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

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24 **Abstract** - Sex-specific chemical secretions has been widely used as diagnostic characters in  
25 chemotaxonomy. The taxonomically confused group of bumblebees has reaped the benefit of  
26 this approach through the analyses of cephalic labial gland secretions (CLGS). Most of  
27 currently available CLGS descriptions concern species from the West-Palearctic region and  
28 few from the New World. Here, the CLGS descriptions of four East-Palearctic species *B.*  
29 *deuteronymus*, *B. filchnerae*, *B. humilis* and *B. exil* (subgenus *Thoracobombus*) are  
30 performed. Our results underline a strong variability in main compounds in *B. exil*. In  
31 contrast, we describe a low differentiation in CLGS compounds between *B. filchnerae* and its  
32 closely phylogenetically related taxon *B. muscorum*. Moreover, the chemical profile of *B.*  
33 *filchnerae* and *B. muscorum* is characterized by a highly concentrated component of the  
34 length C18 while it is a C16 in the other *Thoracobombus* species. It raises a possibility that  
35 the courtship behavior as well as environmental constraints could affect the role of the  
36 bumblebee males cephalic labial glands secretions.

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38 **Key Words** - Courtship behavior, pheromones, cephalic glands

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47 In many animal species, sex-specific chemical secretions are involved in the pre-mating  
48 communication (Patricelli et al. 2003; Hay 2011; Lecocq et al. 2015a). This key role in  
49 reproductive behavior has led to the development of the chemotaxonomy in which such  
50 chemical traits are used as diagnostic characters to assess taxonomic statuses (Quicke 1993;  
51 Bagnères et al. 2003; Pokorny et al. 2014). The systematics of insects has especially reaped the  
52 benefits of this approach (Lockey 1991). Bumblebees give a stunning example of the  
53 improvements raised by chemotaxonomy (Bertsch and Schweer 2011; Lecocq et al. 2015b).  
54 In this taxonomically confused group, morphological traits are often poorly efficient  
55 diagnostic characters because different species can be morphologically highly similar while  
56 conspecific populations can be extraordinarily divergent in their coat color pattern (Michener  
57 1990; Williams 1998; Lecocq et al. 2015b). In contrast, the male cephalic labial gland  
58 secretions (CLGS) are known to be species-specific semiochemicals (Calam 1969; Ayasse  
59 and Jarau 2014). These secretions are produced *de novo* (Luxová et al. 2003; Žáček et al.  
60 2013) and they attract conspecific virgin females (Ayasse and Jarau 2014). They appear to  
61 provide efficient diagnostic characters for species delimitation (Svensson 1979; Bergström  
62 1981; Terzo et al. 2003; Rasmont et al. 2005; Lecocq et al. 2015c).

63 During the last decades, an increasing number of studies has based their taxonomic  
64 conclusions on inter-taxa comparisons of CLGS composition (Lecocq et al. 2015b,d).  
65 However, global taxonomic assessment of bumblebee species through chemotaxonomy  
66 requires an extensive overview of the CLGS composition. Currently the CLGS composition  
67 have been described for approximately 65 species (Terzo et al. 2003; Terzo et al. 2007;  
68 Rasmont et al. 2005; Ayasse and Jarau 2014; Lecocq et al. 2015c,d; Brasero et al. 2015) out  
69 of a total of 250 bumblebee species around the world (Williams 1998).

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

75 Beyond taxonomic matter, more and more ecological questions about the role of compounds  
76 emitted by bumblebees emerge within the scientific community (Lhomme et al. 2015; Brasero  
77 et al. 2017). However, compared to several neural coding studies focused on sex pheromones  
78 in honey bees (Galizia and Menzel 2001; Sandoz 2012; Carcaud et al. 2015), only few studies  
79 have begun to describe neurophysiological structures in bumblebees (Fonta and Masson 1984;  
80 Strube-Bloss et al. 2015).

81 In this study, the CLGS of four East-Palearctic taxa were investigated: *Bombus deuteronymus*  
82 *superequester* (Skorikov 1914) (Fig. 1 A); *B. filchnerae* Vogt 1908 (Fig. 1 B), *B. humilis*  
83 *subbaicalensis* Vogt 1911 (Fig. 1 C) and *B. exil* (Skorikov 1923) (Fig. 1 D) all belonging to  
84 the *Thoracobombus* Dalla Torre, 1880 subgenus. Moreover, we compared the CLGS  
85 composition of *B. filchnerae* with that of its nearest Palearctic taxon; *B. muscorum* (L. 1758)  
86 (Fig. 1 E, F).

## 87 METHODS AND MATERIALS

88 *Sampling.* 65 queens of *B. deuteronymus superequester*, *B. exil*, *B. filchnerae* and *B. humilis*  
89 *subbaicalensis* have been collected in Mongolia in 2014. Twenty were transported alive and  
90 reared in Belgium. In order to produce males, we maintained queens in a wooden box in a  
91 controlled climate, dark room at 26/30°C and 50-60% relative humidity, and fed ad libitum  
92 with salix pollen and sugar syrup (Lhomme et al. 2013). We produced 51 males (Tab. 1): *B.*  
93 *deuteronymus superequester* (n=10), *B. exil* (n=13), *B. filchnerae* (n=15) and *B. humilis*

94 *subbaicalensis* (n=13). We assessed the taxonomic identification of males using the  
95 taxonomical revision of North China bumblebees published by An et al. (2014).  
96 Prior to chemical analyses, all males were killed by freezing at -20°C and their CLGS were  
97 extracted in 400 µl hexane (De Meulemeester et al. 2011). Samples were stored at -40°C prior  
98 to analyses. In order to compare the CLGS compositions of *B. filchnerae*, we used its  
99 phylogenetic sister taxon *B. muscorum* (n=24) partially published (Lecocq et al. 2015d) (Tab.  
100 1).

101 *Chemical Analyses.* We identified the CLGS composition by gas chromatography-mass  
102 spectrometry (GC/MS) using a Focus GC (Thermo Scientific) with a non-polar DB-5 ms  
103 capillary column [5% phenyl (methyl) polysiloxane stationary phase; column length 30 m;  
104 inner diameter 0.25 mm; film thickness 0.25 µm] coupled to DSQ II quadrupol mass analyzer  
105 (Thermo Scientific) with 70 eV electron ionization. We used a splitless injection mode  
106 (220°C) and helium as a carrier gas (1 ml/min). The temperature program of the oven was set  
107 to 70°C for two minutes and then heated up at a rate of 10°C/min to 320°C. The temperature  
108 was then held at 320°C for five minutes. Compounds were identified in Xcalibur™ using the  
109 retention times ( $t_r$ ) and mass spectra of each peak, in comparison to those at National Institute  
110 of Standards and Technology library (NIST, U.S.A). Double bond positions (C=C) were  
111 determined by dimethyl disulfide (DMDS) derivatization (Cvacka et al. 2008).

112 We quantified all samples using a gas chromatograph-flame ionization detector Shimadzu  
113 GC-2010 with a SLB-5ms non-polar capillary column (5% phenyl (methyl) polysiloxane  
114 stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25-µm film thickness) with  
115 the same chromatographic conditions as in GC/MS. Peak areas of compounds were detected  
116 in GCsolution Postrun (Shimadzu Corporation) with automatic peak detection and noise  
117 measurement. We calculated relative amounts (RA in %) of compounds in each sample by  
118 dividing the peak areas of compounds by the total area of compounds in each sample. We

119 discarded all compounds for which RA were lower than 0.1% for all specimens (De  
120 Meulemeester et al. 2011). Moreover, All compounds having a RA equal to or greater than  
121 1% are considered to be abundant compounds. We elaborated the data matrix as the alignment  
122 of each compound between all samples performed with GCAAligner 1.0 (Dellicour and  
123 Lecocq, 2013a,b)(see supplementary file 1).

124 *Statistical Analyses.* Statistical analyses were performed using R (R Development Core Team  
125 2013) to detect CLGS differentiations between taxa. Data consisting of the relative contents  
126 of all compounds were standardized (mean=0, standard deviation=1), to reduce the sample-  
127 concentration effect (Brasero et al. 2015).

128 Clustering method was used to detect the differentiation between taxa. A Pearson correlation  
129 matrix based on CLGS data matrix was computed. An unweighted pair group method with  
130 arithmetic mean (UPGMA) was used as clustering method (R-package ape,(Suzuki and  
131 Shimodaira 2011)). We assessed the uncertainty in hierarchical cluster analysis using *p*-values  
132 calculated via multiscale bootstrap resampling with 50000 bootstrap replications (R-package  
133 pvclust, (Suzuki and Shimodaira 2011)). We calculated CLGS differentiations of taxa by  
134 performing a *multiple response permutation procedure (MRPP)* (R package vegan, (Oksanen  
135 et al. 2011)).

136 To determine compounds specific to each taxa (indicator compounds, IC), we used the  
137 *indicator value (IndVal)* method (Dufrêne and Legendre 1997; Claudet et al. 2006). The value  
138 given is the product of relative abundance and relative frequency of occurrence of a  
139 compound within a group. The statistical significance of an indicator compound (threshold of  
140 0.01) was evaluated with a randomization procedure (Dufrêne and Legendre, 1997).

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

## 144 RESULTS

145 129 compounds were detected in the CLGS of the species belonging to *Thoracobombus*  
146 subgenus: 36 in *B. deuteronymus superequester*, 70 in *B. exil*, 33 in *B. filchnerae*, 35 in *B.*  
147 *muscorum* and 42 in *B. humilis* (Supplementary file 1).

148 The main compounds detected were (i) for *B. deuteronymus superequester*: octadec-11-en-1-  
149 ol (44.40% - 57.05%); (ii) for *B. exil*: hexadec-9-enal (0.04% - 44.35%), octadec-9-enoic acid  
150 (1.36% - 15.20%), icos-11-en-1-ol (0.20% - 8.88%) and hexadecenyl hexadecenoate (0.69% -  
151 10.53%); (iii) for *B. filchnerae* and *B. muscorum*: octadec-9-enyl acetate (40.49% - 75.96%)  
152 and (iv) for *B. humilis subbaicalensis*: octadec-9-en-1-ol (40.12% - 56.64%) (Tab. 2;  
153 supplementary file 1).

154 Our chemical analyses showed qualitative differentiations between all taxa including specific  
155 main compounds (i.e. compounds that have the highest relative amount among all compounds  
156 of CLGS at least in one individual of the taxon) except between *B. filchnerae* and *B.*  
157 *muscorum* (Tab. 2) where only minor compounds diverged (Tab. 3).

158 Our statistical analyses confirmed the differentiations of our sampling in five groups  
159 supported by high multiscale bootstrap resampling values: (i) *B. exil*, (ii) *B. humilis*  
160 *subbaicalensis*, (iii) *B. deuteronymus superequester*, (iv) *B. filchnerae* and (v) *B. muscorum*  
161 (Fig. 2A). Moreover, a multiple response permutation procedure (MRPP) confirmed these  
162 differentiations (all p-values < 0.001): *B. deuteronymus* vs *B. exil* (A= 0.2993); *B.*  
163 *deuteronymus* vs *B. filchnerae* (A=0.3326); *B. deuteronymus* vs *B. humilis* (A=0.3868); *B.*  
164 *deuteronymus* vs *B. muscorum* (A=0.2551); *B. exil* vs *B. filchnerae* (A=0.2876); *B. exil* vs *B.*  
165 *humilis* (A=0.3059); *B. exil* vs *B. muscorum* (A=0.2501); *B. filchnerae* vs *B. humilis*



166 (A=0.3547); *B. filchnerae* vs *B. muscorum* (A=0.186) and *B. humilis* vs *B. muscorum*  
167 (A=0.2648). Several significant indicator compounds were revealed by the IndVal method  
168 (IndVal > 0.70) (Supplementary file 1).

## 169 DISCUSSION

170 *B. humilis subbaicalensis* and *B. deuteronymus superequester*. Based on main and abundant  
171 compounds (Tab. 1), our CLGS analyses underline a differentiation between *B. humilis*  
172 *subbaicalensis* and *B. deuteronymus superequester* (Fig. 2A). This differentiation is also  
173 observed in genetic markers between these two species (Cameron et al. 2007). However, both  
174 genetic and chemical analyses are based on few samples only. This could lead to a  
175 misestimation of the inter-taxon differentiation (Lecocq et al. 2015c). Further studies based on  
176 larger sampling are needed to assess this differentiation.

177 *The intraspecific variability of main compounds in Bombus exil.* *Bombus exil* is characterized  
178 by a high intraspecific variability of its CLGS composition (supplementary file 1). Indeed, *B.*  
179 *exil* displays four main components (Tab. 2) and 45 indicator compounds (supplementary file  
180 1). Moreover, males of *B. exil* have the last three articles of the antennae broadened and  
181 ventrally concave (Tkalců 1974) (Fig. 1D). They press this clubbed part of the antennae  
182 against the lower part of the face of female bees in a characteristic way (P. Williams: com.  
183 pers. based on unpublished personal observations from Moerdaga, Neimenggu, 18/08/2009).  
184 These modified antennae deserve more functional morphology studies and histological  
185 researches dealing particularly on the sensillae in the concave area on the last article.

186 *The case of Bombus filchnerae and B. muscorum questions the role of minor compounds.* As  
187 mentioned in Terzo et al. (2005) for *B. ruderarius*, it is possible that abundant compounds are  
188 absent within a same taxon (i.e. for *B. filchnerae*; Tab. 1). Despite this intraspecific  
189 variability, low interspecific CLGS differentiation is observed between *B. muscorum* and *B.*  
190 *filchnerae* (Supplementary file 1; Tab. 2,3; Fig. 2 A,B). Such a low CLGS differentiation

191 pattern could be explained by (i) the conspecificity of the two taxa or (ii) the lack of role of  
192 CLGS in pre-mating communication. While further taxonomic assessment is needed, we  
193 speculate that the latter hypothesis could explain our results. First, CLGS is a complex  
194 mixture of (mainly aliphatic) compounds (Calam 1969; Coppée et al. 2008) that can be  
195 classified into four groups: (i) C1 to C4 exclusively considered as gas; (ii) C5 to C8  
196 considered as volatile liquids; (iii) C9 to C16 as non-volatile liquids and (iv) C16 and more  
197 considered as solid at a temperature of 20°C (Cicolella 2008). By comparing composition  
198 between these two species and other *Thoracobombus* (*B. exil*, *B. deuteronymus superequester*,  
199 *B. humilis subbaicalensis*, *B. pascuorum*, *B. inexpectatus*, *B. ruderarius* and *B. sylvarum*)  
200 (Terzo et al. 2005; Lecocq et al. 2015b; Brasero et al. 2017), we highlight that the chemical  
201 profile of *B. filchnerae* and *B. muscorum* is characterized by a high concentrated component  
202 of the length C18 in the beginning of the spectra while they are mostly C16 in the other  
203 species of *Thoracobombus*. This could suggest that the CLGS of *B. muscorum* and *B.*  
204 *filchnerae* are not used for long distance intersex attraction but rather could play a recognition  
205 role at a short distance. This is further supported by observed and described aggregations of *B.*  
206 *muscorum* males in close proximity to mature nests entrance in order to mate with virgin  
207 queens (Krüger 1951; Darvill et al. 2007). Considering these observations, a sexual selection  
208 favoring reproductive isolation through CLGS divergence is not expected if reproductive  
209 isolation is mediated by alternative cues such as nest site choice (i.e. the habitat) or nest scent.  
210 Further studies on the pre-mating recognition of bumblebee species are needed to assess this  
211 hypothesis.

212 While most authors only take into account the major compounds in their discussion, our  
213 results show that between *B. filchnerae* and *B. muscorum*, the CLGS differentiation is marked  
214 on minor compounds. This observation questions the role of these minor compounds on

215 bumblebee olfaction. In this perspective, an approach on the neuronal process induced by  
216 odorant information in the bumblebee brain will provide first elements of thought.  
217 Indeed, several neural coding studies in Hymenoptera have shown the capacity of their  
218 olfactory system to grant special properties to mixtures of pheromonal components as well as  
219 to recognize individual components (Galizia and Menzel 2001; Sandoz 2012; Carcaud et al.  
220 2015). In social insects, species use a wide range of different components which induce a  
221 simultaneous activation of several classes of olfactory receptor neurons (Sandoz et al. 2007).  
222 In honey bees, most of the results were obtained using volatile hydrocarbons that differ in  
223 carbon chain length (C5-C10) and functional groups (Sachse et al. 1999; Carcaud et al. 2012).  
224 However, some studies addressed the effect of temperature on neural responses to odorants of  
225 low volatility (Carcaud et al. 2015) and realized that brood and queen compounds (C10 to  
226 C20) were detected only at hive temperature, i.e. around 35°C (Brill et al. 2013; Carcaud et al.  
227 2015).  
228 Since environmental conditions appear to influence the neural response to odorants (Carcaud  
229 et al. 2015), it is difficult to identify which compounds emitted by the CLGS of males induce  
230 an neuronal answer under natural conditions in bumblebees (in or out of the nest). In vivo  
231 calcium imaging or neural representation studies, as developed in Carcaud et al (2012) and  
232 Strube-Bloss et al. (2015), tested in wild conditions (temperature and humidity) would mark a  
233 great first step to go deeper into the biological relevance understanding of the emitted males  
234 message by observing what is or is not detected by females.

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- 244 An J, Huang J, Shao Y, Zhang S, Wang B, Liu X, Wu J, Williams PH (2014) The bumblebees  
245 of North China (Apidae, *Bombus* Latreille). *Zootaxa* 3880 (1): 001-089
- 246 Ayasse M, Jarau S (2014) Chemical ecology of bumble bees. *Annu Rev Entomol* 59: 299–319
- 247 Bagnères AG, Uva P, Clément JL (2003) Description d'une nouvelle espèce de Termite:  
248 *Reticulitermes urbis* n.sp. (Isopt., Rhinotermitidae). *Bull Soc Entomol Fr* 108: 433–435
- 249 Bergström G, Svensson BG, Appelgren M, Groth I (1981) Complexity of bumble bee  
250 marking pheromones: biochemical, ecological and systematical interpretations. *SystAss*  
251 special vo: 175–183
- 252 Bertsch A, Schweer H, Titze A (2004) Analysis of the labial gland secretions of the male  
253 bumblebee *Bombus griseocollis* (Hymenoptera: Apidae). *Z Naturforsch - Section C Journal of*  
254 *Biosciences* 59: 701–707
- 255 Bertsch A, Schweer H, Titze A (2008) Chemistry of the cephalic labial gland secretions of  
256 male *Bombus morrisoni* and *B. rufocinctus*, two North American bumblebee males with  
257 perching behavior. *J Chem Ecol* 34: 1268–1274
- 258 Bertsch A, Schweer H (2011) Labial gland marking secretions of male *Bombus lucorum*  
259 bumblebees from Europe and China reveal two separate species: *B. lucorum* (Linnaeus 1761)  
260 and *Bombus minshanicola* (Bischoff 1936). *Biochem Syst Ecol* 39 (4-6): 587-593
- 261 Brasero N, Martinet B, Urbanová K, Valterová I, Torres A, Hoffmann W, Rasmont P, Lecocq  
262 T (2015) First Chemical Analysis and Characterization of the Male Species-Specific Cephalic  
263 Labial-Gland Secretions of South American Bumblebees. *Chem Biodivers* 12: 1535–1546
- 264 Brasero N, Martinet B, Lecocq T, Lhomme P, Biella P, Valterova I, Urbanova K, Cornalba  
265 M, Hines H, Rasmont P (2017) The cephalic labial gland secretions of two socially parasitic  
266 bumblebees *Bombus hyperboreus* (Alpinobombus) and *Bombus inexpectatus*  
267 (*Thoracobombus*) question their inquiline strategy. *Insect Sci* 00: 1–12
- 268 Brill MF, Rosenbaum T, Reus I, Kleineidam CJ, Nawrot MP, Rössler W (2013) Parallel  
269 processing via a dual olfactory pathway in the honeybee. *J Neurosci* 33: 2443–2456
- 270 Calam DH (1969) Species and Sex-specific Compounds from the Heads of Male Bumblebees  
271 (*Bombus* spp.). *Nature* 221: 856–857
- 272 Cameron SA, Hines HM, Williams PH (2007) A comprehensive phylogeny of the bumble  
273 bees (*Bombus*). *Biol J Linnean Soc* 91: 161–188
- 274 Carcaud J, Hill T, Guirfa M, Sandoz J-C (2012) Differential coding by two olfactory  
275 subsystems in the honeybee brain. *J Neurophysiol* 108: 1106–1121
- 276 Carcaud J, Guirfa M, Sandoz J-C (2015) Differential Combinatorial Coding of Pheromones in  
277 Two Olfactory Subsystems of the Honey Bee Brain. *J Neurosci* 35: 4157–4167

- 278 Cicolella A (2008) Les composés organiques volatils (COV): définition, classification et  
279 propriétés. *Rev Mal Respir* 25: 155–163
- 280 Claudet J, Pelletier D, Jouvenel JY, Bachet F, Galzin R (2006) Assessing the effects of marine  
281 protected area (MPA) on a reef fish assemblage in a northwestern Mediterranean marine  
282 reserve: Identifying community-based indicators. *Biol Conserv* 130: 349–369
- 283 Coppée A, Terzo M, Valterová I, Rasmont P (2008) Intraspecific variation of the cephalic  
284 labial gland secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Chem Biodivers* 5:  
285 2654–2661
- 286 Cvacka J, Kofronová E, Vasícková S, Stránský K, Jiros P, Hovorka O, Kindl J, Valterová I  
287 (2008) Unusual fatty acids in the fat body of the early nesting bumblebee, *Bombus pratorum*.  
288 *Lipids* 43: 441–50
- 289 Dalla Torre KW (1880) Unsere Hummel-(*Bombus*) Arten. *Die Naturhistoriker* 30: 40–41
- 290 Darvill B, Lye GC, Goulson D (2007) Aggregations of male *Bombus muscorum*  
291 (Hymenoptera: Apidae) at mature nest. Incestuous brothers or amorous suitors? *Apidologie*  
292 38: 518–524
- 293 Dellicour S, Lecocq T (2013a) GCALIGNER 1.0 and GCKOVATS 1.0 - Manual of a  
294 software suite to compute a multiple sample comparison data matrix from eco-chemical  
295 datasets obtained by gas chromatography. University of Mons, Mons
- 296 Dellicour S, Lecocq T (2013b) GCALIGNER 1.0: a alignment program to compute a multiple  
297 sample comparison data matrix from large eco-chemical datasets obtained by gas  
298 chromatography. *J Sep Sci* 36: 3206-3209
- 299 De Meulemeester T, Gerbaux P, Boulvin M, Coppée A, Rasmont P (2011) A simplified  
300 protocol for bumble bee species identification by cephalic secretion analysis. *Insectes Soc* 58:  
301 227–236
- 302 Dufrière M, Legendre P (1997) Species assemblages and indicator species: The need for a  
303 flexible asymmetrical approach. *Ecol Monogr* 67: 345–366
- 304 Fonta C, Masson C (1984) Comparative study by electrophysiology of olfactory responses in  
305 bumblebees (*Bombus hypnorum* and *Bombus terrestris*). *J Chem Ecol* 10: 1157–1168
- 306 Galizia CG, Menzel R (2001) The role of glomeruli in the neural representation of odours:  
307 results from optical recording studies. *J Insect Physiol* 47: 115–130
- 308 Hay ME (2011) Crustaceans as powerful models in aquatic chemical ecology. In: Thiel TB  
309 and M (ed) *Chemical communication in crustaceans*. Springer, New York, pp 41–62
- 310 Krüger E (1951) Über die Bahnflüge der Männchen der Gattungen *Bombus* und *Psithyrus*. *Z*  
311 *Tierpsychol* 8: 61–75

- 312 Lecocq T, Coppée A, Mathy T, Lhomme P, Cammaerts-Tricot MC, Urbanová K, Valterová I,  
 313 Rasmont P (2015a) Subspecific differentiation in male reproductive traits and virgin queen  
 314 preferences, in *Bombus terrestris*. *Apidologie* 26: 595–605
- 315 Lecocq L, Brasero N, Martinet B, Valterova I, Rasmont P (2015b) Highly polytypic taxon  
 316 complex: interspecific and intraspecific integrative taxonomic assessment of the widespread  
 317 pollinator *Bombus pascuorum* Scopoli 1763 (Hymenoptera: Apidae). *Syst Entomol* 40: 881–  
 318 890
- 319 Lecocq T, Dellicour S, Michez D, Dehon M, Dewulf A, De Meulemeester T, Brasero N,  
 320 Valterová I, Rasplus JY, Rasmont P (2015c) Methods for species delimitation in bumblebees  
 321 (Hymenoptera, Apidae, *Bombus*): towards an integrative approach. *Zool Scr* 44(3): 1-17
- 322 Lecocq T, Brasero N, De Meulemeester T, Michez D, Dellicour S, Lhomme P, de Jonghe R,  
 323 Valterová I, Urbanová K, Rasmont P (2015d) An integrative taxonomic approach to assess the  
 324 status of Corsican bumblebees: implications for conservation. *Anim Conserv* 18: 236–148
- 325 Lhomme P, Sramkova A, Kreuter K, Lecocq T, Rasmont P, Ayasse M (2013) A method for  
 326 year-round rearing of cuckoo bumblebees (Hymenoptera: Apoidea: *Bombus* subgenus  
 327 *Psithyrus*). *Ann Soc entomol Fr (N.S.)* 49: 37–41
- 328 Lhomme P, Ayasse M, Valterová I, Lecocq T, Rasmont P (2015) A scent shield to survive:  
 329 identification of the repellent compounds secreted by the male offspring of the cuckoo  
 330 bumblebee *Bombus vestalis*. *Entomol Exp Appl* 157: 263–270
- 331 Linné C de (1758) *Systema Naturae. I.* (Laurentii Salvii, Ed.). Holmiae
- 332 Lockey KH (1991) Insect hydrocarbon classes: Implications for chemotaxonomy. *Insect*  
 333 *Biochem* 21: 91–97
- 334 Luxová A, Valterová I, Stránský K, Hovorka O, Svatoš A (2003) Biosynthetic studies on  
 335 marking pheromones of bumblebee males. *Chemoecology* 13: 81–87
- 336 Michener CD (1990) Classification of the Apidae (Hymenoptera). *Univ Kans publ, Mus Nat*  
 337 *Hist* 54: 75–164
- 338 Oksanen FJ, Blanchet G, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL,  
 339 Solymos P, Stevens MHH, Wagner H (2011) *Vegan: Community Ecology Package*
- 340 Patricelli GL, Uy JAC, Borgia G (2003) Multiple male traits interact: Attractive bower  
 341 decorations facilitate attractive behavioural displays in satin bowerbirds. *Proc R Soc Lond B*  
 342 *Biol Sci* 270: 2389–2395
- 343 Pokorny T, Lunau K, Quezada-Euan JJG (2014) Cuticular hydrocarbons distinguish cryptic  
 344 sibling species in *Euglossa* orchid bees. *Apidologie* 45: 276–283
- 345 Quicke DL (1993) Chemotaxonomy and related topics. In: *Principles and Techniques of*  
 346 *Contemporary Taxonomy*. Springer, Netherlands, pp 146-154

347 R Development Core Team (2013) R: a language and environment for statistical computing.  
348 Vienna: R Foundation for Statistical Computing. ISBN 3-900051-07-0  
349 <http://www.R-project.org> (accessed ••)  
350

351 Rasmont P, Terzo M, Aytakin AM, Hines H, Urbanová K, Cahliková L, Valterová I (2005)  
352 Cephalic secretions of the bumblebee subgenus *Sibiricobombus* Vogt suggest *Bombus*  
353 *niveatus* Kriechbaumer and *Bombus vorticosus* Gerstaecker are conspecific (Hymenoptera,  
354 Apidae, *Bombus*). *Apidologie* 36: 571–584

355 Sachse S, Rappert A, Galizia CG (1999) The spatial representation of chemical structures in  
356 the antennal lobe of honeybees: steps towards the olfactory code. *J Neurosci* 11: 3970–3982

357 Sandoz JC, Deisig N, de Brito Sanchez MG, Giurfa M (2007) Understanding the logics of  
358 pheromone processing in the honeybee brain: from labeledlines to across-fiber patterns. *Front*  
359 *Behav Neurosci* 1: 5

360 Sandoz JC (2012) Olfaction in honey bees: From molecules to behavior. In: Galizia G,  
361 Eisenhardt D and Giurfa M (ed), *Honeybee neurobiology and behavior. A tribute to Randolf*  
362 *Menzel.* Springer, Dordrecht, the Netherlands, pp 235–252

363 Skorikov AS (1914) Les formes nouvelles des bourdons (Hymenoptera, Bombidae). V.  
364 *Russkoe éntomologicheskoe Obozrênie* 14: 119–129

365 Skorikov AS (1923) [Palaeartic bumblebees. Part I. General biology (including  
366 zoogeography)]. *Izv Severn Obl Stantsii Zashch Rast Vredit* 4: 1–160

367 Strube-Bloss MF, Brown A, Spaethe J, Schmitt T, Rössler W (2015) Extracting the  
368 Behaviorally Relevant Stimulus: Unique Neural Representation of Farnesol, a Component of  
369 the Recruitment Pheromone of *Bombus terrestris*. *PloS one* 10: e0137413

370 Suzuki R, Shimodaira H. (2011) Pvcust: Hierarchical Clustering with P-values via Multiscale  
371 Bootstrap Resampling. Contributed package. Version 1-1.10. R Foundation for Statistical  
372 Computing, Vienna.  
373 <http://www.R-project.org>

374 Svensson BG, Bergström G (1979) Marking pheromones of *Alpinobombus* males. *J Chem*  
375 *Ecol* 5 (4): 603-615

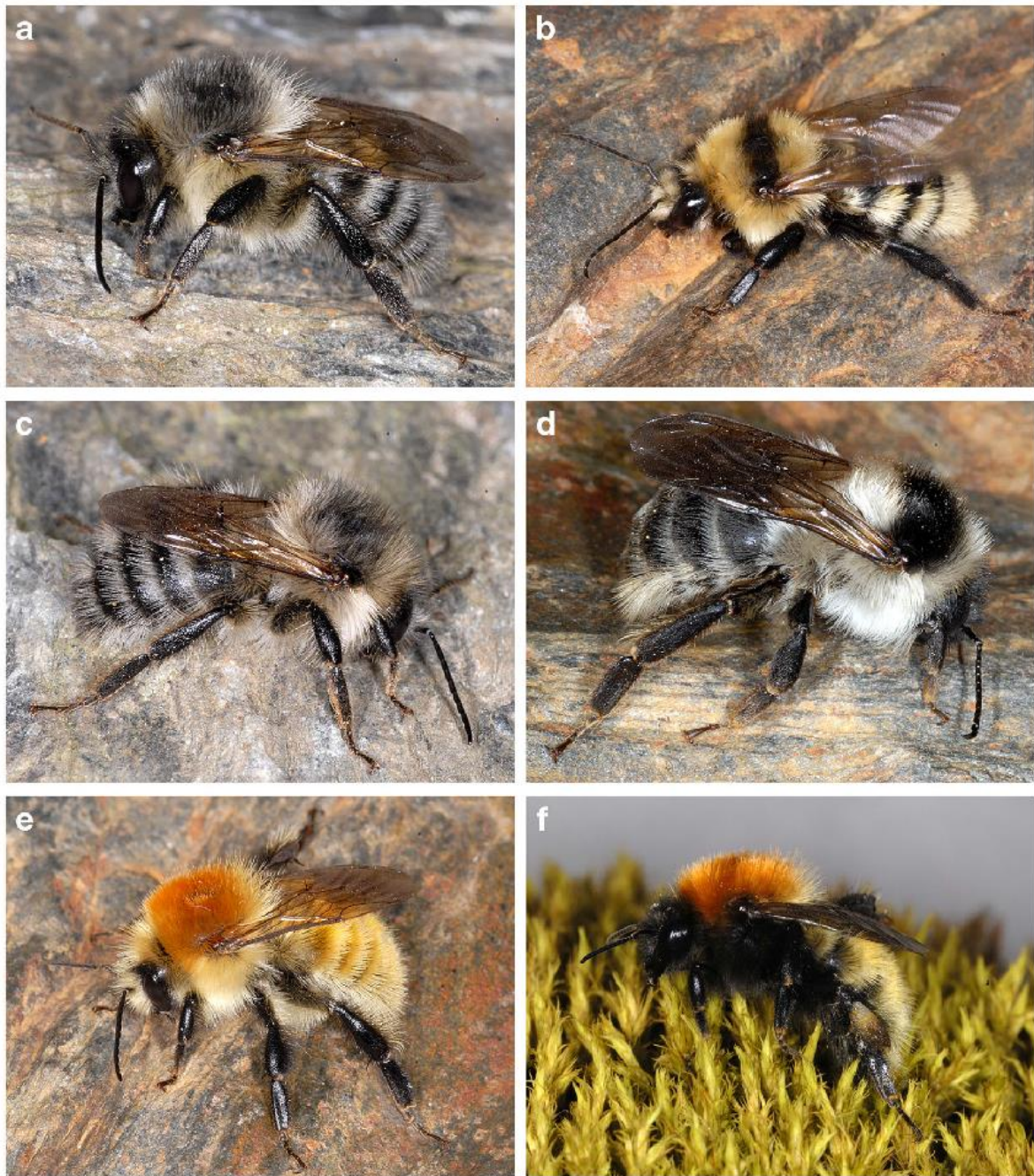
376 Terzo M, Valterová I, Urbanová K, Rasmont P (2003) De la nécessité de redécrire les  
377 phéromones sexuelles des mâles de bourdons [Hymenoptera: Apidae, Bombini] publiées  
378 avant 1996 pour leur utilisation en analyse phylogénétique. *Phytoprotection* 84: 39–49

379 Terzo M, Urbanová K, Valterová I, Rasmont P (2005) Intra and interspecific variability of the  
380 cephalic labial glands' secretions in male bumblebees: the case of *Bombus* (*Thoracobombus*)  
381 *runderarius* and *B.* (*Thoracobombus*) *sylvarum* Hymenoptera, Apidae. *Apidologie* 36: 85–96

382 Terzo M, Valterová I, Rasmont P (2007) Atypical secretions of the male cephalic labial  
383 glands in bumblebees: The case of *Bombus* (*Rhodobombus*) *mesomelas*  
384 Gerstaecker(Hymenoptera, Apidae). *Chem Biodivers* 4: 1466–1471

- 385 Tkalčů B (1974) Ergebnisse der 1. und 2. mongolisch-tschechoslowakischen entomologisch-  
386 botanischen Expedition in der Mongolei Nr. 29: Hymenoptera, Apoidea, Bombinae. – Acta  
387 faunistica entomologica Musei Nationalis Pragae 15: 25-58
- 388 Venables WN, Ripley BD (2002) Modern Applied Statistics with S. Springer, Queensland
- 389 Vogt O (1908) Bombi (Hummeln). Wissenschaftliche Ergebnisse von Expedition Filchner  
390 nach China und Thibet 1903-1905, X. Band - I. Teil, 1. Abschnitt: Zoologische Sammlungen,  
391 Berlin.100–101
- 392 Vogt O (1911) Studien über das Artproblem. 2. Mitteilung. über das Variieren der Hummeln.  
393 2. Teil (Schluss). *Schriften der berlinischen Gesellschaft Naturforschender, Freunde, Berlin*  
394 1911: 31–74
- 395 Williams PH (1998) An annotated checklist of bumble bees with an analysis of patterns of  
396 description (Hymenoptera: Apidae, Bombini). *Bulletin of The Natural History Museum*  
397 (*Entomology*) 67: 79–152
- 398 Žáček P, Prchalová-Hornáková D, Tykva R, Kindl J, Vogel H, Svatoš A, Pichová I, Valterová  
399 I (2013) De novo biosynthesis of sexual pheromone in the labial gland of bumblebee males.  
400 *Chembiochem* 14: 361–371
- 401
- 402
- 403
- 404
- 405
- 406
- 407
- 408
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- 410
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- 412
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417 **Fig. 1** A: *Bombus deuteronymus superequester* from Mongolia (photo: P. Rasmont). B: *B.*  
418 *filchnerae* from Mongolia (photo: P. Rasmont). C: *B. humilis subbaicalensis* from Mongolia  
419 (photo: P. Rasmont). D: *B. exil* from Mongolia (photo: P. Rasmont). E: *B. muscorum*  
420 *muscorum* from Holland. F: *B. muscorum liepetterseni* from Norway

421



432 **Supplementary file**

433 **Supplementary file 1** - Data matrix of cephalic labial gland secretions (CLGS) (relative  
 434 concentration of each compound), list of the identified compounds, kovats indice and IndVal  
 435 analysis with specific compounds in *B. deuteronymus superequester*, *B. exil*, *B. filchnerae*, *B.*  
 436 *muscorum* and *B. humilis subbaicalensis*. U indicates undetermined compounds.

437 *Table*

438 Table 1. SAMPLING

| Taxa   | Collecting sites                      | Coordinates                  | N  |
|--|---------------------------------------|------------------------------|----|
| <b><i>Thoracobombus</i> subgenus</b>                     |                                       |                              |    |
| <i>B. deuteronymus superequester</i><br>(Skorikov, 1914) | Mongolia, Tov, Terelj                 | 47°59'28.0"N 107°22'25.7"E   | 10 |
| <i>B. filchnerae</i> Vogt, 1908                          | Mongolia, Tov, Terelj                 | 47°59'28.0"N 107°22'25.7"E   | 15 |
| <i>B. muscorum muscorum</i> (L., 1758)                   | Sweden, Ystad, Mossbystrand           | 55°25'10"N 13°38'55"E        | 1  |
|  | Poland, Olsztynski, Wójtowo           | 53°47'11"N 20°35'33"E        | 1  |
|  | Siberia, Irkutsk Obl., Ust'-Ordinskiy | 52°54'51.98"N 105°04'29.96"E | 1  |
|  | Ireland, Clare, Ennis                 | 52°51'37"N 09°02'12"W        | 1  |
|  | France, Oriental Pyrenees, Corbère    | 42°39'34"N 02°40'07"E        | 1  |
|  | Holland, Zuid-Beijerland, Tiengemeten | 51°44'37.1"N 04°19'04.8"E    | 1  |
|  | Denmark, Viborg, Moldrup              | 56°37'01.37"N 09°30'01.34"E  | 2  |
| <i>B. muscorum pereziellus</i> (Skorikov 1922)           | France, Corse, Palneca                | 42°42'42"N 09°11'27"E        | 10 |
| <i>B. muscorum liepetterseni</i> Löken 1973              | Norway, Flatanger                     | 64°28'11"N 10°43'16"E        | 5  |
| <i>B. muscorum allenellus</i> Stelfox, 1933              | Ireland, Galway, Inishmore            | 53°07'42"N 09°43'54"W        | 1  |
| <i>B. humilis subbaicalensis</i> Vogt, 1911              | Mongolia, Tov, Terelj                 | 47°59'45.7"N 107°19'46.1"E   | 4  |
|  | Mongolia, Arkhangai, Tsenker          | 47°07'40.4"N 101°57'04.5"E   | 1  |
|  | Mongolia, Arkhangai, Kharkhorin       | 47°03'50.3"N 102°12'23.6"E   | 8  |
| <i>B. exil</i> (Skorikov 1923)                           | Mongolia, Tov, Terelj                 | 47°59'45.7"N 107°19'46.1"E   | 13 |

439 Collecting sites and number (N) of bred specimens for *Bombus deuteronymus superequester*,  
 440 *B. filchnerae*, *B. humilis subbaicalensis* and *B. exil*, *B. muscorum* were integrated in the study

441 in order to check their CLGS composition with their East-Palearctic subspecies or closely  
442 related species. Coordinate are given with the reference to the WGS84.

Table 2. LIST OF MAIN COMPOUNDS

| Compounds                  | Carbons | MW  | <i>B. deuteronymus superequester</i> |       |       | <i>B. exil</i> |      |       | <i>B. filchnerae</i> |       |       | <i>B. muscorum</i> |       |       | <i>B. humilis subbaicalensis</i> |       |       |
|----------------------------|---------|-----|--------------------------------------|-------|-------|----------------|------|-------|----------------------|-------|-------|--------------------|-------|-------|----------------------------------|-------|-------|
|                            |         |     | Min                                  | M     | Max   | Min            | M    | Max   | Min                  | M     | Max   | Min                | M     | Max   | Min                              | M     | Max   |
| Octadec-11-en-1-ol         | C18     | 268 | 44.40                                | 47.09 | 57.05 | 0.00           | 0.00 | 0.00  | 0.00                 | 5.00  | 9.15  | 0.00               | 0.00  | 0.00  | 4.83                             | 8.84  | 10.81 |
| Hexadec-9-enal             | C16     | 238 | 0.00                                 | 0.00  | 0.00  | 0.04           | 2.05 | 44.35 | 0.00                 | 0.00  | 0.00  | 0.00               | 0.00  | 0.00  | 0.00                             | 0.00  | 0.00  |
| Octadec-9-enoic acid       | C18     | 282 | 0.21                                 | 0.32  | 0.56  | 1.36           | 8.67 | 15.20 | 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  | 0.20           | 4.21 | 8.88  | 0.02                 | 0.06  | 0.28  | 0.00               | 0.00  | 0.00  | 0.00                             | 0.00  | 0.00  |
| Hexadecenyl hexadecenoate1 | C32     | 476 | 0.27                                 | 0.40  | 0.50  | 0.69           | 6.81 | 10.53 | 0.00                 | 0.00  | 0.00  | 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  | 40.49                | 65.96 | 75.96 | 50.45              | 65.38 | 88.38 | 0.06                             | 0.29  | 2.65  |
| Octadec-9-en-1-ol          | C18     | 268 | 0.00                                 | 0.00  | 0.00  | 0.64           | 1.39 | 2.90  | 10.69                | 17.06 | 34.53 | 4.14               | 26.34 | 31.16 | 40.12                            | 44.71 | 56.64 |

Main compounds (grey background) identified in cephalic labial gland secretions of *Bombus deuteronymus superequester*, *B. exil*, *B. filchnerae*, *B. muscorum* and *B. humilis subbaicalensis*. Carbons: the number of carbon atoms; MW: the molecular weight; M: median of relative concentration of compound (%). Abundant compounds are on black background. All identified compounds in Supplementary file1.

Table 3. LIST OF COMPOUNDS CALCULATED WITH THE INDICATOR VALUE (INDVAL) METHOD FOR *B. MUSCORUM* AND *B. FILCHNERAE*

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

List of compounds identified in cephalic labial gland secretions of *B. muscorum* and *B. filchnerae* after the identification of characteristic compounds (grey background) calculated with the Indicator Value (IndVal) method (>0.70). MW: the molecular weight; M: median of relative concentration of compound (%).

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