First Chemical Analysis and Characterization of the Male Species-Specific Cephalic Labial-Gland Secretions of South American Bumblebees

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The evolution of signals and reproductive traits involved in the pre-mating recognition has been in focus of abundant research in several model species, such as bumblebees (genus *Bombus*). However, the most-studied bumblebee reproductive trait, the male cephalic labial gland secretions (CLGS), remains unknown among bumblebee species from South America. In this study, the CLGS of five South American bumblebees of the subgenera *Thoracobombus* (*Bombus excellens* and *B. atratus*) and *Cullumanobombus* (*B. rubicundus*, *B. hortulanus*, and *B. melaleucus*) were investigated, by comparing the chemical compositions of their secretions to those of closely related European species. The results showed an obvious interspecific differentiation in both subgenera. The interspecific differentiation among the species of the *Thoracobombus* subgenus involved different compounds present at high contents (main compounds), while those of the *Cullumanobombus* subgenus shared the same main components. This suggests that among the species of the *Cullumanobombus* subgenus, the differentiation in minor components could lead to species discrimination.

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

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

recognition [13–15][26], as well as they are useful chemo-taxonomic characters [22][27–29].

Despite the usefulness of the CLGS as model in evolutionary biology and taxonomy, the male chemical secretions of a great number of species remain unknown [13]. In fact, the CLGS have been described for 50 species of a total of *ca*. 250 bumblebee species [30]. In several biogeographic regions such as South America, no studies have been published. Indeed, the CLGS chemical analyses require fresh samples [31], which are most of the time unavailable for uncommon taxa. Here, the CLGS of males of five South American bumblebees were investigated. *Bombus melaleucus* HANDLIRSCH, 1888 and *B. hortulanus* FRIESE, 1904 are taxonomically close species belonging to the '*Robustus* group' (= *Robustobombus sensu* RICHARDS, 1968 [32]), while *B. rubicundus* SMITH, 1854 (*Fig. 1*,*d*) belongs to the '*Rubicundus* group' (= *Rubicundobombus sensu* RICHARDS, 1968 [32]), these species-groups being now included in the *Cullumanobombus* subgenus [33]. *B. atratus* FRANKLIN, 1913 (*Fig. 1*,*b*) and *B. excellens* SMITH, 1879 (*Fig. 1*,*a*) both belong to the '*Fervidus* group' (= *Fervidobombus sensu* RICHARDS, 1968 [32]), which is now comprised in the *Thoracobombus* subgenus [33]. The ultimate aim was to compare the CLGS composition of these five South American *Bombus* species with those of related species from the same subgenus, *viz*., *B.* (*Thoracobombus) muscorum liepetterseni* LØKEN, 1973, *B.* (*Thoracobombus*) *pascuorum melleofacies* VOGT, 1911, *B.* (*Cullumanobombus*) *cullumanus apollineus* SKORIKOV, 1910 (*Fig. 1*,*c*), and *B. (Cullumanobombus) semenoviellus* SKORIKOV, 1910.

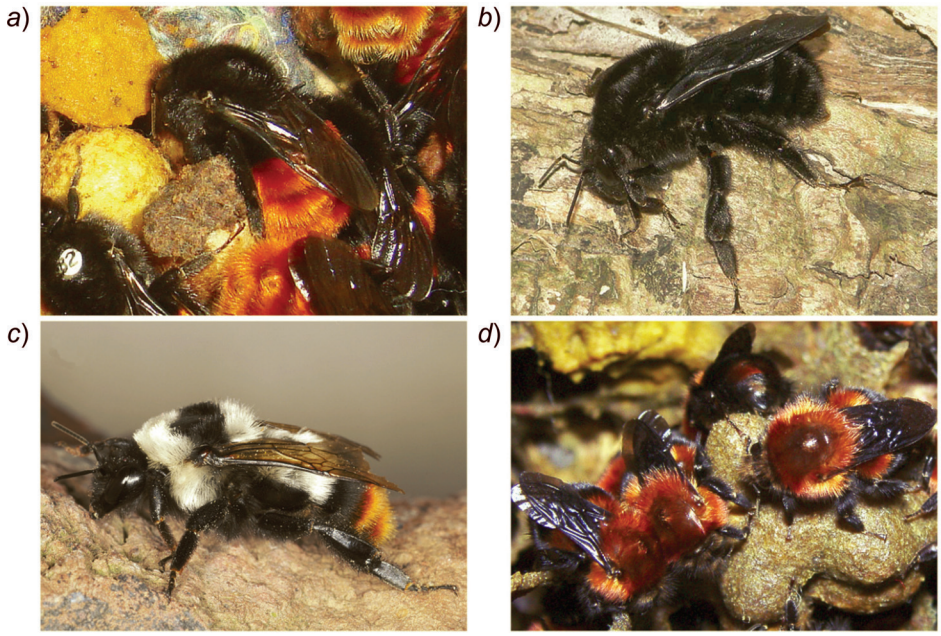

Fig. 1. *Photos of the studied bumblebees:* a) Bombus excellens *from Colombia* (photo: *W. H.*), b) B. atratus *from Argentina* (photo: *Nicolas R. Chimento*), c) B. cullumanus apollineus *from Turkey* (photo: *P. R.*), *and* d) B. rubicundus *from Colombia* (photo: *W. H.*)

Results and Discussion. – *Composition of Cephalic Labial Gland Secretions.* The chemical analyses allowed the detection in the CLGS of 146 compounds in total (33 from *B. excellens*, 35 from *B. atratus*, 53 from *B. pascuorum*, 19 from *B. muscorum*, 16 from *B. rubicundus*, 22 from *B. hortulanus*, 39 from *B. melaleucus*, 22 from *B. cullumanus*, and 44 from *B. semenoviellus*; *cf. Table 1*). The results showed that all species of the *Thoracobombus* subgenus were differentiated in their CLGS composition (seven compounds shared by all species and 17 compounds shared between the two South American species), including differences in the main compounds, *e.g.*, octadec-11-enol (29–46%) was the main compound in the *B. excellens* CLGS, while that of *B. atratus* was dominated by octadec-9-enol (54–91%; *Table 1*). Among the European taxa, the major compounds were hexadec-7-enol (10–31%) and octadec-9-enyl acetate (88%) in the *B. pascuorum* and *B. muscorum* CLGS, respectively (*Table 1*). As main (or major) compounds of a taxon were considered all compounds that had the highest relative amount within the CLGS in at least one individual. Other differences between the South American *Thoracobombus* CLGS were mainly qualitative differences in minor compounds (*Table 1*). Similarly, the *Cullumanobombus* species displayed qualitative differences (only five compounds shared by all species and six compounds shared by the three South American species of the *Cullumanobombus* subgenus), but the main compound, geranylgeranyl acetate, was in common to all *Cullumanobombus* CLGS analyzed here: 81–94% in *B. rubicundus*, 76–83% in *B. hortulanus*, 18–59% in *B. melaleucus*, 51–92% in *B. cullumanus*, and 37–60% in *B. semenoviellus*. Qualitative variations in minor components differentiated these species (*Table 1*).

Stastistical Analysis. For both the species of the *Thoracobombus* and the *Cullumanobombus* subgenera, the statistical analyses of the CLGS composition confirmed the interspecific differentiation (*Fig. 2*). This chemical differentiation is supported by high values of multiscale bootstrap resampling ($>80\%$, *Fig. 2*). The interspecific differentiations between all consubgeneric species observed for the present results are in agreement with the species-specificity of the CLGS compositions observed for other bumblebee groups (*e.g.*, [18][22][27][29]). Similarly to our results obtained for species of the *Thoracobombus* subgenus, the comparison of CLGS between closely related bumblebee taxa with a commonly recognized species status allowed suggesting that the interspecific differentiation involves the change in main compounds *e.g.*, [18][29][34]). These main compound differentiations can be *i*) a significant shift (production of new types of compounds, *e.g.*, *B. pascuorum vs. B. muscorum*) consecutive to switching off and on the respective metabolic pathway (*e.g.*, [7]), *ii*) a large increase of a minor compound shared with closely related species (*e.g.*, octadec-9-enyl acetate between *B. muscorum* and *B. atratus*) [35], as well as *iii*) a difference in the C=C bond position arisen by the activity of different enzymes (*e.g.*, Δ11-desaturase and Δ9-desaturase, introducing a C=C bond in octadecenol in *B. excellens* and *B. atratus*, *e.g.*, [8]) or by activation of a non-functional enzyme gene transcript present in a common ancestor, as observed in moths [9] and in other bumblebees [15]. In contrast, all *Cullumanobombus* species displayed the same main component (geranylgeranyl acetate) as observed in the North American *B.* (*Cullumanobombus*) *rufocinctus* Cresson, 1863 [20] and *B.* (*Cullumanobombus*) *griseocollis* [38]. Geranylgeranyl acetate was also a main component of *B.* (*Alpigenobombus*) *wurflenii* [36] and

Table 1. *Chemical Composition of the Cephalic Labial Gland Secretions of the Studied* Bombus *Taxa Belonging to the* Thoracobombus *and* Cullumanobombus *Subgenera*

Compound[a])	MW[b])	Relative content [%][c])								
		B. atr	*B. exc*	*B. pas*	*B. mus*	*B. hor*	*B. mel.*	*B. rub*	*B. cul*	*B. sem*
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
β-Springene	272	–	–	–	–	–	1.42	0.08	–	0.48
Hexadecenoic acid	254	–	–	5.85	–	–	–	–	–	–
Hexadec-9-enoic acid	254	–	–	–	–	–	–	–	–	6.49
α-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	–	–	–	–	–	–	–	–

Table 1 (cont.)

Compound[a])	MW[b])	Relative content [%][c])								
		B. atr	*B. exc*	*B. pas*	*B. mus*	*B. hor*	*B. mel.*	*B. rub*	*B. cul*	*B. sem*
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	–
U8	?	–	–	0.08	–	–	–	–	–	–
U9	?	–	–	0.05	–	–	–	–	–	–
Ethyl octadecenoate	310	–	–	–	–	–	–	0.50	0.11	–
Geranyl geranial	288	–	–	–	–	–	0.19	0.29	–	–
Geranyl decanoate	308	–	–	–	–	–	–	–	–	0.31
Octadecadienoic acid	280	–	–	–	0.06	–	0.40	–	–	–
Octadecenoic acid	282	–	–	2.84	–	–	–	–	–	–
Octadec-9-enoic acid	282	–	–	–	–	–	–	–	–	1.73
Octadec-11-enoic acid	282	–	–	–	–	–	21.41	–	–	–
Docosane	310	–	0.13	0.24	–	–	0.19	0.08	0.18	–
U10	?	–	–	–	–	–	0.45	–	–	–
U11	?	–	–	–	–	–	0.07	–	–	–
Unidentified terpene 1	?	–	–	–	–	–	–	–	–	0.09
U12	?	–	0.07	–	–	–	–	–	–	–
Ethyl octadecatrienoate	306	–	3.92	–	–	–	0.23	–	0.07	–
U13	?	–	0.93	–	–	–	–	–	–	–
Ethyl octadec-9-enoate	310	0.00	–	–	–	–	–	–	–	–
Octadec-9-enyl acetate	310	1.16	–	–	88.19	–	2.12	–	–	–
Octadec-11-enyl acetate	310	0.16	5.39	–	–	–	–	–	–	–

Table 1 (cont.)

Compound[a])	MW[b])	Relative content [%][c])								
		B. atr	*B. exc*	*B. pas*	*B. mus*	*B. hor*	*B. mel.*	*B. rub*	*B. cul*	*B. sem*
Octadecadienyl acetate	308	0.02	–	–	–	–	–	–	–	–
Octadecenyl acetate	310	–	–	0.47	–	–	–	–	–	–
Icosenal	294	–	0.12	–	–	–	–	–	–	–
Octadecadienyl acetate 1	308	–	0.29	–	–	–	–	–	–	–
Octadecadienyl acetate 2	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 acetate 1	338	0.03	–	–	–	–	–	–	–	–
Icosenyl acetate 2	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
Hexacosene 1	364	–	–	–	–	–	–	–	0.10	–
Dodecyl dodecanoate	368	–	–	–	–	–	–	–	–	4.85
Hexacosene 2	364	1.71	–	0.09	–	–	–	–	–	–
Hexacosane	366	–	–	0.07	–	–	–	–	–	0.07
Docosenyl acetate	366	–	–	–	0.19	–	–	–	–	–
Heptacosadiene 1	376	–	–	–	–	–	–	–	0.14	–

Table 1 (cont.)

Compound[a])	MW[b])	Relative content [%][c])								
		B. atr	*B. exc*	*B. pas*	*B. mus*	*B. hor*	*B. mel.*	*B. rub*	*B. cul*	*B. sem*
Heptacosadiene 2	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 terpene 3	?	–	–	–	–	–	–	–	–	0.34
U14	?	–	–	–	–	–	–	–	–	0.05
Dodecyl tetradecanoate	396	–	–	–	–	–	–	–	–	1.19
Squalene	410	–	–	–	–	–	–	–	0.05	–
Nonacosadiene 1	404	–	–	–	–	–	–	–	0.03	–
Nonacosadiene 2	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
Triacontene 1	422	–	–	0.02	–	–	–	–	–	–
Triacontene 2	422	–	–	0.04	–	–	–	–	–	–
Triacontane	424	–	–	0.06	–	–	–	–	–	–
Dodecyl hexadec-9-enoate	422	–	–	–	–	–	–	–	–	8.04
Dodecyl hexadecanoate	424	–	–	–	–	–	–	–	–	7.63
Geranylgeranyl decanoate	444	–	–	–	–	–	0.06	–	–	–
Hentriacont-9-ene	434	0.04	0.19	0.85	–	–	0.11	–	–	0.12
Hentriacontane	436	0.05	–	0.18	–	–	–	–	–	–
Hexadecenyl tetradecenoate	448	–	–	0.09	–	–	–	–	–	–
Tetradecyl hexadecenoate	450	–	–	0.10	–	–	–	–	–	–
Octadecenyl dodecenoate	448	–	0.09	–	–	–	–	–	–	–
Dotriacontene	448	–	–	0.12	–	–	–	–	–	–
Dotriacontane	450	–	–	0.06	–	–	–	–	–	–
U15	?	–	–	1.29	–	–	–	–	–	–

Table 1 (cont.)

Compound[a])	MW[b])	Relative content [%][c])								
		B. atr	*B. exc*	*B. pas*	*B. mus*	*B. hor*	*B. mel.*	*B. rub*	*B. cul*	*B. sem*
U16	450	–	–	0.23	–	–	–	–	–	–
Tritriacontene	462	–	–	0.07	–	–	–	–	–	–
Dodecyl octadecenoate 1	450	–	–	–	–	–	–	–	–	0.31
Dodecyl octadecenoate 2	450	–	–	–	–	–	–	–	–	0.53
Hexadecyl tetradecanoate	452	–	–	–	–	–	–	–	–	0.21
Octadecenyl tetradecenoate/tetradecanoate	476/478	–	0.12	–	–	–	–	–	–	–
Hexadecenyl hexadecenoate	476	–	–	16.19	–	–	0.06	–	–	–
Hexadecyl hexadec-9-enoate	478	–	–	–	–	–	–	–	–	2.90
Hexadecyl hexadecanoate	480	–	–	–	–	–	–	–	–	0.51
Hexadecenyl octadecatrienoate	500	–	–	0.33	–	–	0.11	–	–	–
Octadecadienyl hexadecenoate	502	–	1.66	–	–	–	–	–	–	–
Hexadecenyl octadecenoate	504	–	–	1.10	–	–	–	–	–	–
Octadecenyl hexadecenoate	504	–	3.92	–	–	–	–	–	–	–
Hexadecyl octadecenoate	506	–	–	0.04	–	–	0.06	–	–	0.09
Octadecenyl hexadecanoate 1	506	0.23	–	–	0.05	–	–	–	–	–
Octadecenyl hexadecanoate 2	506	0.13	–	–	–	–	–	–	–	–
U17	?	0.07	–	–	–	–	–	–	–	–
Geranylgeranyl hexadecanoate	528	–	–	–	–	–	0.41	–	–	–
U18	?	–	–	–	–	–	0.17	–	–	–
Octadecadienyl octadecenoate	530	–	–	2.02	–	–	–	–	–	–
Octadecenyl octadecenoate	532	–	–	3.37	0.57	–	–	–	–	–
Octadecenyl octadecanoate	534	2.18	–	–	0.20	–	–	–	–	–
Octadecenyl octadecatrienoate	528	1.04	–	–	–	–	–	–	–	–

[a]) Unidentified compounds are listed as U1–U18. [b]) MW: Molecular weight. [c]) The median values of the relative contents are given; for the species abbreviations, their subgenus classification, and the number of samples, *cf. Table 2*; for each species, the contents of the main compounds are highlighted with a dark-gray background, and those of the characteristic compounds, calculated with the indicator-value (IndVal) method (> 0.70), are highlighted with a light-gray background; a full table with first and fourth quartile (Q1 and Q4 [%]) can be obtained as *Supplementary Material*[1]).

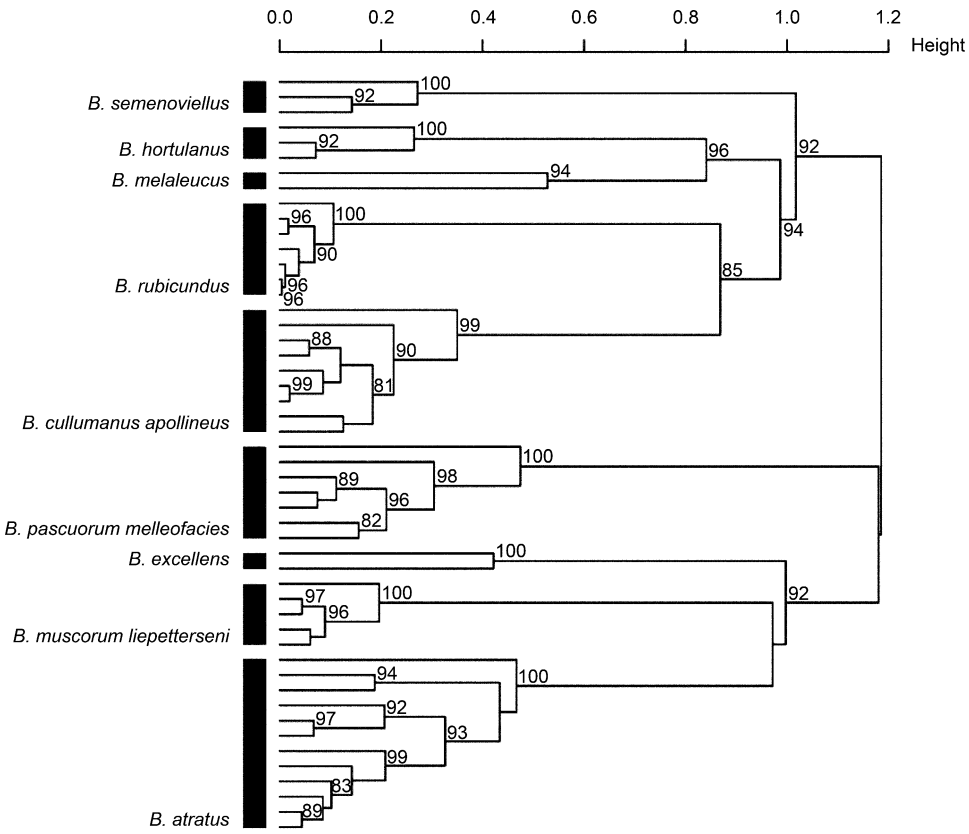

Fig. 2. *Dendrogram obtained by hierarchical clustering using the unweighted pair-group method with arithmetic mean, based on a correlation matrix calculated from a matrix of cephalic labial gland secretions of* Bombus atratus, B. excellens, B. pascuorum melleofacies, B. muscorum liepetterseni, B. hortulanus, B. melaleucus, B. rubicundus, B. cullumanus apollineus, *and* B. semenoviellus. Values above branches represent multiscale bootstrap resampling (only values $>80\%$ are given).

B. (*Kallobombus*) *soroeensis* [37], meaning that this component could not be considered as autapomorphic of the subgenus *Cullumanobombus*. Assuming the key role of CLGS in pre-mating recognition, this suggests that differentiation in minor compounds could lead to species discrimination. Moreover, for each CLGS group, the IndVal method revealed several significant indicator compounds[1]).

Conclusions. – The CLGS of males are widely acknowledged as a useful and practical diagnostic trait for bumblebee species. Among the European bumblebee fauna, several taxonomically doubtful taxa groups have been solved owing to this reproductive trait. The present study is the first one to examine this trait among South

[1]) A table presenting the results obtained with the IndVal method can be obtained as *Supplementary Material* from the authors.

American bumblebee species. This paves the way to a massive taxonomic assessment of South American bumblebees.

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Experimental Part

Biological Material. In total, 50 males of bumblebees belonging to two subgenera were used for this study (*Table 2*). The test group (South American bumblebees) comprised *Bombus melaleucus* ($n=2$), *B. hortulanus* ($n=3$), *B. rubicundus* ($n=7$), *B. atratus* ($n=12$), and *B. excellens* ($n=2$). As comparison group representing the species of the *Thoracobombus* subgenus, specimens already described by *Lecocq et al.* [28] were used, *i.e.*, *B. muscorum liepetterseni* ($n=5$) and *B. pascuorum melleofacies* ($n=7$). *B. cullumanus apollineus* ($n=9$) and *B. semenoviellus* ($n=3$), already described by *Hovorka et al.* [39], comprised the comparison group of the *Cullumanobombus* subgenus. The individual bumblebee specimens were killed by freezing at $-20°$, and the cephalic labial gland secretions (CLGS) were extracted with 400 ml of hexane, according to the method described by *De Meulemeester et al.* [31]. Samples were stored at $-40°$ prior to analysis.

Table 2. *Studied* Bombus *Taxa, Classified According to the Subgenus, Their Abbreviations, Collection Sites, and Number of Samples Collected* (*N*)

Bombus Taxon	Abbreviation	Collection site	Coordinates[a])	*N*
Thoracobombus subgenus				
B. atratus	*B. atr*	Colombia, Pamplona	7°22′N 72°39′W	4
		Colombia, Tenjo	4°52′N 74°09′W	8
B. excellens	*B. exc*	Colombia, Cundinamarca	4°34′N 74°20W	1
		Colombia, Pamplona	7°22′N 72°39′W	1
B. pascuorum melleofacies	*B. pas*	Italy, Torre d'Isola	45°13′N 9°02′E	4
		Italy, Cellara	39°13′N 16°20′E	3
B. muscorum liepetterseni	*B. mus*	Norway, Flatanger	64°28′N 10°43′E	5
Cullumanobombus subgenus				
B. hortulanus	*B. hor*	Colombia, Pamplona	7°21′N 72°41′W	2
		Colombia, Cundinamarca	4°34′N 74°01′W	1
B. melaleucus	*B. mel*	Colombia, Pamplona	7°32′N 72°37′W	2
B. rubicundus	*B. rub*	Colombia, Pamplona	7°20′N 72°37′W	7
B. cullumanus apollineus	*B. cul*	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
B. semenoviellus	*B. sem*	Czech Republic, Krkonoše	50°41′N 15°51′E	3

[a]) Coordinates are given according to the WGS84.

GC/MS Analysis. The qualitative composition of the CLGS was determined by GC/MS analysis using a *Finnigan GCQ* quadrupol system equipped with a nonpolar *DB-5ms* cap. column (5% phenyl (methyl) polysiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temp. was programmed isothermal at 70° for 2 min, then rising from 70 to 320° at 10°/min, and finally held isothermal at 320° for 5 min; injector temp., 220°; carrier gas, He (constant velocity of 50 cm/s); injection vol., 1 μl (splitless mode). Compounds were identified using the retention times (t_R) and mass spectra of each peak. The positions of the C=C bonds were determined by dimethyl disulfide (DMDS) derivatization [40].

GC-FID Analysis. All samples were quantified by GC-FID analysis using a *Shimadzu GC-2010* system equipped with a nonpolar *SLB-5ms* cap. column (5% phenyl (methyl) polysiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm). The chromatographic conditions were the same as described above (*cf. GC/MS Analysis*).

Peak areas of compounds were processed with GCsolution Postrun (*Shimadzu Corporation*) with automatic peak detection and noise measurement. The relative contents (RC) of the compounds in each sample, expressed as percentage, were calculated by dividing the peak areas of the compounds by the total peak area of all compounds in each sample, without using correction factors. The compounds for which RC < 0.1% were recorded, were discarded for all specimens [31]. The data matrix was elaborated as the alignment of each compound between all samples, performed with GCAligner 1.0 [41][42] (*cf. Supplementary Material*[1]).

Statistical Analyses. Statistical analyses were performed using R [43], to detect CLGS differentiations between specimen groups. 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, and the data matrix was then standardized (mean = 0, standard deviation = 1), to reduce the sample-concentration effect [22]. A clustering method was used to detect the divergence between taxa. A *Pearson* Phi Correlation matrix based on the CLGS data matrix (RC of each compound) was computed. The unweighted pair-group method with average (UPGMA) was used as clustering method (R-package ape [44]). The uncertainty in hierarchical cluster analysis was assessed using p-values calculated *via* multiscale bootstrap resampling with 50.000 bootstrap replications (R-package pvclust [44]). To determine characteristic compounds of each taxa (indicator compounds), the indicator-value (IndVal) method was used [45][46]. The value given is the product of relative abundance and relative frequency of occurrence of a compound within a group. The statistical significance of a compound as an indicator at the 0.01 level was evaluated with a randomization procedure.

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