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1 **First chemical analysis and characterization of the male species-specific cephalic labial**
2 **gland secretions of South American bumblebee**

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15 *Abstract*

16 The evolution of signals and reproductive traits involved in the pre-mating recognition has
17 been in focus of abundant research in several model species group such as bumblebees (genus
18 *Bombus*). However, the most studied bumblebee reproductive trait, the male cephalic labial
19 gland secretions (CLGS), remains unknown among bumblebee species from South America.
20 In this study we investigated the CLGS of five South American bumblebees of the subgenera
21 *Thoracobombus* (*Bombus excellens*, *B. atratus*) and *Cullumanobombus* (*B. rubicundus*, *B.*
22 *hortulanus* and *B. melaleucus*) comparing their secretions to those of European closely related
23 species. Our results show an obvious interspecific differentiation in all subgenera. The

24 interspecific differentiation in *Thoracobombus* involves different compounds with a large
25 abundance (main compounds) while all *Cullumanobombus* share the same main components.
26 This suggests that in *Cullumanobombus* subgenus differentiation in minor components could
27 lead to species discrimination.

28 *Keywords*

29 Cephalic labial gland, Chemical reproductive traits, South America, bumblebees

30 **Introduction**

31 The pre-mating communication is of particular interest for evolutionary biologists because
32 of its key role in sexual selection [1,2]. Therefore, the evolution of signals and reproductive
33 traits involved in the pre-mating recognition has been widely studied [3] in several model
34 species groups such as birds (e.g., [4–6]), moths (e.g. [7–9]) flies (e.g., [10–12]), or more
35 recently the bumblebees (e.g., [13–15]).

36 The pre-mating recognition of bumblebees (genus *Bombus*) includes both chemical and
37 behavioral features [16]. Most bumblebee males patrol along paths (i.e. patrolling behavior)
38 where they scent-mark objects with their cephalic labial gland secretions (CLGS) [17,18] that
39 attract conspecific virgin females [13,19]. Other species exhibit less common behavior as
40 perching behavior, in which males wait individually at prominent places and dart passing
41 queens or other moving objects [20]. The CLGS is a complex mixture of (mainly aliphatic)
42 compounds[18,21,22] produced *de novo* by the cephalic labial glands (acinar gland) [23] from
43 saturated fatty-acids [24,25]. The resulting composition is species-specific [18]. The CLGS
44 are thus an excellent tool for exploring interspecific and intraspecific variations of pre-mating
45 recognition [13–15,26] as well as they are useful chemo-taxonomic characters [22,27–29].

46 Despite the usefulness of the CLGS as model in evolutionary biology and taxonomy, the
47 male chemical secretions of a great number of species remain unknown [13]: the CLGS have
48 been described for 50 species of a total of approximately 250 bumblebee species [30]. In
49 several biogeographic regions such as South America, no studies have been published.
50 Indeed, the CLGS chemical analyses require fresh samples [31] that are most of the time
51 unavailable for uncommon taxa. In this paper, we investigated, the CLGS of males of five
52 South American bumblebees: *Bombus melaleucus* Handlirsch, 1888 and *B. hortulanus* Friese,
53 1904 are near species belonging to the "*Robustus* group" (= *Robustobombus* sensu Richards,
54 1968 [32]) while *B. rubicundus* Smith, 1854 (Fig. 1 D) belongs to "*Rubicundus* group" (= *Rubicundobombus*
55 *sensu* Richards, 1968 [32]), these species-groups being now included in
56 *Cullumanobombus* subgenus [33]. *B. atratus* Franklin, 1913 (Fig. 1 B) and *B. excellens*
57 Smith, 1879 (Fig. 1 A) both belong to the "*Fervidus*-species group" (= *Fervidobombus sensu*
58 Richards, 1968 [32]) now all included in *Thoracobombus* subgenus [33]. We ultimately aim
59 to compare their CLGS composition with those of related species from the same subgenus: *B.*
60 (*Thoracobombus*) *muscorum liepetterseni* (L. 1758), *B. (Thoracobombus) pascuorum*
61 *melleofacies* Vogt, 1911, *B. (Cullumanobombus) cullumanus apollineus* Skorikov, 1910 (Fig.
62 2 C) and *B. (Cullumanobombus) semenoviellus* Skorikov, 1910.

63 **Results and discussion**

64 *Composition of cephalic labial gland secretions*

65 Chemical analyses detected 146 compounds (33 from *B. excellens*, 35 from *B. atratus*, 53
66 from *B. pascuorum*, 19 from *B. muscorum*, 16 from *B. rubicundus*, 22 from *B. hortulanus*, 39
67 from *B. melaleucus*, 22 from *B. cullumanus* and 44 from *B. semenoviellus*) (see *Table 1*). The
68 results showed that all *Thoracobombus* species were differentiated in their CLGS composition
69 (seven compounds shared by all species and 17 compounds shared between the two South

70 American species), including changes in main compounds (octadec-11-enol (29% - 46%) in
71 *B. excellens* and octadec-9-enol (54% - 91%) in *B. atratus* (Table 1). Inside the European
72 taxa, hexadec-7-enol (10% - 31%) in *B. pascuorum* and octadec-9-enyl acetate in *B.*
73 *muscorum* were the major compounds (Table 1). By main compounds of a taxon, we mean
74 every compound that has the highest relative amount within the CLGS at least in one
75 individual. Other differences between the South American *Thoracobombus* were mainly
76 qualitative differences in minor compounds (Table 1). Similarly, the *Cullumanobombus*
77 species displayed qualitative differences (only five compounds shared; six compounds shared
78 between the three South American *Cullumanobombus*), but the main compound,
79 geranylgeranyl acetate, is in common to all *Cullumanobombus* analyzed here: 81% - 94% in
80 *B. rubicundus*, 76% - 83% in *B. hortulanus*, 18% - 59% in *B. melaleucus*, 51% - 92% in *B.*
81 *cullumanus* and 37% - 60% in *B. semenoviellus*. Qualitative variations differentiated species
82 in minor components (Table 1).

83 *Statistical analysis*

84 For both *Thoracobombus* and *Cullumanobombus* groups, the statistical analyses of the CLGS
85 confirmed the interspecific differentiation (Fig. 2). This chemical differentiation is supported
86 by high values of multiscale bootstrap resampling (> 80%, Fig. 2). The interspecific
87 differentiations between all consubgeneric species observed in our results are in agreement
88 with the species-specificity of CLGS observed in other bumblebee groups (e.g.,
89 [18,22,27,29]). Similarly to our results on *Thoracobombus* species, the comparison of CLGS
90 between closely related bumblebee taxa with a commonly recognized species status allow
91 suggesting that the interspecific differentiation involves the change in main compounds (e.g.,
92 [18,29,34]). These main compound differentiations can be (i) a significant shift (production of
93 new types of compounds, e.g., *B. pascuorum* versus *B. muscorum*) consecutive to switching
94 off and on the respective metabolic pathway (e.g., [7]), (ii) large increase of a minor

95 compound shared with closely related species (e.g., octadec-9-enyl acetate between *B.*
96 *muscorum* versus *B. atratus*) [35], as well as (iii) difference in double bond position arisen
97 by activity of different enzymes (e.g., $\Delta 11$ -desaturase and $\Delta 9$ -desaturase introducing a double
98 bound in octadecenol in *B. excellens* and *B. atratus*, e.g., [8]) or by activation of a non-
99 functional enzyme gene transcript present in a common ancestor, as observed in moths [9] and
100 in other bumblebees [15]. In contrast, all *Cullumanobombus* species display the same main
101 component (geranylgeranyl acetate) as observed in the North American *B.*
102 (*Cullumanobombus*) *rufocinctus* Cresson, 1863 [20]. The geranylgeranyl acetate is also a
103 main component of *Bombus* (*Alpigenobombus*) *wurflenii* [36], *B.* (*Kallobombus*) *soroensis*
104 [37] and *B.* (*Separatobombus*) *griseocollis* [38] meaning that this component could not be
105 considered as autapomorphic of *Cullumanobombus*. Assuming the key role of CLGS in pre-
106 mating recognition, this suggests that differentiation in minor compounds could lead to
107 species discrimination. Moreover, for each CLGS group, the IndVal method revealed several
108 significantly indicator compounds (see supplementary file 1).

109 **Conclusion**

110 The cephalic labial gland secretions of males are widely acknowledge as a useful and
111 practical diagnostic trait for bumblebee species. Among the European bumblebee fauna,
112 several taxonomically doubtful taxa groups have been solved thank to this reproductive trait.
113 The present study is the first study to examine this trait among South American bumblebee
114 species. This paves the way to a massive taxonomic assessment of South American
115 bumblebees.

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133 *liepeterseni* males and to Nicolas R. Chimento (Laboratorio de Anatomía Comparada y
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148 **Experimental part**

149 *Biological material*

150 Fifty males from two subgenera were used for this study (*Table 2*): *B. melaleucus* (n=2), *B.*
151 *hortulanus* (n=3), *B. rubicundus* (n=7), *B. atratus* (n=12), and *B. excellens* (n=2). As
152 comparison group of *Thoracobombus* we used specimens already described in Lecocq *et al.*
153 [28]: *B. muscorum liepetterseni* (n=5), *B. pascuorum melleofacies* (n=7). We used *B.*
154 *cullumanus apollineus* (n=9) and *B. semenoviellus* (n=3) already described in [39] as
155 comparison group of *Cullumanobombus*. We killed specimens by freezing at -20°C. The
156 CLGS were extracted in 400 ml of hexane [31]. Samples were stored at -40°C prior to the
157 analyses.

158 *Chemical analyses*

159 We identified the CLGS composition by gas chromatography-mass spectrometry (GC/MS) on
160 a quadrupol Finigan GCQ, with a DB-5ms non-polar capillary column (5% phenyl (methyl)
161 polysiloxane stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25- μ m film
162 thickness). One μ l was injected in splitless mode at the injector temperature set to 220°C. The
163 temperature of the column was initially held to 70°C for two minutes and then increased at a
164 rate of 10°C/min to 320°C. The temperature was then held at 320°C for five minutes. The
165 carrier gas was helium at a constant velocity of 50 cm/s. Compounds were identified using the
166 retention times and mass spectra of each peak. Double bond positions were determined by
167 DMDS (dimethyl disulfide) derivatization [40].

168 We analyzed all samples by a gas chromatograph-flame ionization detector Shimadzu GC-
169 2010 with a SLB-5ms non-polar capillary column (5% phenyl (methyl) polysiloxane

170 stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25- μ m film thickness) with
171 the same chromatographic conditions as in GC/MS.

172 Peak areas of compounds were detected in GCsolution Postrun (Shimadzu Corporation) with
173 automatic peak detection and noise measurement. We calculated relative amounts (RA in %)
174 of compounds in each sample by dividing the peak areas of compounds by the total area of
175 compounds in each sample. We did not use any correction factor to calculate the RA of
176 individual compounds. We discarded all compounds for which RA were recorded as less than
177 0.1% for all specimens [31]. We elaborated the data matrix as the alignment of each
178 compound between all samples performed with GCAligner 1.0 [41,42](see supplementary
179 file 1).

180 *Statistical analyses*

181 Statistical analyses were performed using R [43] to detected CLGS differentiations between
182 specimen groups. Data consisting of the relative proportion of all compounds were
183 transformed ($\log(x-1)$) to reduce the large difference of abundance between highly and
184 slightly concentrated compounds, and we then standardized (mean= 0, standard deviation= 1)
185 the data matrix to reduce the sample concentration effect [22]. Clustering method was used to
186 detect the divergence between taxa. A Pearson Phi Correlation matrix based on CLGS data
187 matrix (relative abundance of each compound) was computed. An unweighted pair group
188 method with arithmetic mean (UPGMA) was used as clustering method (R-package ape,[44]).
189 We assessed the uncertainty in hierarchical cluster analysis using p -values calculated via
190 multiscale bootstrap resampling with 50.000 bootstrap replications (R-package pvclust,[44]).
191 Only bootstrap values greater than 85% were considered significant. To determine
192 compounds of each taxa (indicator compounds), we used the indicator value (IndVal) method
193 [45,46]. The value given is the product of relative abundance and relative frequency of

194 occurrence of a compound within a group. We evaluated the statistical significance of a
195 compound as an indicator at the 0.01 level with a randomization procedure.

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305 *Table*

306 Table 1. *List of the identified compounds in the Thoracobombus and Cullumanobombus subgenera.* Molecular Weight (MW), median (M [%]) of
307 the 146 identified compounds. For each species, main compounds are shown on black background, characteristic compounds calculated with the
308 Indicator value (IndVal) method (>0.70) are shown in grey background. The compounds we were not able to determine are indicated as *Ux*. The
309 full table with first and fourth quartile (Q1 and Q4 [%]) are in the supplementary file 1

310

Compounds	MW	<i>B. atratus</i>	<i>B. excellens</i>	<i>B.pascuorum</i>	<i>B. muscorum</i>	<i>B. hortulanus</i>	<i>B. melaleucus</i>	<i>B. rubicundus</i>	<i>B. cullumanus</i>	<i>B. semenoviellus</i>
		M	M	M	M	M	M	M	M	M
Octanoic acid	144	-	-	-	-	0.40	0.10	-	-	-
Decanoic acid	172	-	-	-	-	-	0.30	-	-	-
Dodecanol	186	-	-	-	-	-	-	-	-	0.67
U1	?	-	-	-	-	0.16	-	-	-	-
Dodecanoic acid	200	-	-	-	-	-	-	-	-	0.07
Tetradecenol	212	-	-	0.05	-	-	-	-	-	-
Tetradecanol	214	-	-	0.07	-	-	-	-	-	-
Tetradecanoic acid	228	-	-	0.01	-	-	-	-	-	-

Dodecyl acetate	228	-	-	-	-	0.09	-	-	-	-
Hexadec-7-enal	238	-	-	22.49	-	-	-	-	-	-
Hexadec-7-enol	240	-	-	24.12	-	-	-	-	-	-
Heptadecane	240	-	-	-	-	-	0.07	-	-	-
Hexadecanol	242	-	-	0.31	-	-	-	-	-	-
U2	?	-	-	-	-	0.10	-	-	-	-
Tetradecyl acetate	256	-	-	-	-	1.52	0.13	-	-	-
Farnesyl acetate	264	-	-	-	-	1.49	0.10	-	0.11	0.31
Hexadecanol	242	-	-	-	-	-	-	-	-	3.32
Beta-Springene	272	-	-	-	-	-	1.42	0.08	-	0.48
Hexadecenoic acid	254	-	-	5.85	-	-	-	-	-	-
Hexadec-9-enoic acid	254	-	-	-	-	-	-	-	-	6.49
Alpha-Springene	?	-	-	-	-	-	-	-	-	0.19
Hexadecanoic acid	256	-	-	0.32	-	-	3.03	-	-	2.40
U3	?	-	-	-	-	-	-	0.48	-	-
U4	?	-	-	-	-	0.15	-	-	-	-
Octadecanal	268	-	-	-	-	-	-	0.12	-	-

Ethyl hexadec-9-enoate	282	-	-	-	-	0.09	-	-	-	-
Hexadecenyl acetate	282	-	-	1.07	-	-	-	-	-	-
Hexadec-9-enyl acetate	282	-	-	-	-	9.39	23.53	-	-	-
Hexadecyl acetate	284	-	-	0.03	-	1.76	1.28	-	-	0.12
U5	?	0.01	-	-	-	-	-	-	-	-
Octadec-9-enal	266	0.41	-	-	0.11	-	-	-	-	-
Octadec-11-enal	266	0.13	2.42	-	-	-	-	-	-	-
Octadec-13-enal	266	-	0.89	-	-	-	-	-	-	-
U6	?	-	0.99	-	-	-	-	-	-	-
U7	?	-	0.40	-	-	-	-	-	-	-
Octadecanal	268	-	-	-	-	0.02	-	-	-	-
Octadec-9-enol	268	74.04	-	1.25	4.14	-	-	-	-	-
Octadec-11-enol	268	4.72	37.59	-	-	-	-	-	-	-
Octadec-13-enol	268	-	0.07	-	-	-	-	-	-	-
Icosane	282	-	-	0.04	-	-	-	-	-	-
Heneicosene	294	-	-	-	-	-	0.11	-	-	-
Heneicosane	296	0.05	0.30	0.10	0.19	-	0.23	3.78	3.66	-

Octadec-9-enyl acetate	310	1.16	-	-	88.19	-	2.12	-	-	-
Octadec-11-enyl acetate	310	0.16	5.39	-	-	-	-	-	-	-
Octadecadienyl acetate	308	0.02	-	-	-	-	-	-	-	-
Octadecenyl acetate	310	-	-	0.47	-	-	-	-	-	-
Icosenal	294	-	0.12	-	-	-	-	-	-	-
Octadecadienyl acetate1	308	-	0.29	-	-	-	-	-	-	-
Octadecadienyl acetate2	308	-	0.17	-	-	-	-	-	-	-
Dodecyl octanoate	312	-	-	-	-	-	-	-	-	0.14
Geranylgeranyl acetate	322	-	-	-	-	82.93	38.42	91.77	87.94	48.08
Tricos-9-ene	322	0.53	17.77	0.13	-	0.14	0.16	-	-	-
Tricos-7-ene	322	0.48	4.13	0.08	-	-	0.13	-	-	-
Tricos-5-ene	322	0.07	-	-	-	-	-	-	-	-
Tricosane	324	2.91	8.72	3.46	2.63	-	1.06	-	-	-
Unidentified terpene 2	?	-	-	-	-	-	-	-	-	0.00
Geranyl dodecanoate	336	-	-	-	-	-	-	-	-	0.99
Tetracos-9-ene	336	0.00	2.91	0.02	-	-	-	-	-	-
Tetracosene	336	-	-	0.07	-	-	-	-	-	-

Tetracosane	338	0.09	-	0.11	0.21	-	0.06	-	0.07	0.11
Icosenyl acetate1	338	0.03	-	-	-	-	-	-	-	-
Icosenyl acetate2	338	0.02	-	-	-	-	-	-	-	-
Icosyl acetate	338	-	0.88	-	-	-	-	-	-	-
Dodecyl decanoate	340	-	-	-	-	-	-	-	-	1.22
Pentacosadiene	348	-	-	-	-	-	-	-	0.07	0.00
Pentacos-9-ene	350	0.75	0.43	3.49	0.47	-	0.19	0.09	0.09	0.17
Pentacos-7-ene	350	0.78	0.75	0.00	0.36	0.07	0.15	-	0.94	0.50
Pentacosane	352	0.13	2.61	2.09	1.78	0.65	1.48	1.71	2.45	2.10
Geranylgeranyl butyrate	360	-	-	-	-	0.28	0.15	-	-	0.08
Hexacosene1	364	-	-	-	-	-	-	-	0.10	-
Dodecyl dodecanoate	368	-	-	-	-	-	-	-	-	4.85
Hexacosene2	364	1.71	-	0.09	-	-	-	-	-	-
Hexacosane	366	-	-	0.07	-	-	-	-	-	0.07
Docosenyl acetate	366	-	-	-	0.19	-	-	-	-	-
Heptacosadiene1	376	-	-	-	-	-	-	-	0.14	-
Heptacosadiene2	376	-	-	-	-	-	-	-	0.09	-

Heptacos-9-ene	378	0.12	0.24	1.61	0.32	0.12	-	-	0.12	0.19
Heptacos-7-ene	378	0.08	0.87	-	0.13	0.28	0.22	0.16	1.92	0.67
Heptacosane	380	0.13	0.69	0.80	0.36	0.47	1.05	0.68	1.15	1.02
Geranyl hexadec-7-enoate	390	-	-	-	-	-	-	-	-	0.24
Octacosene	392	-	-	0.05	-	-	-	-	-	-
Octacosane	394	-	-	0.06	-	-	-	-	-	-
Unidentified terpene3	?	-	-	-	-	-	-	-	-	0.34
U14	?	-	-	-	-	-	-	-	-	0.05
Dodecyl tetradecanoate	396	-	-	-	-	-	-	-	-	1.19
Squalene	410	-	-	-	-	-	-	-	0.05	-
Nonacosadiene1	404	-	-	-	-	-	-	-	0.03	-
Nonacosadiene2	404	-	-	-	-	-	-	-	0.11	-
Nonacos-9-ene	406	0.06	0.15	0.94	0.12	0.07	-	0.10	0.50	0.21
Nonacos-7-ene	406	0.03	0.18	-	-	0.24	0.22	0.19	-	0.44
Nonacosane	408	0.06	-	0.57	0.08	0.24	0.40	0.26	0.23	0.21
Unidentified terpene 4	?	-	-	-	-	-	-	-	-	0.09
Triacotene1	422	-	-	0.02	-	-	-	-	-	-

311 Table 2. Data on Collection of Biological Material. Collecting sites and number (N) of
 312 samples collected for *Bombus atratus*, *B. excellens*, *B. pascuorum melleofacies*, *B. muscorum*
 313 *liepetterseni*, *B. hortulanus*, *B. melaleucus*, *B. rubicundus*, *B. cullumanus apollineus* and *B.*
 314 *semenoviellus*. Coordinate are given with the reference to the WGS84.

Taxa	Collecting sites	Coordinates	N
<i>Thoracobombus</i> subgenus			
<i>B. atratus</i>	Colombia, Pamplona	7°22'N 72°39'W	4
	Colombia, Tenjo	4°52'N 74°09'W	8
<i>B. excellens</i>	Colombia, Cundinamarca	4°34'N 74°20'W	1
	Colombia, Pamplona	7°22'N 72°39'W	1
<i>B. pascuorum melleofacies</i>	Italy, Torre d'Isola	45°13'N 9°02'E	4
	Italy, Cellara	39°13'N 16°20'E	3
<i>B. muscorum liepetterseni</i>	Norway, Flatanger	64°28'N 10°43'E	5
<i>Cullumanobombus</i> subgenus			
<i>B. hortulanus</i>	Colombia, Pamplona	7°21'N 72°41'W	2
	Colombia, Cundinamarca	4°34'N 74°01'W	1
<i>B. melaleucus</i>	Colombia, Pamplona	7°32'N 72°37'W	2
<i>B. rubicundus</i>	Colombia, Pamplona	7°20'N 72°37'W	7
<i>B. cullumanus apollineus</i>	Turkey, Kars, Çıldır gölü	40°55'N 43°16'E	2
	Turkey, Kars, Göldalı	40°58'N 43°18'E	5
	Turkey, Kars, Çanaksu	41°00'N 43°18'E	2
<i>B. semenoviellus</i>	Czech Republic, Krkonoše	50°41'N 15°51'E	3

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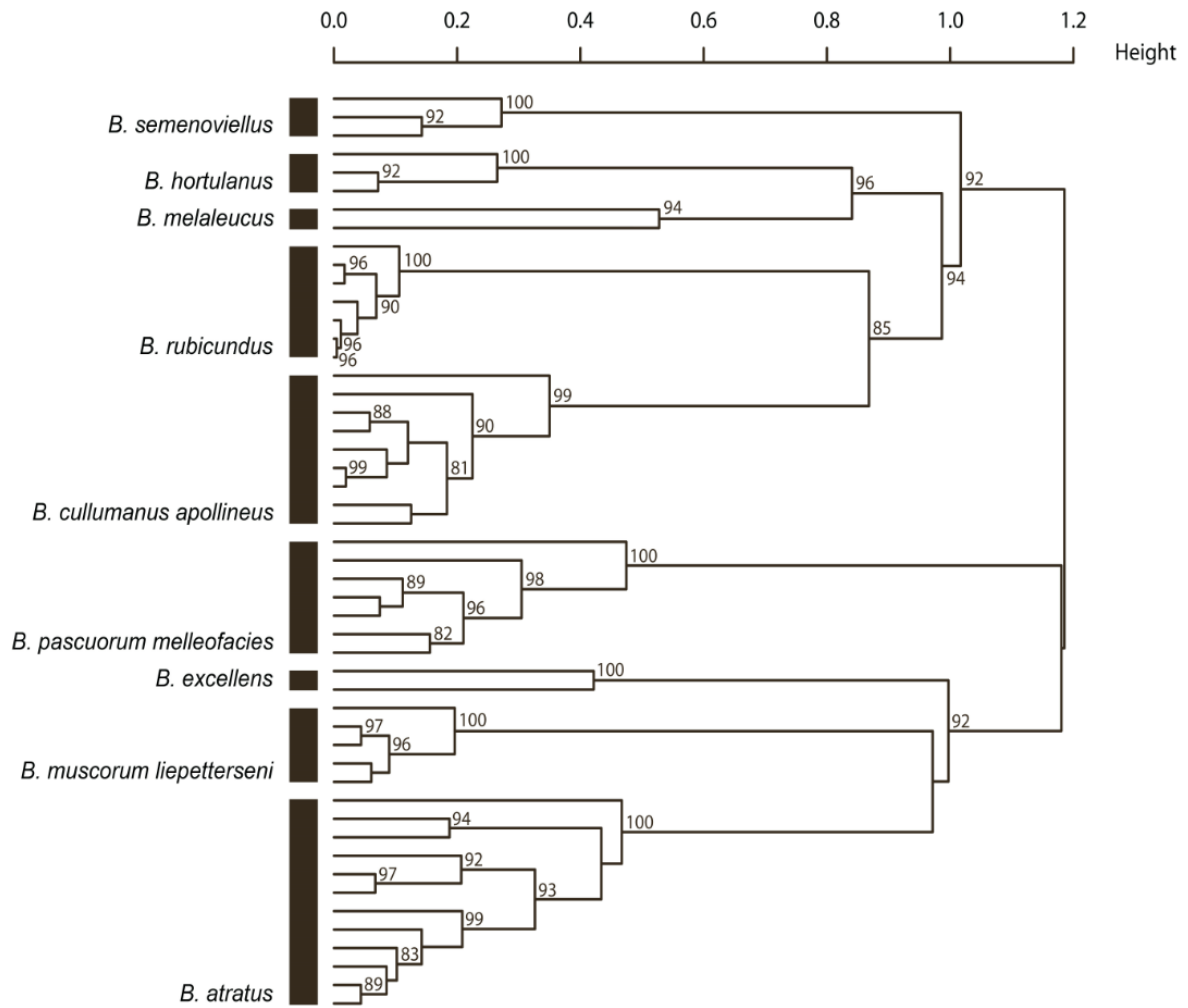
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319 Figure 1. A: *Bombus excellens* from Colombia (photo: W. Hoffmann). B: *Bombus atratus*
320 from Argentina (photo: N. Chimento). C: *Bombus cullumanus apollineus* from Turkey
321 (photo: P. Rasmont). D: *Bombus rubicundus* from Colombia (photo: W. Hoffmann)

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323

324 Figure 2. Unweighted pair group method with arithmetic mean cluster based on a correlation
 325 matrix calculated from matrix of cephalic labial gland secretions of *Bombus atratus*, *B.*
 326 *excellens*, *B. pascuorum melleofacies*, *B. muscorum liepetterseni*, *B. hortulanus*, *B.*
 327 *melaleucus*, *B. rubicundus*, *B. cullumanus apollineus* and *B. semenoviellus*. Values above
 328 branch represent multiscale bootstrap resampling (only values >80% are given)