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RESEARCH ARTICLE

A simplified protocol for bumble bee species identification by cephalic secretion analysis

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Abstract Studies of bumble bee male sex pheromones are often based on hexane extraction of cephalic labial gland secretions. Here, we propose an alternative extraction procedure based on whole head extractions. To test this method, we compared cephalic parts of the labial glands (CLG) to whole head extractions using high-sensitivity gas chromatography coupled to mass spectrometry (GC–MS) analysis. Two species were analyzed: *Bombus terrestris dalmatinus* and *Bombus ignitus ignitus*. We used correlation coefficients, cluster analysis and the two-group *k*-means method to test the similarity between the two extractions of the two species. We also used the indicator value (IndVal) method to characterize the indicator compounds of each procedure. Statistical analyses reveal a similar compound extraction using both methods. This simplified sample preparation gives reliable results and is more suitable for broad-scale sampling both by specialists and by amateur collectors. Using this method, we provide the first description of the male pheromonal secretions of *B. ignitus ignitus* and demonstrate that these secretions are very similar to the pheromonal blend of *B. terrestris dalmatinus* where 44/51 compounds are shared.

Keywords Bumble bees · *Bombus terrestris dalmatinus* · *Bombus ignitus ignitus* · Sex pheromones · Labial gland secretion

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Introduction

Chemical taxonomy can greatly aid systematists in the discovery of new species and the identification of cryptic species (Reynolds, 2007; Bagnères and Wicker-Thomas, 2010). Among social insects, hydrocarbon analyses have already been applied in wasps (e.g., Bruschini et al., 2007), ants (e.g., Lucas et al., 2002; Dahbi et al., 2008; Martin et al., 2008), bees (e.g., Blum et al., 2000), and termites (e.g., Haverty et al., 1988, 2005). Despite their efficiency, chemical characters have not been extensively used in large revisions of wide groups of insects, because they require fresh specimens and complicated extraction procedures.

Traditional description and identification of bumble bee species (*Bombus*) is often based on subtle differences in morphological characters, making the description and systematic characterization of complex or cryptic species difficult [e.g., at least 9 taxa would be involved in the "*lucorum* complex" (Williams, 1998)]. In part, these uncertainties can be resolved by investigative methods such as DNA analysis (Estoup et al., 1996; Ellis et al., 2005, 2006; Murray et al., 2008), morphometric analysis (Ito, 1987; Williams and Hernandez, 2000; Aytekin et al., 2007) and pheromone analysis (Svensson, 1979; Bertsch, 1997; Terzo et al., 2003, 2005; Bertsch et al., 2005; Rasmont et al., 2005; Coppée et al., 2008).

Sex pheromones are of great interest to bumble bee systematists, because they provide information on the specific status of the studied taxa. These pheromones can be isolated from the secretions of the cephalic portions of the labial glands (CLGs) in males and act as attractants for unmated queens (Kullenberg et al., 1973). Sex pheromones play a major role in the specific mate recognition system and therefore allow the application of the Patterson's "Species recognition concept" (Patterson, 1985, 1993). According to this concept, conspecific individuals sharing the same recognition signals are supposed to recognize each other as sexual partners (Calam, 1969; Agren et al., 1979; see review by Terzo et al., 2003).

Early studies of *Bombus* sex pheromones relied on whole head extractions (Bergstrom and Svensson, 1973; Bergstrom et al., 1973) prior to gas chromatography (GC), and thus extractions from several pooled male heads were required to increase the chromatographic signal. Later, it was discovered that the sex pheromones were synthesized in the CLGs, and the glands themselves were analyzed (Svensson and Bergström, 1977; Svensson, 1980). Nevertheless, the available technology did not allow a comprehensive description of the CLG secretions, and so these previous studies were limited to examination of only the major pheromonal compounds. Since 1996, new technologies have considerably increased the sensitivity of instruments, and use of CLGs individually extracted with hexane is now commonly applied (Terzo et al., 2003). In recent studies, the CLGs are dissected from fresh specimens prior to the compound extraction (Kindl et al., 1999; Rasmont et al., 2005; Terzo et al., 2005). The solvent used for extraction is typically hexane or pentane. Despite its reliability, this sample preparation method is cumbersome, and thus neither suitable for the amateur collector nor even for entomologists who are not specialized in Hymenoptera dissection. Moreover, this method is difficult to apply in the field away from laboratory conditions.

In this study, we propose an alternative procedure to extract bumble bee cephalic secretions. This alternative protocol is based on whole head extractions, which is more suitable for broad-scale sampling by amateurs and casual collectors. The aim of the present paper is to compare high-sensitivity gas chromatography coupled to mass spectrometry (GC–MS) analysis of compound extraction from dissected CLG to whole head pheromonal extracts to determine if the two extraction methods can produce similar results.

Materials and methods

Extraction of bumble bee cephalic secretions

To prevent any degradation of the glands and their secretions, males were kept alive in ventilated plastic vials prior to sample preparation. Immediately before dissection, the males were frozen briefly at −20°C.

CLG dissection method

Following the procedure of Terzo et al. (2005, 2007a), the eyes were removed with a micro-scalpel. Both cephalic parts of the labial glands were then carefully dissected out with tweezers. The CLGs were placed in a glass vial with 200 µl of hexane. Latex gloves were used to prevent any contamination of the sample.

Whole head extraction method

The specimens were decapitated using clean tweezers or a scalpel. The whole head was then placed in a glass vial with 400 µl of hexane for extraction. To prevent contamination of the sample, the experimenter avoided touching the head directly.

Extraction and conservation

Vials containing the solvent and the glands or the head were kept for 24 h at room temperature (20°C) to fulfill the extraction. The vials were then stored in deep-freezing conditions (−20°C) until use for chemical analysis. We stored the remaining bumble bee body in ethanol or as a dried pinned specimen. Each extract (with the head) and the corresponding bodies were stored as a voucher and were carefully labeled with a unique code to maintain the integrity of the sample.

Materials

A total of 20 males of *Bombus terrestris dalmatinus* Dalla Tore and 20 males of *B. ignitus ignitus* Smith were analyzed. The *B. t. dalmatinus* strain originated from Rhodos (Greece), while the *B. i. ignitus* strain was from Sapporo (Japan). Choice of the species was motivated by their breeding availability and by their close phylogenetic relationship (Cameron et al., 2007). For both species, individuals that emerged from only one colony (provided by commercial bee breeders Biobest bvba) were killed at the same age (7 days), and dissected at the same time to maximize variation due to the extraction protocol and minimize the intraspecific variability. For each species, ten males were prepared following the whole head extraction protocol and ten males following the CLG extraction protocol.

Chemical analyses

Mass spectrometry analysis

GC–MS analyses were performed using a Waters GCT Premier instrument based on an orthogonal injection time-

of-flight analyzer. For all the measurements, 1 µl of hexane solution was injected . The gas chromatograph was equipped with a Restek Rtx-5Sil MS column (30-m length, 0.25-mm ID and 0.25-µm DF). Typical GC conditions were: injector temperature, 250°C; splitless mode; Helium carrier gas flow rate, 1 ml/min; interface temperature: 250°C. The temperature program was as follows: initial temperature, 55°C (hold 2 min); 10°C/min ramp; final temperature, 300°C (hold 2 min). Electron ionization (EI) source conditions were: source temperature, 200°C; electron energy, 70 eV; trap current, 200 µA; emission current, 400 µA. All ions were transmitted into the pusher region of the time-of-flight analyzer where they were mass analyzed with a 1-s integration time. Data were acquired in continuum mode. The GCT Premier instrument is a highly sensitive instrument and, for instance, in EI positive ionization mode, 1 pg of hexachlorobenzene gives a signal-to-noise ratio of S/N > 10:1 while acquiring full spectra over a mass range up to *m*/*z* 800.

Sample preparation and GC–MS data analysis

Each sample was diluted ($1/10^2$ v/v) in hexane. For each sample, 1 µl of the diluted hexane solution was injected into the GC. The relative concentration (%) of the individual component was calculated based on GC peak areas observed without using correction factors. The MS fragmentation pattern was compared with those of the pure compound, by matching the MS fragmentation patterns with National Institute of Standards and Technology (NIST) MS Search/version 4.0 mass spectra libraries and with those given in the literature.

Statistical analysis

A data matrix was assembled based on the original GC output (record of molecule peaks, Fig. 1) as variables. Molecule peaks were integrated with MassLynx version 4.1 (2006 Waters Inc.). We used automatic peak detection and noise measurement and then manually checked the integration. Molecule peaks were expressed as relative abundance (RA) by specimen (summarized in Table 1). Following Terzo et al. (2007a), we only retained molecules for which relative abundances were recorded as more than 0.1% for at least one specimen.

Before statistical analysis, the data matrix (specimens × RA) was first transformed ($\log(x + 1)$) to reduce the great difference of abundance between highly and slightly concentrated compounds. We then standardized each compound (mean = 0, standard deviation = 1) to reduce the sample concentration effect.

Species discrimination was based on a UPGMA cluster analysis using a correlation distance matrix (Rasmont et al., 2005). In addition to the hierarchical cluster analysis, a two-group *k*-means method was performed (MacQueen, 1967). This heuristic method splits the data set into two groups in which each specimen belongs to the group with the nearest centroid. The method was repeated 50,000 times to avoid local *k*-means solutions, and a consensus cluster was used to display the results (UPGMA clustering based on the Euclidean distance of *k*-means solutions).

A Spearman's rank correlation coefficient was calculated between the median RA of compounds observed in the gland extraction and in the head extraction. This coefficient is based on a monotonic function and evaluates the relationship between the pheromonal secretions of paired groups. A coefficient of +1 or −1 describes a perfect correlation between the secretions. The coefficient was calculated separately for both species and the obtained values were compared to the interspecific correlation coefficient (calculated between the median RA of compounds observed in the head extraction of the two species).

To assess the effects of sample preparation, we re-sampled the original data set (RA matrix) into two sub-data sets comprising data belonging, respectively, to *B. t. dalmatinus* (Head + GLC) and *B. i. ignitus* (Head + GLC). Normalization of the data was done again as previously described. The effect of the extraction procedure was evaluated on each sub-data set by hierarchical cluster analysis (UPGMA based on a correlation distance matrix) and by a two-group *k*-means method (repeated 50,000 times). Results of *k*-means were displayed by a consensus cluster as previously described.

The effects of sample preparation were also characterized by compounds using the indicator value (IndVal) method (Dufrene and Legendre, 1997; Claudet et al., 2006) on both sub-data sets. The value given is the product of relative abundance and relative frequency of occurrence of a compound within a group. A high value is obtained when the compound is specific and regular to a particular group compared to the whole set of observations. The statistical significance of a compound as an indicator at the 0.05 level was evaluated using a randomization procedure.

Statistical analyses were performed using R version 2.10.1 (R Development Core Team, 2009).

Results

Chemical analysis

Typical gas chromatograms for the head and cephalic labial gland secretions of males of *B. t. dalmatinus* and *B. i. ignitus* are given in Fig. 1. One specimen of *B. t. dalmatinus* was excluded because the extraction failed. The data matrix comprises 39 specimens and 51 molecules (Table 1). Two

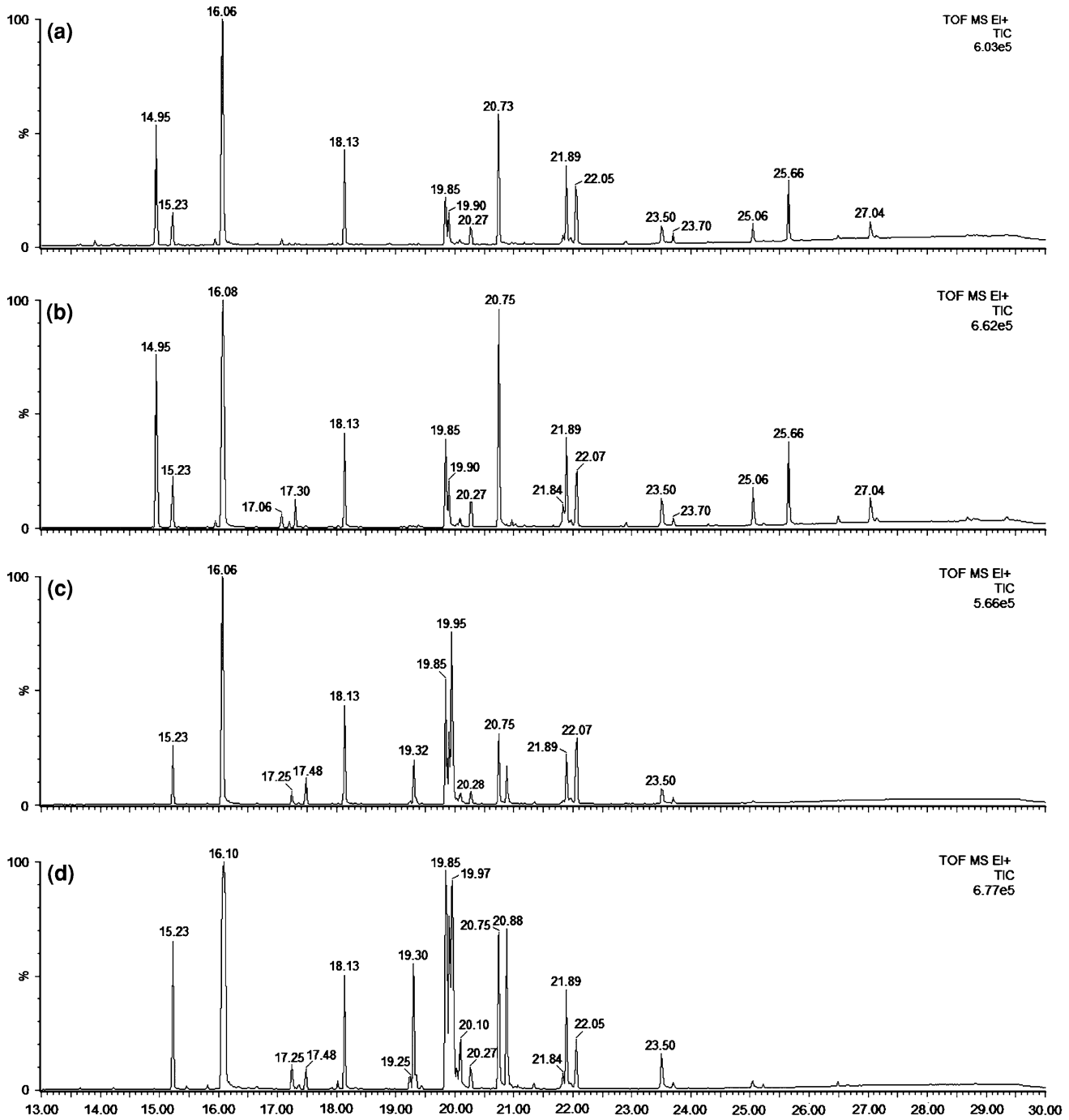

Fig. 1 Typical gas chromatogram of (**a**) CLG and (**b**) head extraction for males of *B. t. dalmatinus*; (**c**) CLG and (**d**) head extraction for males of *B. i. ignitus*

molecules are unique to *B. t. dalmatinus* [tetradecanoic acid ethyl ester (RT = 17.20); hexadecanol acetate (RT = 19.38)]. Seven molecules are unique to *B. i. ignitus* [hexadecenal (RT = 17.25); octadecenal (RT = 19.43); octadecenol (RT = 19.95); geranylcitronellyl acetate (RT = 20.88); octadecenol acetate (RT = 21.05); octadecanoic acid ethyl ester (RT = 21.10); dihydrofarnesyl heptanoate (RT = 24.57)]. The chemical composition of these two species is quite similar: they share 42/51 compounds. Among the 51 detected compounds, 20 are determined as trace molecules (all RA <1%).

Species discrimination

Based on the cluster analysis (UPGMA on a correlation distance matrix), it appears that species can easily be

Table 1 Compounds of the cephalic secretions of *B. t. dalmatinus* and *B. i. ignitus* from CLGs and head extractions

RT	Compounds	MM	*B. terrestris dalmatinus*						*B. ignitus ignitus*					
			CLG ($n = 10$)			Head ($n = 9$)			CLG ($n = 10$)			Head ($n = 10$)		
			Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
14.01	Pentadecene	210	–	0.003	0.013	–	–	0.007	0.003	0.011	0.135	0.002	0.006	0.013
14.23	Pentadecane	212	0.022	0.055	0.904	0.011	0.015	0.613	0.008	0.049	1.355	0.006	0.034	0.098
14.34	Hexadecene	224	0.011	0.030	0.142	0.005	0.008	0.103	0.005	0.013	0.186	0.003	0.009	0.021
14.94	Ethyl dodecanoate	228	**5.151**	**10.029**	**12.060**	**2.334**	**7.740**	**11.936**	–	0.005	0.030	–	0.002	0.012
15.03	Hexadecane	226	–	–	0.134	–	–	–	0.014	0.026	0.125	0.005	0.015	0.027
15.21	Dihydrofarnesal	222	2.386	3.360	4.886	2.531	3.309	5.065	2.163	3.299	4.501	2.758	4.272	6.182
15.93	Tetradecanol	214	0.060	0.410	0.847	0.076	0.360	1.177	–	0.006	0.035	–	0.004	0.015
16.06	Dihydrofarnesol	224	**28.091**	**31.814**	**39.274**	**22.103**	**26.527**	**30.683**	**21.366**	**23.756**	**33.738**	**11.689**	**18.647**	**23.051**
16.18	Heptadecane	240	–	–	0.661	–	–	–	–	–	0.594	–	–	–
17.06	Tetradecenoic acid, ethyl ester	254	0.212	0.492	1.734	0.138	0.476	0.774	–	0.027	0.098	–	0.016	0.057
17.20	Tetradecanoic acid, ethyl ester	256	0.138	0.230	0.511	0.006	0.124	0.329	–	–	–	–	–	–
17.25	Hexadecenal	238	–	–	–	–	–	–	0.375	0.649	1.034	0.725	1.065	2.429
17.30	Dihydrofarnesyl acetate	266	0.039	0.246	1.309	0.138	1.489	6.315	–	–	–	–	0.045	0.857
17.35	Hexadecenal	238	–	0.035	0.159	–	–	0.125	0.056	0.112	0.199	0.080	0.185	0.420
17.48	Hexadecanal	240	0.079	0.115	0.226	0.045	0.097	0.286	0.903	1.655	3.247	0.677	1.476	2.261
18.01	Hexadecenol	240	0.023	0.060	0.192	0.017	0.097	0.164	0.054	0.088	0.199	0.131	0.231	0.407
18.13	Hexadecanol	242	0.920	5.220	8.256	2.472	4.015	6.478	2.567	6.402	10.093	2.874	6.059	7.107
18.41	Nonadecane	268	0.065	0.151	0.313	–	0.060	0.201	0.021	0.056	0.269	0.073	0.088	0.686
18.85	Icosane	282	–	0.069	0.244	–	0.038	0.165	–	0.058	0.372	0.022	0.035	0.058
19.23	Octadecadienal	264	–	0.025	0.078	0.064	0.100	0.152	0.187	0.241	0.718	0.580	0.744	2.816
19.30	Octadecatrienal	262	0.050	0.096	0.311	0.069	0.117	0.225	**2.710**	**3.415**	**5.698**	**5.006**	**5.947**	**7.875**
19.38	Hexadecanol acetate	284	0.014	0.115	0.223	0.024	0.131	0.186	–	–	–	–	–	–
19.43	Octadecenal	266	–	–	–	–	–	–	0.043	0.077	0.251	0.091	0.156	0.798
19.83	Octadecadienol	266	0.621	3.014	5.446	1.027	3.999	5.607	**6.221**	**8.199**	**12.528**	**5.452**	**8.534**	**11.436**
19.90	Octadecatrienol	264	0.498	1.931	2.538	0.811	1.904	2.789	4.783	5.584	7.125	2.831	6.692	8.435
19.95	Octadecenol	268	–	–	–	–	–	–	**8.913**	**13.333**	**15.608**	**9.823**	**11.585**	**12.744**
20.08	Geranylcitronellal	290	0.656	0.798	1.261	0.677	0.863	1.493	0.935	1.423	3.707	2.121	2.527	6.281
20.27	Henicosane	296	0.917	2.362	4.005	0.872	1.790	4.318	0.886	1.454	2.646	0.792	1.821	2.928
20.73	Geranylcitronellol	292	**12.083**	**16.798**	**20.675**	**13.267**	**18.074**	**27.685**	**4.046**	**7.106**	**9.656**	**5.309**	**7.349**	**9.613**
20.88	Geranylcitronellyl acetate	310	–	–	–	–	–	–	0.865	3.143	8.584	2.058	4.447	7.093
20.95	Octadecadienol acetate	308	–	0.173	0.425	0.128	0.257	0.522	–	–	0.112	–	0.184	1.812
21.02	Octadecatrienoic acid ethyl ester	306	–	0.053	0.258	–	0.130	0.202	–	–	0.023	–	0.019	0.087
21.05	Octadecenol acetate	310	–	–	–	–	–	–	–	0.023	0.132	0.098	0.294	1.394
21.10	Octadecanoic acid, ethyl ester	312	–	–	–	–	–	–	–	–	0.100	–	0.004	0.042
21.18	Docosane	310	0.095	0.274	0.416	0.051	0.137	0.494	0.061	0.161	0.327	–	0.086	0.597
21.33	Eicosenal	294	–	0.009	0.101	–	–	0.181	0.099	0.156	0.454	0.130	0.324	0.858
21.83	Tricosene	322	0.681	1.339	1.902	0.867	1.369	2.649	0.027	0.265	0.598	0.561	0.804	3.285
21.88	Docosenol	296	2.455	3.561	6.971	2.617	5.293	5.731	2.318	3.969	6.372	2.443	3.583	6.649
21.94	Docosanol	298	–	0.307	1.560	0.147	0.319	0.460	–	0.267	0.902	–	0.435	2.917
22.05	Tricosane	324	3.239	6.543	10.559	1.727	4.990	8.697	4.884	7.919	10.946	1.702	3.914	5.353
22.89	Tetracosene	338	0.077	0.232	0.656	0.099	0.258	0.646	0.038	0.092	0.200	0.035	0.089	0.448
23.50	Tetracosenol	324	0.998	1.547	2.094	1.408	1.994	2.451	0.962	1.568	2.504	1.566	2.939	4.620
23.70	Pentacosane	352	0.357	0.953	1.631	0.242	0.862	1.505	0.395	1.025	2.643	0.222	0.632	2.192

Table 1 continued

RT	Compounds	MM	*B. terrestris dalmatinus*						*B. ignitus ignitus*					
			CLG ($n = 10$)			Head ($n = 9$)			CLG ($n = 10$)			Head ($n = 10$)		
			Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
24.29	Hexacosene	364	0.059	0.136	0.213	0.113	0.148	0.207	–	0.007	0.041	–	0.015	0.065
24.57	Dihydrofarnesyl heptanoate	488	–	–	–	–	–	–	–	0.001	0.017	–	–	0.181
25.05	Heptacosene	378	0.866	1.280	3.015	1.623	2.608	4.059	0.094	0.285	0.716	0.395	0.625	1.325
25.22	Heptacosane	380	–	0.048	0.123	0.110	0.237	0.292	–	0.024	0.065	0.047	0.145	0.572
25.65	Dihydrofarnesyl dodecanoate	406	1.948	3.013	4.968	0.021	6.244	7.277	–	–	0.014	–	0.001	0.011
26.49	Nonacosene	406	0.090	0.205	0.580	0.397	0.539	1.240	0.016	0.040	0.116	0.113	0.181	0.454
27.04	Dihydrofarnesyl tetradecenoate	432	0.449	0.674	1.653	0.552	1.906	3.970	–	–	0.017	–	0.006	0.037
27.14	Dodecanoic acid, hexadecyl ester	424	0.034	0.228	0.395	–	0.352	0.620	–	–	0.018	–	–	0.008

Retention time (RT), molecular mass (MM), minimum (min), median (med) and maximum relative abundance (max) of the 51 identified compounds. The species' main compounds (minimal RA >5%) are highlighted in bold

diagnosed using cephalic secretions. The two species we tested are clearly distinct as also shown in the two-group *k*-means consensus cluster (Fig. 2). The two groups correspond, respectively, to *B. t. dalmatinus* and *B. i. ignitus* without sub-groups due to sample preparation. Moreover, *k*-means clustering did not suggest clustering due to the extraction method in spite of the grouping by species.

Effect of sample preparation of species sub-data set

Based on Spearman's rank correlation coefficient, the median RAs of compounds observed in the two sample preparations are highly correlated within the two species (*B. t. dalmatinus*: Spearman's $\rho = 0.97$; *B. i. ignitus*: Spearman's $\rho = 0.93$). These coefficient values are compared to the coefficient observed between species for head extraction (Spearman's $\rho = 0.32$). These values show a clear specific distinction of the pheromonal secretions and a close relationship between the sample preparations.

The sub-data set of *B. t. dalmatinus* comprises 19 specimens as objects and 44 molecules as descriptors. Two trace molecules are absent in head extractions of *B. t. dalmatinus* [hexadecane (RT = 15.03) and heptadecane (RT = 16.18), both found only in 3 samples of CLG extraction]. The cluster analysis was not able to discriminate between the two sample preparations (Fig. 3). Two major groups cluster separately. Both groups comprise samples from head extractions and CLG extractions. This clustering reveals that variations between extraction procedures are lower than variations that occur naturally between individuals and between injection processes (measurement errors). The clustering also shows that there are no particular common compounds that would suggest clustering based on the extraction method. The consensus cluster based on the two-group *k*-means also splits the data set into two groups, both of which comprise mixed samples of head extraction and

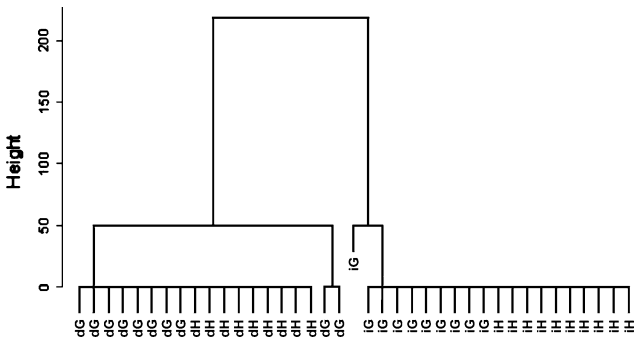

Fig. 2 Consensus cluster obtained from the solutions of the two-group *k*-means (repeated 50,000 times) on pheromonal secretions of species. Consensus is performed by UPGMA clustering based on the Euclidean distance. Secretions of *B. t. dalmatinus* from GLC (dG) and head extraction (dH) and of *B. i. ignitus* from GLC (iG) and head extraction (iH)

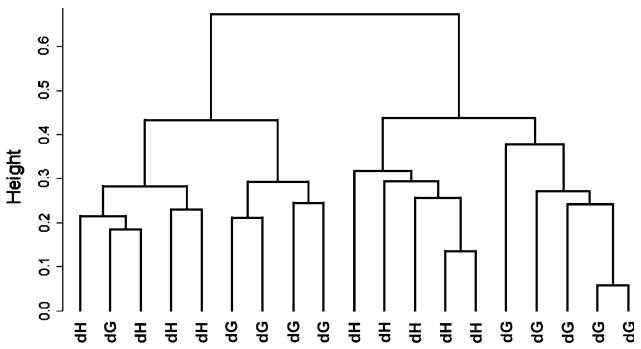

Fig. 3 UPGMA clustering of *B. t. dalmatinus* pheromonal secretions. Clustering is based on a correlation distance matrix. Code labels as mentioned in Fig. 2

Table 2 IndVal values of the eight significant indicator compounds of *B. t. dalmatinus* CLG (dG) and head (dH) extractions, minimal (G min, H min) and maximal RA (G max, H max) of the indicator compounds

Significant indicator compounds	dG	dH	G min	G max	H min	H max
dG						
Dihydrofarnesol (16.06)	0.83	0.02	28.09	39.27	22.10	30.68
dH						
Dihydrofarnesyl acetate (17.30)	0.01	0.52	0.04	1.31	0.14	6.31
Octadecadienal (19.23)	**0.02**	**0.95**	–	**0.08**	**0.06**	**0.15**
Tetracosenol (23.50)	0.07	0.59	1.00	2.09	1.41	2.45
Heptacosene (25.05)	0.06	0.71	0.87	3.02	1.62	4.06
Heptacosane (25.22)	0.00	0.89	–	0.12	0.11	0.29
Dihydrofarnesyl dodecanoate (25.65)	0.01	0.73	1.95	4.97	0.02	7.28
Nonacosene (26.49)	**0.01**	**0.91**	**0.09**	**0.58**	**0.40**	**1.24**

IndVal values and RAs of the best indicator compounds (indicator value greater than 0.90) are highlighted in bold

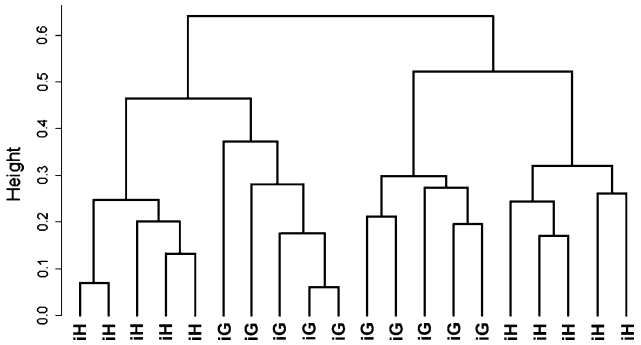

Fig. 4 UPGMA clustering of *B. i. ignitus* pheromonal secretions. Clustering is based on a correlation distance matrix. Code labels as mentioned in Fig. 2

CLG extraction. Once again, *k*-means does not suggest clustering due to the extraction method.

The IndVal method reveals one significant indicator compound for the gland extraction procedure and seven significant indicator compounds for the head extraction procedure (Table 2). Among them, only two compounds characterize the head extraction procedure with an indicator value higher than 0.90 (octadecadienal, nonacosene). Except for a slight difference in dihydrofarnesyl acetate abundance, the ranges of RA of the significant indicator compounds are considered to be similar.

The sub-data set of *B. i. ignitus* comprises 20 specimens and 49 molecules. One trace molecule is absent in CLG extraction of *B. i. ignitus* [dihydrofarnesyl acetate (RT = 17.30), found in 5 samples of head extractions] and one trace molecule is absent in head extraction of *B. i. ignitus* (heptadecane (RT=16.18), found only in 1 sample of CLG extraction]. As found in *B. t. dalmatinus*, neither cluster analysis nor the two-group *k*-means method are able to discriminate between the two sample preparations (Fig. 4) and no particularity is found to suggest clustering due to extraction method.

Table 3 IndVal values of the 19 significant indicator compounds of *B. i. ignitus* CLG (iG) and head (iH) extractions, minimal (G min, H min) and maximal RA (G max, H max) of the indicator compounds

Significant indicator compounds	iG	iH	G min	G max	H min	H max
iG						
Hexadecane (15.03)	0.49	0.00	0.01	0.12	0.01	0.03
Dihydrofarnesol (16.06)	**0.94**	**0.01**	**21.37**	**33.74**	**11.69**	**23.05**
Octadecenol (19.95)	0.74	0.02	8.91	15.61	9.82	12.74
Tricosane (22.05)	**0.90**	**0.00**	**4.88**	**10.95**	**1.70**	**5.35**
iH						
Dihydrofarnesal (15.21)	0.02	0.73	2.16	4.50	2.76	6.18
Hexadecenal (17.25)	0.02	0.74	0.37	1.03	0.73	2.43
Dihydrofarnesyl acetate (17.30)	0.00	0.50	–	–	–	0.86
Hexadecenal (17.35)	0.02	0.55	0.06	0.20	0.08	0.42
Hexadecenol (18.01)	0.00	0.67	0.05	0.20	0.13	0.41
Octadecadienal (19.23)	0.00	0.87	0.19	0.72	0.58	2.82
Octadecatrienal (19.30)	**0.01**	**0.93**	**2.71**	**5.70**	**5.01**	**7.88**
Geranylcitronellal (20.08)	0.03	0.61	0.93	3.71	2.12	6.28
Octadecadienol acetate (20.95)	0.00	0.50	–	0.11	–	1.81
Octadecenol acetate (21.05)	0.00	0.50	–	0.13	0.10	1.39
Tricosene (21.83)	0.00	0.70	0.03	0.60	0.56	3.28
Tetracosenol (23.50)	0.01	0.76	0.96	2.50	1.57	4.62
Heptacosene (25.05)	0.03	0.63	0.09	0.72	0.39	1.33
Heptacosane (25.22)	0.00	0.80	–	0.06	0.05	0.57
Nonacosene (26.49)	**0.00**	**0.90**	**0.02**	**0.12**	**0.11**	**0.45**

IndVal values and RAs of the best indicator compounds (indicator value greater than 0.90) are highlighted in bold

The IndVal method reveals 4 significant indicator compounds for the gland extraction procedure and 15 for the head extraction procedure (Table 3). Among them, two compounds characterize the gland extraction procedure

with an indicator value higher than 0.90 (dihydrofarnesol, tricosane) and two compounds characterize the head extraction procedure with an indicator value higher than 0.90 (octadecatrienal, nonacosene). As found in *B. t. dalmatinus*, except for a slight difference in some compound abundances (dihydrofarnesol, tricosane, octadecatrienal, geranylcitronellal and tricosene), the ranges of RA of the significant indicator compounds are considered to be similar.

Discussion

Species characterization

As far as we know, the present results include the first description of the male CLG secretions of *B. i. ignitus*. The secretions of this Far-East species appear surprisingly very close to those of *B. t. dalmatinus* (Fig. 1). Among the 51 identified compounds in Table 1, 44 are common to both species. These results provide an interesting comparison to the variation observed within pheromonal secretions of the *B. terrestris* species complex (Coppée et al., 2008), where the two west-continental subspecies of *B. terrestris* (*B. t. terrestris* and *B. t. lusitanicus*) share only 19 common compounds among the 54 recorded. However, the present results do confirm that there are indeed species-specific differences between *B. t. dalmatinus* and *B. i. ignitus*. These two species are well discriminated by the statistical analyses and show great differences in the relative abundance of their common compounds. However, the large number of shared compounds between these two species could indicate a possible attractant effect between *B. t. dalmatinus* and *B. i. ignitus*. In fact, interspecific matings have already been observed under laboratory conditions (Kanbe et al., 2008), although *B. ignitus* queens were not recorded as mating with *B. terrestris* under field conditions (Kondo et al., 2009).

Extraction protocol of *Bombus* CLG secretions

Head extraction as an alternative protocol was already suggested by Svensson (1980), who found no difference among analyses of the head and the CLG extracts. This author also noted that "[head extraction] was convenient as sampling of pheromones could be performed in the mountains with easy transports of extracts". However, as highlighted by Terzo et al. (2003), these similarities could simply have resulted from a low sensitivity of the instrument. Therefore, re-examination of the results of studies published prior to the year 1996 was needed. Using highly sensitive instruments for chemical analysis, the present results (Fig. 1, Table 1) confirmed the utility and accuracy of this protocol and have validated Svensson's findings (1980).

Both protocols are able to discriminate between closely related bumble bee species. Sample preparation does not seem to affect the ability to discriminate species, as evidenced by the *k*-means clustering analysis. Moreover, cluster analyses (hierarchical and *k*-means) pinpoint no significant differences in extraction methods for both *B. t. dalmatinus* and *B. i. ignitus*. Spearman's rank correlation coefficient also shows a close relationship between extractions using the two procedures. Some analytical variations that could be due to measurement errors are found to be higher than variations due to the extraction procedure, splitting the species sub-data set clusters into two groups that comprise mixed samples of the two preparation methods. The IndVal method finds some significant indicator compounds for the different sample preparations. Nonetheless, RA differences between groups are much slighter than variations that can be observed among individuals in field conditions. The inter-individual variation of secretion components within a single species can be related to geographical origin, seasonal variation or age of the male (Terzo et al., 2005; Sobotnik et al., 2008; Zacek et al., 2009; Agren et al., 1979; Svensson and Bergström, 1977). These studies mentioned a great qualitative and quantitative inter-individual variability (e.g., the RA of the major compound of *B. ruderarius* ranges from 0 to 75%). Our results show anecdotal qualitative differences between sample preparations. The molecules that were detected by only one extraction procedure are all trace molecules found in very few specimens. Moreover, all molecules identified as EAD active compounds for *B. terrestris* [Šobotnik et al. 2008; Žácek et al. 2009: ethyl dodecanoate (14.94), dihydrofarnesal (15.21), dihydrofarnesol (16.06), hexadecanol (18.13), octadecatrienol (19.90) and geranylcitronellol (20.73)] are all detected both in CLG and head extractions with similar RAs.

The quantitative differences observed between sample preparations are very low. All compounds share a similar range of RAs between the compared groups. The significance of results may be related to outliers or small sample sizes. These slight variations do not seem to affect the description of the composition of the secretions.

Bumble bee CLGs are voluminous glands (about 5.90 mm^3) and take up a very large part of the head (more than half) (Terzo et al., 2007b). All other glands (e.g., hypopharyngeal and mandibular glands) are very tiny in comparison to the CLGs, and their secretions represent only 1% of total head secretions (Agren et al., 1979). Furthermore, it appears that components from other head parts, cuticle and/or pollen are very low in quantity (under peak consideration of 0.1%). Thus, we argue that head extraction can be used as well as CLG extraction to characterize species-specific pheromonal secretions. This extraction procedure has been avoided for a long time because of the

pursuit of precision, which has led researchers to employ more laborious techniques despite any significant contribution to the description of sexual pheromone secretions.

We suggest using the proposed protocol for a broad-scale sampling of bumble bee taxa, as well as for specialist and amateur entomologists. Amateurs and collaborators provide important help to scientists despite the difficult taxonomy of bumble bees. If they used a routine collecting method allowing biogeography (by recording GPS position and altitude), DNA extraction (by storing at least one leg of the specimen in ethanol) and pheromone extraction (by storing the head in hexane), this would considerably increase knowledge in bumble bee systematics.

A simplified protocol of chemical extraction promotes chemical taxonomy and comparison of various arguments in the frame of integrative taxonomy approaches (Schlick-Steiner et al., 2010). Moreover, simplified protocols could aid revisions of widespread groups or declining (or nearly extinct) taxa. For these types of studies, utilizing this novel extraction method will enable the use of field collections by casual collectors.

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