without contact with females (Djegham et al. 1994; Felicioli 1998; Romani et al. 2003). Thereby, these modified antennae deserve more chemical and functional morphology studies as well as histological research dealing particularly with the concave area on the last article (Felicioli 1998; Yin et al. 2013).

Bombus filchnerae and B. muscorum differentiation. As mentioned in Terzo et al. (2005) for *B. ruderarius*, abundant compounds may be absent within the same taxon (i.e. for

B. filchnerae; Table 2). Reproductive traits are shaped by selective pressure to maximize encounter rates between conspecific mates (Wyatt 2003) resulting in isolation between heterospecific individuals (Paterson 1993; Symonds et al. 2009). Such selective pressure most likely leads to the strong species-specificity of CLGS (Lecocq et al. 2011; Lecocq et al. 2015d). This species-specificity has been highlighted in all studied bumblebee taxa (Svensson and Bergström 1977; Žáček et al. 2009). Nevertheless, some intraspecific differences related to age or

geography occur (e.g. Lecocq et al. 2015a, d), while remaining lower than interspecific differentiation (Martinet et al. 2017). Low CLGS differentiation is observed between *B. muscorum* and *B. filchnerae* (Supplementary file 1; Tables 2 and 3; Fig. 2a, b). Such a low CLGS differentiation pattern could be explained by (i) the conspecificity of the two taxa (i.e. they are the same species) or (ii) the minor role of CLGS in pre-mating communication relaxing selective pressure on species-specificity. The strong morphological differentiation (i.e. genitalia) (Williams 1998) of the two taxa and their genetic isolation (Cameron et al. 2007; Lecocq et al. 2015d) suggests their heterospecificity, which supports the latter hypothesis. In both *B. muscorum* and *B. filchnerae*, the CLGS include only very low concentrations of the C16 compound and no compounds with shorter carbon chains (Supplementary file 1). Since alkanes with more than 16 carbon atoms are not volatile at a temperature of 20 °C (Cicolella 2008), the CLGS of *B. muscorum* and *B. filchnerae* could not be involved in long distance intersex attraction but rather could play a role at a short distance. Aggregations of *B. muscorum* males in close proximity to mature nest entrances waiting to mate with virgin queens have been reported (Darvill et al. 2007; Krüger 1951). This suggests that the long distance pre-mating recognition could be mediated by cues other than the CLGS, such as nest site choice (i.e. the habitat) or nest scent. Therefore, we advocate that CLGS play a minor role in pre-mating recognition of *B. muscorum* and *B. filchnerae*. Further studies on the pre-mating recognition of bumblebee species are needed to assess this hypothesis.

In the other *Thoracobombus* studied here (*B. exil, B. deuteronymus superequester* and *B. humilis subbaicalensis*) we found a high concentration of the C16 compound and other lighter compounds (Supplementary file 1). These have also been described in other *Thoracobombus* (Brasero et al. 2017; Lecocq et al. 2015b; Terzo et al. 2005) and other bumblebee subgenera (Ayasse and Jarau 2014; Lecocq et al. 2015c, d; Rasmont et al. 2005; Terzo et al. 2003). Indeed, we find no values greater than 2.15% for a C16 component in *B. muscorum* and *B. filchnerae*, whereas values such as 42.88%, 44.35% and 36.10% were found in *B. deuteronymus superequester, B. exil,* and *B. humilis subbaicalensis*, respectively (Supplementary file 1).

This suggests that the CLGS of *B. exil, B. deuteronymus superequester* and *B. humilis subbaicalensis* play the same role in their specific mate recognition systems (Paterson 1993) as in most other bumblebees.

B. humilis subbaicalensis *and B. deuteronymus superequester differentiation.* Based on main and abundant compounds (Table 1), our CLGS analyses demonstrate clear differentiation between *B. humilis subbaicalensis* and *B. deuteronymus superequester* (Fig. 2a). This differentiation is also observed in genetic markers between these two species (Cameron et al. 2007).

The Role of Minor Compounds While most authors only take into account the major compounds in their discussion, our results show that between *B. filchnerae* and *B. muscorum*, differences in their CLGS are specified by minor compounds. This observation questions the role of these minor compounds in bumblebee olfaction. From this perspective, research on the neuronal processes induced by odorant information in the bumblebee brain would provide initial insights.

Indeed, several neural coding studies in Hymenoptera have shown the capacity of their olfactory system to both grant special properties to mixtures of pheromonal components and recognize individual components (Carcaud et al. 2015; Galizia and Menzel 2001; Sandoz 2012). In social insects, species use a wide range of different components which induce a simultaneous activation of several classes of olfactory receptor neurons (Sandoz et al. 2007). In honey bees, most of the results were obtained using volatile hydrocarbons that differ in carbon chain length (C5-C10) and functional groups (Carcaud et al. 2012; Sachse et al. 1999). However, some studies have addressed the effect of temperature on neural responses to odorants of low volatility (Carcaud et al. 2015) and indicated that brood and queen compounds (C10 to C20) were detected only at hive temperatures, i.e. around 35 °C (Brill et al. 2013; Carcaud et al. 2015).

Since environmental conditions appear to influence the neuronal response to odorants (Carcaud et al. 2015), it is difficult to identify which compounds emitted from the CLGS of males induce neuronal responses under natural conditions in bumblebees (in or out of the nest). *In vivo* calcium imaging (Carcaud et al. 2012) or electrophysiological recordings (Strube-Bloss et al. 2015), tested in similar conditions as in the wild (temperature and humidity), would mark a great first step towards a deeper understanding of the biological relevance of the messages emitted by males by observing what are and what are not detected and then responded to by females.

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