Intraspecific Variation of the Cephalic Labial Gland Secretions in *Bombus terrestris* (L.) (Hymenoptera: Apidae)

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Variations of secretions of the cephalic part of the labial glands from four different subspecies of *Bombus terrestris*, *B. t. terrestris, B. t. lusitanicus, B. t. sassaricus*, and *B. t. dalmatinus,* were investigated. 95 compounds were detected in the whole data set: 54 in *B. t. terrestris*, 54 in *B. t. lusitanicus,* 48 in *B. t. sassaricus*, and 44 in *B. t. dalmatinus.* The (*E*)-2,3-dihydrofarnesol is the main compound in *B. t. dalmatinus* and *B. t. sassaricus*, while it is dihydrofarnesyl dodecanoate in *B. t. terrestris* and *B. t. lusitanicus.* A principal component analysis produced a pattern showing three well distinct groups corresponding to *dalmatinus, sassaricus*, and *terrestris* + *lusitanicus.*

Introduction. – The specific mate recognition system (SMRS *sensu Paterson* 1985 [1]) of bumblebees is largely based on the specificity of pheromonal secretions. Unmatted bumblebee queens (gynes) are attracted to species-specific pheromone blends synthesized in the cephalic part of the labial glands (CLG) of conspecific males [2–7]. These pheromones have, for a long time, been considered as species-specific with a low geographical variability.

Until recently, the few variations observed in male CLG secretions were thought to be related to seasons rather to place of collection [8]. However, individual variability reported in that paper was hidden by the necessity of pooling glands from several specimens to reach the sensitivity level of measuring instruments. Modern instruments are considerably more sensitive, allowing analysis of the secretions from the glands of a single individual [7]. Re-analysis of CLG secretions from previously studied bumblebee species (*i.e.*, before 1996) shows that the individual variability is important, and that the concentration of the major compounds can vary considerably [9].

Such individual variability in the pheromonal blend is already known for several insects including solitary bees (*Colletes cunicularius* (L.) Hymenoptera: Colletidae [10]), ants (Hymenoptera: Formicidae [11]), fruitflies (Diptera: Tephritidae [12–15]) and moths (Lepidoptera: Noctuidae [16–20]). Indeed, pheromonal variability in *Colletes cunicularius* is highly correlated with the distance between sampling localities [10], suggesting that specific recognition systems should be more effective with increasing distance between the geographical origins of potential mates.

Bombus terrestris (L.) is widely distributed in West-Palaearctic region. Its distribution is typically Mediterranean extending from the Canary Islands in the West

to the Altai to the East, and from the Antiatlas Mountains of Morocco in the South to Southern Finland in the North [21–23]. Within its wide distribution, there are important subspecific differences in morphological characters, *e.g.*, coat color [21][22][24–27], and behavior, *e.g.*, innate color preference [28][29] and learning performance [30] which underline the genetic differentiation among subspecies. Of these subspecies, the insular subspecies, *e.g.*, *Bombus terrestris sassaricus*, are the most genetically differentiated [23][30].

While the CLG secretions of *B. terrestris* have been previously studied [2][4][6][31–33], the extent to which pheromone blends vary among subspecies is unknown. In a more recent study, which deals with a population from the Netherlands, *Bergman* identified 23 compounds, including six isoprenoids (*Table 1*).

Hence, the aim of this study was to investigate the potential for variation in CLG secretions among *B. terrestris* males from four different subspecies.

Results and Discussion. – *Chemical Analysis.* 95 compounds were detected in the whole *B. terrestris* data set (*Table 1*): 54 from *B. t. terrestris*; 54 from *B. t. lusitanicus*; 48 from *B. t. sassaricus*, and 44 from *B. t. dalmatinus*. The four subspecies share only 14 compounds in common (*Table 1*), but the two continental subspecies (*B. t. terrestris* and *B. t. lusitanicus*) share 19 specific compounds. The (*E*)-2,3-dihydrofarnesol (DHF; *Fig. 1*), previously suggested to be the main pheromone component for the whole species [4], is not present in the same proportions across all studied subspecies. While it was the main compound found in the CLG extracts from *B.t. dalmatinus* and *B.t. sassaricus* (19–93%), it is the dihydrofarnesyl dodecanoate that was the most abundant compound (14–47%) for *B.t. terrestris* and *B.t. sassaricus*. The other differences between the four subspecies were principally due to the presence or absence of minor compounds.

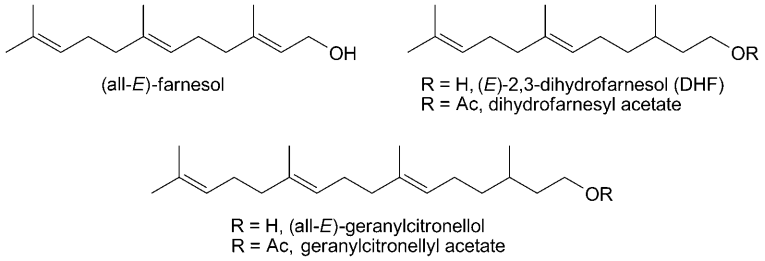

Fig. 1. *Chemical structures of the sesqui- and diterpenes together with their trivial names*

Statistical Analysis. Principal component analysis (PCA) of the data matrix, including data from all 95 compounds, leads a distinct pattern showing three distinct groups of specimens (*Fig. 2*): left to right *1*) *sassaricus, 2*) *dalmatinus,* and *3*) *terrestris* + *lusitanicus*. The first axis separates *terrestris* + *lusitanicus* from the two other subspecies groups, and the second axis separates clearly *dalmatinus* from both other groups.

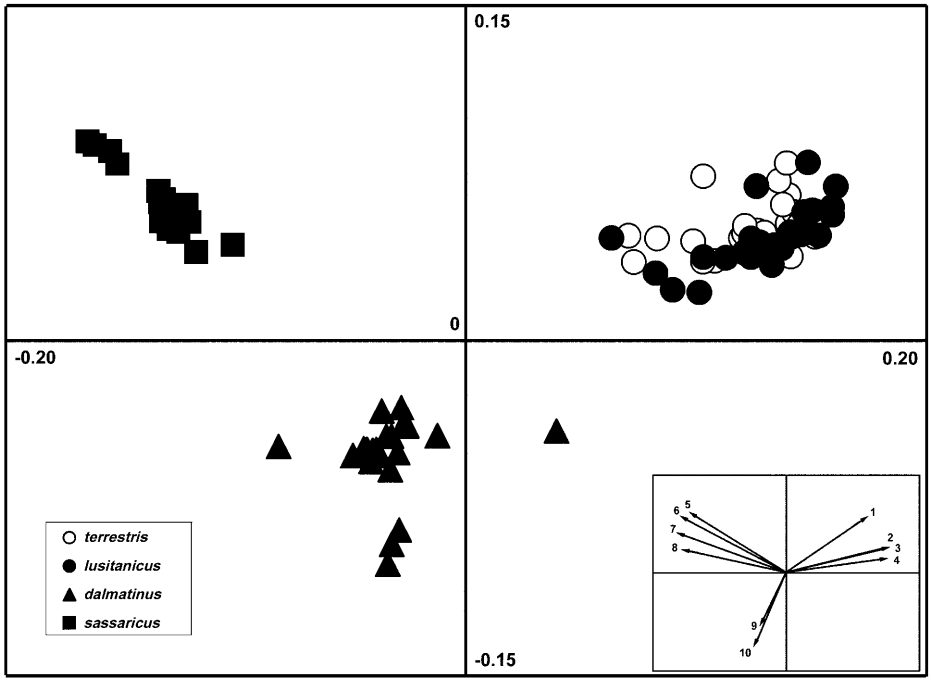

Fig. 2. *Two first axis projection of the PCA based on 95 compounds × 100 specimens.* Populations of *B. t. terrestris* ($n = 27$) and *B. t. lusitanicus* ($n = 27$) are widely overlapping (*top right*). Samples of *B. t. dalmatinus* ($n = 25$; *bottom*) and *B. t. sassaricus* ($n = 20$; *top left*) form two well-separated clouds. The ten most influential compounds in determinig the subspecies positions within the scatterplot are: *1*) farnesol; *2*) octadecatrienyl dodecanoate/octadecyl dodecanoate/hexadecyl tetradecanoate; *3*) hexadecyl dodecanoate; *4*) dihydrofarnesyl dodecanoate; *5*) tricosene 2; *6*) docosenol; *7*) octadecadienol; *8*) hexadecanol; *9*) tetradecanal; *10*) 2,3-dihydrofarnesal.

The *B. t. sassaricus* group is discriminated by the presence of tricosene 2, docosenol, octadecadienol, and hexadecanol. The *B. t. dalmatinus* group is discriminated by tetradecanal and 2,3-dihydrofarnesal. The *B. t. terrestris* + *sassaricus* group is discriminated by the amounts of farnesol, octadecatrienyl dodecanoate/octadecyl dodecanoate/hexadecyl tetradecanoate, hexadecyl dodecanoate, and dihydrofarnesyl dodecanoate.

The analysis of CLG secretions of four *B. terrestris* subspecies shows that, among 95 compounds identified, only 14 are common to the studied taxa. The geographical distributions of *B. t. terrestris* and *B. t. lusitanicus* overlap broadly in Southwest France. These two subspecies share 19 compounds among 54 in *B. t. terrestris* and in *B. t. lusitanicus*. The PCA shows three groups corresponding to: *B. t. dalmatinus*, *B. t. sassaricus* and *B. t. terrestris* + *B. t. lusitanicus*. Our study indicates that differences in CLG secretions exist among populations of *B. terrestris*. These differences affect the composition of secretions both qualitatively and quantitatively. Our results indicate that the divergence between the CLG secretions increases with the geographic distance. The subspecies (*B. t. terrestris* and *B. t. lusitanicus*) with the most similar CLG secretions overlap in their geographic range, while the other two subspecies, with distinct patterns of CLG secretions (*B. t. dalmatinus* and *B. t. sassaricus*), are also

Table 1. *List of the Identified Compounds.* Retention time (t_R [min]), median (M [%]), first and fourth quartile (Q1 and Q4, resp.) of the 95 identified compounds. The compounds we were not able to determine are indicated as U*x*. The 14 common compounds are shown on grey background. The main compounds are shown on black background. A: 2,3-dihydrofarnesyl tetradecadienoate + 2,3-dihydrofarnesyl tetradecenoate; B: 2,3-dihydrofarnesyl hexadecanoate + hexadecyl tetradecanoate + tetradecyl hexadecanoate; C: octadecatrienyl dodecanoate/octadecyl dodecanoate/hexadecyl tetradecanoate.

	t_R	*B. t. terrestris*			*B. t. lusitanicus*			*B. t. dalmatinus*			*B. t. sassaricus*		
		Q1	M	Q4	Q1	M	Q4	Q1	M	Q4	Q1	M	Q4
U1	11.30	0.08	0.14	1.82	0.07	0.11	0.58	–	–	–	–	–	–
Methyl dodecanoate	12.04	0.07	0.16	0.52	0.01	0.19	0.54	–	–	–	–	–	–
Dodecanoic acid	12.66	0.49	1.27	8.3	0.09	0.97	8.86	–	–	–	–	–	–
Ethyl dodecanoate	12.97	3.15	4.88	12.08	3.02	5.19	17.5	–	–	–	–	–	–
Dihydrofarnesene	13.06	–	–	–	–	–	–	0	0	0	–	–	–
Farnesol[a])	13.22	2.25	2.56	5.13	2.22	2.56	3.88	–	–	–	1.03	1.23	2.72
Hexadecene	13.34	–	–	–	–	–	–	0	0	3.37	–	–	–
Ethyl dodecanoate[b])	13.13	–	–	–	–	–	–	0	0	24.5	–	–	–
Ethyl dodecanoate 2	13.21	–	–	–	–	–	–	0.16	0.31	6.25	–	–	–
Tetradecanal	13.40	–	–	–	–	–	–	0.42	1.01	8.44	–	–	–
Isopropyl dodecanoate[b])	13.30	0.1	0.13	0.79	–	–	–	–	–	–	–	–	–
2.3-Dihydrofarnesal[b])	13.46	–	–	–	–	–	–	1.6	1.87	10.3	–	–	–
U2	13.96	0	0	0.59	–	–	–	–	–	–	–	–	–
Dodecyl acetate	14.16	–	–	–	–	–	–	0.09	0.13	1.18	–	–	–
DHF[a])[b])	14.12	4.77	8.35	17.61	4.24	8.41	19.01	26.45	45.02	93.01	19.24	20.67	26.43
2.3-Dihydrofarnesyl acetate[a])	15.15	–	–	–	–	–	–	0.06	0.09	2.21	–	–	–
7-Methylhexadecane	14.30	0.07	0.13	0.33	0.06	0.1	0.71	0.12	0.24	62.16	–	–	–
U3	14.72	–	–	–	0	0	0.07	–	–	–	–	–	–
Tetradecenoic acid	14.84	0.35	0.62	3.11	0.25	0.47	1.72	–	–	–	0.14	0.18	0.98
Tetradecanoic acid	14.92	0.27	0.48	4.78	0.36	0.56	2.57	–	–	–	–	–	–
U4	14.98	–	–	–	–	–	–	–	–	–	0.14	0.21	0.73
Ethyl tetradecenoate	15.09	0.24	0.44	1.91	0.22	0.39	4.7	–	–	–	–	–	–
Ethyl tetradecanoate	15.22	0.36	0.55	0.99	0.45	0.58	1.73	–	–	–	–	–	–
Dihydrofarnesyl acetate	15.34	1.24	1.77	10.52	1.75	2.43	10.99	–	–	–	0.05	0.1	0.34
U5	15.36	–	–	–	–	–	–	–	–	–	0.01	0.01	0.05
Hexadecenal	15.54	–	–	–	–	–	–	0.16	0.24	2.31	–	–	–
Hexadecanal	15.49	0.24	0.34	2.04	0.23	0.29	1.59	0.01	0.13	2.28	0.24	0.31	0.92
Pent-4-en-1-yl dodecanoate	16.11	–	–	–	–	–	–	0	0	0.13	–	–	–
Hexadecenol	16.24	–	–	–	–	–	–	0	0	0.43	0.21	0.27	0.54
Hexadecanol[b])	16.18	0.25	0.85	2.23	0.27	0.65	4.84	1.42	2.86	18.29	7.9	8.46	11.7
Heptadecane	16.50	0	0	0.23	–	–	–	–	–	–	–	–	–
Hexadecenoic acid	16.82	0.14	0.26	1.99	0.21	0.41	1.16	–	–	–	0.51	0.71	1.69
Hexadecenoic acid 2	16.90	0.19	0.31	3.26	0.21	0.3	1.84	–	–	–	0.14	0.18	0.84
Hexadecanoic acid	17.11	–	–	–	0.09	0.2	2.92	0	0	0.05	0.11	0.13	0.28
Octadecadienal	17.08	–	–	–	–	–	–	0	0	0.44	0.28	0.33	1.14
Octadecatrienal	17.34	0.28	0.38	0.89	–	–	–	0	0.06	1.78	0.49	0.6	1.75
Icosane	17.37	–	–	–	–	–	–	0	0	0.12	–	–	–
Nonadecadienal	17.43	–	–	–	–	–	–	0	0.08	3.09	0.02	0.03	0.08
Hexadecyl acetate	17.44	0.76	1.04	2.26	0.71	1.22	1.89	0.07	0.15	0.79	–	–	–

Table 1 (cont.)

	t_R	*B. t. terrestris*			*B. t. lusitanicus*			*B. t. dalmatinus*			*B. t. sassaricus*		
		Q1	M	Q4	Q1	M	Q4	Q1	M	Q4	Q1	M	Q4
U6	17.58	–	–	–	–	–	–	0	0	0.28	–	–	–
Octadecadienol[b])	18.04	–	–	–	–	–	–	0.30	0.81	6.41	9.76	11.21	15.31
Octadecatrienol[b])	18.09	–	–	–	–	–	–	–	–	–	3.67	4.39	13.62
Geranylcitronellal[b])	18.18	1.77	2.30	7.36	1.19	1.80	5.28	0.08	0.88	5.22	0.83	1.42	1.79
Henicosane[b])	18.36	1.34	1.86	9.49	1.09	1.34	2.47	1.19	2.19	23.63	2.08	2.37	2.99
3-Methylicosane	18.50	0.02	0.06	0.59	0.00	0.06	0.80	–	–	–	–	–	–
Geranylcitronellol[a])[b])	18.89	8.95	10.61	28.89	7.20	9.79	20.78	0.53	1.78	12.07	9.29	9.74	13.92
Ethyl octadecenoate	18.97	0.97	1.41	12.09	0.83	1.12	4.89	–	–	–	–	–	–
Octadecadienyl acetate	19.07	0.75	0.99	4.47	0.58	0.67	3.34	0.00	0.00	1.46	0.28	0.39	0.86
Octadecatrienyl acetate	19.13	0.81	1.08	2.13	0.83	1.08	6.46	–	–	–	0.20	0.29	0.63
Docosane	19.28	0.42	0.53	1.29	0.38	0.47	1.00	–	–	–	0.38	0.41	0.55
Octadecyl acetate	19.44	0.00	0.07	1.89	–	–	–	–	–	–	0.04	0.06	0.07
Icosadienol	19.43	–	–	–	–	–	–	–	–	–	0.07	0.10	0.25
Geranylcitronellyl acetate[a])	19.79	0.19	0.32	4.53	0.31	0.59	2.34	–	–	–	–	–	–
Tricosene[b])	19.18	0.75	1.01	2.30	0.62	0.75	1.80	0.09	0.54	6.55	2.57	2.88	3.88
Tricosene 2	19.97	0.40	0.90	2.83	0.00	0.35	0.70	–	–	–	9.10	10.19	15.09
U7	20.02	–	–	–	–	–	–	–	–	–	0.00	0.00	3.13
Tricosane[b])	20.17	5.74	6.36	8.87	4.55	5.54	8.21	0.55	4.72	34.09	5.48	6.08	7.83
U8	20.22	–	–	–	–	–	–	–	–	–	0.05	0.05	0.10
Tetracosene	20.81	–	–	–	–	–	–	0.00	0.00	5.75	–	–	–
Tetracosane	20.95	0.00	0.00	0.39	0.00	0.00	0.62	–	–	–	–	–	–
Icosenyl acetate	20.47	0.17	0.38	0.81	0.23	0.43	1.50	–	–	–	0.01	0.02	0.04
Icosenyl acetate 2	20.95	1.11	1.79	3.81	1.27	1.96	4.88	–	–	–	0.15	0.18	0.27
Icosyl acetate	21.05	0.00	0.18	0.92	0.00	0.23	0.85	–	–	–	–	–	–
Docosenol	21.10	–	–	–	–	–	–	–	–	–	7.55	8.50	12.01
Pentacosene[b])	21.75	0.97	1.22	2.64	0.69	1.16	4.14	0.00	0.08	3.17	–	–	–
Pentacosane[b])	21.67	2.20	2.37	3.70	1.72	2.22	3.48	0.04	0.43	5.46	1.14	1.34	2.20
Methylpentacosane	21.87	–	–	–	0.00	0.00	1.93	0.00	0.00	0.34	–	–	–
Hexacosene	22.15	0.11	0.13	0.45	0.11	0.14	0.96	0.00	0.00	0.60	0.19	0.27	0.38
Docosenyl acetate	22.46	0.72	1.49	4.04	1.39	1.73	5.37	–	–	–	0.02	0.04	0.08
Docosenyl acetate 2	22.56	–	–	–	–	–	–	0.00	0.00	0.48	–	–	–
Hexacosane	22.59	0.12	0.20	0.48	0.00	0.21	0.65	–	–	–	0.01	0.02	0.02
Docosyl acetate	22.65	0.00	0.07	0.45	0.01	0.12	0.46	–	–	–	–	–	–
Heptacosene[b])	22.72	1.89	2.33	3.18	1.60	2.04	10.32	0.10	1.22	12.03	2.77	3.27	4.23
Heptacosane	23.25	0.49	0.59	1.31	0.56	0.72	2.04	0.00	0.00	2.60	0.24	0.37	0.57
U9	23.41	–	–	–	–	–	–	–	–	–	0.40	0.51	0.80
Octacosene	23.40	–	–	–	–	–	–	–	–	–	0.06	0.07	0.12
Dihydrofarnesyl[b]) dodecanoate	23.95	14.32	18.39	31.46	16.72	21.32	47.49	0.30	0.64	17.62	–	–	–
Nonacosene	23.92	0.70	0.84	1.95	0.87	1.00	3.07	0.00	0.08	3.56	0.63	0.74	1.04
U10	24.71	–	–	–	–	–	–	–	–	–	0.17	0.25	0.40
Nonacosane	24.68	–	–	–	0.07	0.11	0.54	–	–	–	0.15	0.27	0.48
Dihydrofarnesyl tetradecanoate[b])	24.88	1.84	2.42	6.63	2.19	3.10	10.88	–	–	–	0.21	0.26	0.52
Hexadecyl dodecanoate	25.25	1.76	2.52	4.01	2.24	2.49	6.73	–	–	–	–	–	–
Hexacosenyl acetate	25.35	0.00	0.09	0.20	0.10	0.12	0.31	–	–	–	–	–	–
Hexacosenyl acetate 2	25.53	–	–	–	0.00	0.03	0.25	–	–	–	–	–	–

Table 1 (cont.)

	t_R	*B. t. terrestris*			*B. t. lusitanicus*			*B. t. dalmatinus*			*B. t. sassaricus*		
		Q1	M	Q4	Q1	M	Q4	Q1	M	Q4	Q1	M	Q4
A	25.62	–	–	–	–	–	–	0.13	0.51	11.83	–	–	–
Hentriacontene	26.04	–	–	–	–	–	–	–	–	–	0	0.02	0.04
B	26.21	–	–	–	–	–	–	0	0.04	2.32	0.04	0.05	0.12
Octadecadienyl dodecanoate	26.52	0.31	0.5	1.44	0.39	0.57	10.38	–	–	–	–	–	–
Dihydrofarnesyl hexadecenoate	26.53	–	–	–	–	–	–	0.02	0.14	3.39	–	–	–
Dihydrofarnesyl hexadecanoate	26.59	–	–	–	–	–	–	–	–	–	0.1	0.11	0.18
C	26.60	1.73	2.04	3.96	1.7	2.1	4.32	–	–	–	–	–	–
Geranylcitronellyl dodecanoate	26.94	0.95	1.51	2.56	1.2	1.86	4.89	–	–	–	–	–	–
Dihydrofarnesyl octadecenoate	27.40	–	–	–	–	–	–	0	0	1.34	–	–	–
Geranylcitronellyl tetradecanoate	27.63	–	–	–	–	–	–	0	0.08	9.41	–	–	–

[a]) Identified by *Bergmann* [6]. [b]) Chemical structures given in *Fig. 1.*

geographically isolated form one another and the other subspecies pair. Moreover, the main compound identified by *Bergman* [6] in *B. t. terrestris* from the Netherlands is the DHF (20.6%). These results suggest a North-South gradient within the *B. t. terrestris* subspecies, corresponding to the genetic gradient observed [23]. 17 of the 23 compounds identified by *Bergman* [6] are present in *Table 1*. On the one hand, all the isoprenoids listed by the latter author are detected here. On the other hand, seven fatty acid derivatives are missing: isopropyl dodecanoate, tetradecanol, tetradecanal, isopropyl tetradecenoate, icosanol, icosenol, and docosenol.

At present, we are not able to connect the phenetical characters of CLG secretions with the phylogeny of *B. terrestris* populations [23][30]. This analysis would be possible when the CLG secretions of most *B. terrestris* subspecies will be known.

The variations found in the CLG secretions of *B. terrestris* could reflect the geographical distance between subspecies, as formerly demonstrated in other taxa. In turnip moth *Agrotis segetum* DENIS and SCHIFFERMÜLLER (Lepidoptera Noctuidae), *e.g.*, various authors also found a geographic variation in the female sex pheromonal secretions and in the sensitive sensilla of males' antenna [18–20][34]. In the same way, in *Polistes dominulus* CHRIST (Hymenoptera Vespidae), *Dapporto et al.* [35] found significant differences in cuticular hydrocarbons from island and continental populations from the Tyrrhenian area. The same observations were made in *Colletes cunicularius* [10], in which different pheromonal 'dialects' are found in females originating from distant origins. However, in this latter case, *C. cunicularius* males are more attracted to the females of the most distinct populations. As we do not know if the *Bombus terrestris* virgin queens are more or less attracted to males from more or less distant origin, we cannot make further inference on the SMRS (Specific Mate

Recognition System) variation. We are currently conducting behavioral experiments to provide data about it.

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Experimental Part

Biological Material. Four taxa were used for this study: *B. t. terrestris* (L.), *B. t. lusitanicus* KRÜGER, *B. t. sassaricus* TOURNIER, and *B. t. dalmatinus* DALLATORRE (*Table 2*). *B. t. terrestris* (27 males) and *B. t. lusitanicus* (27 males) of unknown age were collected in Southwestern France. Queens of *B. t. sassaricus* ($N=2$) from North Sardinia were reared in the laboratory of Prof. *L. Chittka* (Queen Mary University of London, UK) and ten males from each colony were used (age 4–27 d); *B. t. dalmatinus* (25 males, age 5–25 d) was obtained from one commercial bee breeders colony (*Biobest bvba*, Westerlo, Belgium).

Table 2. *Data on Collection of Biological Material.* Collecting sites and number (N) of samples collected for *Bombus terrestris terrestris, B. t. lusitanicus*, *B. t. dalmatinus*, and *B. t. sassaricus.* Coordinates are given with the reference to the WGS84.

Subspecies	Collecting sites	*N*
	France	
B. t. terrestris	Corbère, 42°39′N 2°40′E	4
	Millas, 42°42′N 2°42′E	13
	Ille-sur-Têt, 42°40′N 2°38′E	10
B. t. lusitanicus	Camélas, 42°39′N 2°42′E	1
	Corbère, 42°39′N 2°40′E	4
	Banyuls-dels-Aspres, 42°33′N 2°52′E	1
	Millas, 42°42′N 2°42′E	8
	Ille-sur-Têt, 42°40′N 2°38′E	13
	Greece	
B. t. dalmatinus	Rhodos (Biobest)	25
	Sardinia	
B. t. sassaricus	Luogo Santo, 41°01′N 09°12′E	20

CLG Extracts were prepared according to a protocol adapted from [9]. Secretions were extracted in hexane (200 µl).

Chemical Analysis. Gas chromatography/mass spectrometry (GC/MS) was used to identify chemical compounds (an 'ion trap' *Finigan GCQ*, with a *DB-5ms* non-polar cap. column (5% phenyl(methyl)polysiloxane stationary phase; 30-m column length; 0.25-mm inner diameter; 0.25-µm film thickness). Solns. (1 µl) were injected in splitless mode at the injector temp. set to 220°. The temp. of the column was initially held at 70° for 2 min, rising to 320° at 10°/min, and held at 320° for the last 5 min. The carrier gas was He at a constant velocity of 50 cm/s. Mass spectra were obtained in electron impact mode 'full scan (300–600)'. Compounds were identified using the retention times and mass spectra of each peak.

Statistical Analysis. The NTSYS [36] was used for statistics. The data set was analysed by Principal Component Analysis (PCA). The matrix consisted in records of molecules peaks (expressed as percentage relative abundance) by specimen in GC/MS.

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