Analysis of the Labial Gland Secretions of the Male Bumble Bee *Bombus perplexus* Cresson (Hymenoptera: Apidae) from North America

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The labial gland secretions from males of the bumble bee *Bombus (Pyrobombus) perplexus* Cresson were analysed by gas chromatography/mass spectrometry (GC/MS) in the electron impact and positive ion chemical ionization mode. The major compound of the complex mixture of alkenols, alkenals, fatty acids, hydrocarbons, wax type esters and steroids is 3,7,11,15-tetramethyl-2,6,10-hexadecatrien-1-ol (geranylcitronellol), considerable amounts of hexadecan-1-ol and *Z*-9-hexadecen-1-ol were also found. All alcohols were present as esters of the detected acids. In older samples both the acids and the alcohols sometimes could not be detected in the GC; therefore, the possibility to check the detected acid-alcohol pattern by interpreting the wax type ester peaks is very instructive. Moreover, the labial gland contains a rich mixture of mono- and di-unsaturated straight chain hydrocarbons. The similarity in composition of the labial glands of the North American *B. (Pyrobombus) perplexus* with the Eurasian species *B. (Pyrobombus) hypnorum* corroborates the assumption that the two species are conspecific. The likely supposition that the hydrocarbons could play an essential role in the chemical communication in bumble bees is discussed.

Key words: Bombus perplexus, Labial Glands, Hydrocarbons, Steroids

Introduction

The labial glands of male bumble bees are the source of a complex mixture of compounds used as marking pheromones (Kullenberg *et al.*, 1973). The secretion of these glands is usually species-specific and consists of a mixture of acyclic mono-, sesqui- and diterpenes (alcohols and aldehydes) and various straight-chain fatty acid derivatives (alcohols, esters, aldehydes, and hydrocarbons) (Bergström *et al.*, 1981). Up to date the males of about 40 European species of bumble bees (*Bombus* and *Psithyrus*) have been investigated (Bergström *et al.*, 1981; Valterová and Urbanová, 1997). Only recently, first results from North American species [*B. (Fervidobombus) sonorus* Say, *B. (Pyrobombus) huntii* Greene and *Psithyrus insularis* Smith] have been published (Bergström *et al.*, 1996).

For the first time Genin *et al.* (1984) reported a complex mixture of mono- and di-unsaturared hydrocarbons from the labial glands of *B. (Pyrobombus) hypnorum* (males, females and workers). Bergman and Bergström (1997) detected considerable amounts (8%) of pentacosadiene in labial glands of male *B. (Pyrobombus) pratorum* L. They discussed the possible role of this hydrocarbon for communication purposes. 7,17-Pentacosadiene was also described as a medium-abundant (5%) component in *B. (Confusibombus) confusus* (Kindl *et al.*, 1999). In *B. (Rhodobombus) pomorum* (Valterová *et al.*, 2001) it was found an unusually high amount (up to 12%) of alkadienes (chain length $C_{27}-C_{31}$). This finding makes it very probable, that unsaturated hydrocarbons play a role in chemical communication in bumble bees.

It is well established that complex mixtures of cuticular hydrocarbons are the primary chemical cue involved in species and kin recognition systems (Howard and Blomquist 1982; Bonavita-Cougourdan *et al.*, 1987). Cuticular hydrocarbons are also implicated in chemical camouflage or chemical mimicry systems of inquilines and parasitoids (Van der Meer and Wojcik, 1982; Howard *et al.*, 1990, 2001; Dettner and Lippert, 1994; Schiestl *et al.*, 1999; Ayasse *et al.*, 2000). If compounds in male labial gland secretions of bumble bees are chemical signals of species recognition systems it seems most probable that hydrocarbons are involved.

If the composition of the labial gland secretion of male bumble bees is species-specific the chemis-

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try of gland secretions could also be used to investigate the possible relationship of species from Eurasia with species from America. *B. (Pyrobombus) perplexus* Cresson of North America is said to be closely related (Williams, 1991) or maybe conspecific to the European *B. (Pyrobombus) hypnorum* L., whose labial glands secretion is well studied (Kullenberg *et al.*, 1970; Svensson and Bergström, 1977; Genin *et al.*, 1984). The labial gland secretion of males of the North American *B. (Pyrobombus) perplexus* has been investigated to compare it with the compounds of *B. (Pyrobombus) hypnorum* as a test for conspecificity of these species.

Materials and Methods

Materials

Females of *B. (Pyrobombus) perplexus* Cresson have been collected in spring in the surroundings of Bridgewater (Massachusetts, USA), where this species is abundant. In a greenhouse artificial colonies were developed, from which the males have been taken for GC/MS investigations. The cephalic part of the labial glands was dissected from the head of males in frozen condition and placed in vials (glands from 5 males per vial) containing 0.2 ml pentane.

GC/MS

A Finnigan MAT TSQ700 gas chromatograph/tandem mass spectrometer was employed. Gas chromatography was carried out on a Hewlett Packard Ultra 1 (50 m, 0.2 mm i.d., 0.11 μm film thickness) in the splitless mode with helium as carrier gas at an inlet pressure of 300 kPa. Initial temperature of 120 °C was held for 1 min, then increased at 8 °/min to 280 °C, at 3 °/min to 310 °C and at 1 °/min to 320 °C. This temperature was held for 10 min. Mass spectrometer conditions were: interface temperature 300 °C, source temperature 130 °C, electron energy 70 eV, emission current 0.2 mA, and electron multiplier 1400 V. In the positive ion chemical ionization mode ammonia CI gas pressure was 70 Pa. Dimethyl disulfide adducts were prepared as described by Buser *et al.* (1983). Compounds were identified by comparing their mass spectra with those of the NIST '02 Library (National Institute of Standards and Technology, USA) and coinjection with commercially available standards.

Results

A typical chromatogram for the labial gland secretions of *B. (Pyrobombus) perplexus* is given in Fig. 1 and the compounds are summarized in Table

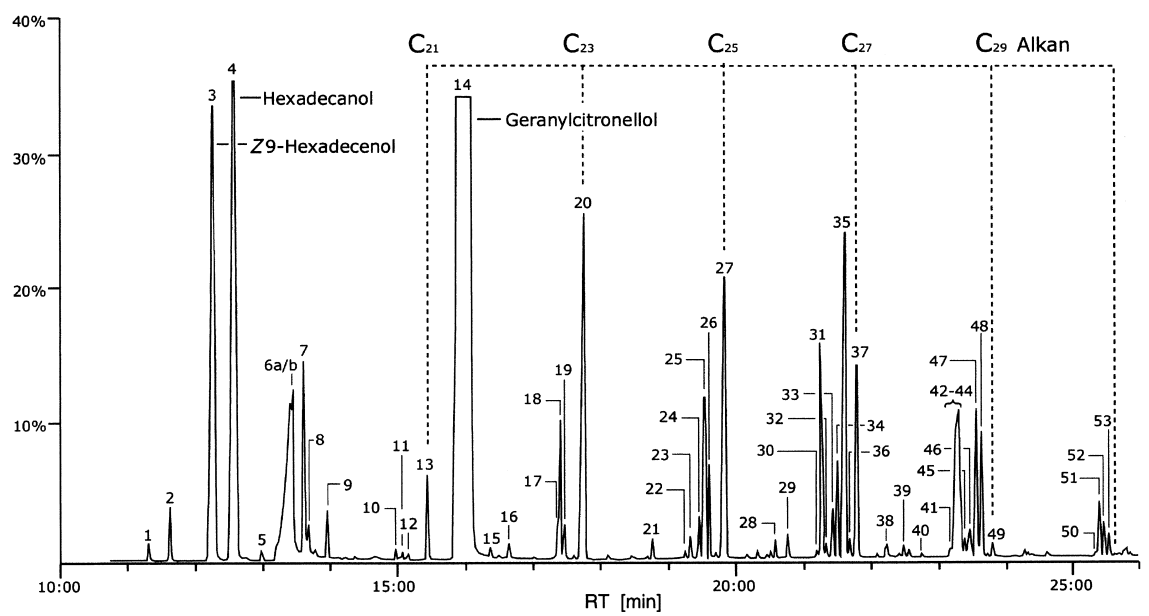

Fig. 1. Gas chromatogram of the labial gland secretion of *Bombus (Pyrobombus) perplexus* males up to 26 min retention time.

Table I. Compounds of the labial glands of *Bombus (Pyrobombus) perplexus* males and structural evidence of unsaturated compound.

Compound	No.	RT[a]	$M^{+\bullet}$	Structural evidence
9-Hexadecenal	1	11:20	238	GC/MS, coinjection with standard
Hexadecanal	2	12:38	240	
9-*Z*-Hexadecen-1-ol	3	12:18	240	145, 189, $M^{+\bullet}$ 334
Hexadecan-1-ol	4	12:37	242	
Nonadecan	5	12:59	268	
9-Hexadecenoic acid	6a	13:24	254	173, 175, $M^{+\bullet}$ 348
7-Hexadecenoic acid	6b	13:28	254	145, 203, $M^{+\bullet}$ 348
Ethyl hexadecenoate	7	13:38	282	
Hexadecanoic acid	8	13:41	256	
Ethyl hexadecanoate	9	13:58	284	
Geranylcitronellal	10	14:59	290	
9-Heneicosene	11	15:04	294	
7-Heneicosene	12	15:08	294	
Heneicosane	13	15:26	296	
9-Octadecenoic acid		15:55	282	173, 203, $M^{+\bullet}$ 376
7-Octadecenoic acid		15:57	282	145, 231, $M^{+\bullet}$ 376
Geranylcitronellol	14	16:02	292	
Octadecanoic acid		16:05	284	
Ethyl 9-octadecenoate		16:06	310	173, 231, $M^{+\bullet}$ 404
Ethyl octadecanoate	15	16:22	312	
Docosane	16	16:35	310	
11-Tricosene	17	17:21	322	
9-Tricosene	18	17:24	322	173, 243, $M^{+\bullet}$ 416
7-Tricosene	19	17:27	322	145, 271, $M^{+\bullet}$ 416
Eicosanol			298	CI
Tricosane	20	17:46	324	
Tetracosane	21	18:44	338	
?-?-Pentacosadiene	22	19:13	348	
?-?-Pentacosadiene	23	19:18	348	
11-Pentacosene	24	19:27	350	
9-Pentacosene	25	19:33	350	173, 271, $M^{+\bullet}$ 444
7-Pentacosene	26	19:35	350	145, 299, $M^{+\bullet}$ 444
Docosanol			326	CI
Pentacosane	27	19:50	352	
9-Hexacosene		20:29	364	
7-Hexacosene	28	20:33	364	145, 313, $M^{+\bullet}$ 458
Hexacosane	29	20:44	366	
Heptacosadiene	30	21:14	376	
Heptacosadiene mixture[c]	31	21:16	376	$M^{+\bullet}$ 470
Heptacosadiene	32	21:19	376	
11-Heptacosene	33	21:25	378	
9-Heptacosene	34	21:29	378	173, 299, $M^{+\bullet}$ 472
7-Heptacosene	35	21:37	378	145, 327, $M^{+\bullet}$ 472
?-Heptacosene	36	21:40	378	
Tetracosanol			336	CI
Heptacosane	37	21:47	380	
?-?-Octacosadiene	38	22:14	390	
9-Octacosene	39	22:28	392	173, 313, $M^{+\bullet}$ 486
7-Octacosene		22:32	392	145, 341, $M^{+\bullet}$ 486
Octacosane	40		394	
Nonacosatriene	41	23:09	402	
7,17-Nonacosadiene	42	23:14	404	A: 145, 353 B: 215, 283, $M^{+\bullet}$ 592
7,19-Nonacosadiene	43	23:16	404	A: 145, 353 B: 187, 311, $M^{+\bullet}$ 498[d]
9,19-Nonacosadiene	44	23:17	404	A: 173, 325 B: 187, 311, $M^{+\bullet}$ 592
?,?-Nonacosadiene	45	23:22	404	
13-Nonacosene		23:24	406	
11-Nonacosene	46	23:27	406	
9-Nonacosene	47	23:33	406	173, 327, $M^{+\bullet}$ 500

Table I. (continued)

Compound	No.	RT[a]	$M^{+\bullet}$	Structural evidence
7-Nonacosene	48	23:37	406	145, 355, $M^{+\bullet}$ 500
Nonacosane	49	23:47	408	
7,17-Hentriacontadiene	50	25:21	432	
9,17-Hentriacontadiene	51	25:24	432	
9,19-Hentriacontadiene	52	25:29	432	
9-Hentriacontene	53	25:44	434	173, 355, $M^{+\bullet}$ 528
Steroids				
Campesterol		27:15	400	367, 382, 385, 400
Sitosterol		28:24	414	381, 396, 399, 414
Fucosterol		28:33	412	281, 296, 299, 314, 397, 412
Wax esters	CN[b]			
Hexadecenyl 7-hexadecenoate	32	28:47	476	173, 397, $M^{+\bullet}$ 570
Hexadecenyl 9-hexadecenoate	32	28:50	476	145, 425, $M^{+\bullet}$ 570
Hexadecyl hexadecenoate	32	29:04	478	145, 427, $M^{+\bullet}$ 572
Hexadecenyl hexadecanoate	32	29:09	478	[*222*, 257]
Hexadecyl 7-hexadecenoate	32	29:09	478	157, 173, $M^{+\bullet}$ 572
Hexadecyl hexadecanoate	32	29:19	480	[*224*, 257]
Hexadecenyl 7-octadecenoate	34	31:23	504	[*222*, 283]
Hexadecenyl 9-octadecenoate	34	31:36	504	[*222*, 283], 173, 427, $M^{+\bullet}$ 600
Hexadecenyl octadecanoate	34	31:42	506	[*222*, 285]
Hexadecyl octadecanoate	34	31:55	508	[*224*, 285]
Geranylcitronellyl 7-hexadecenoate	32	32:20	528	[69, 136]
Geranylcitronellyl 9-hexadecenoate	32	32:23	528	[69, 136]
Geranylcitronellyl hexadecanoate	32	32:37	530	[69, 136]
Eicosanol 7-hexadecenoate	36	34:34	534	[*280*, 255]
Eicosanol 9-hexadecenoate	36	34:41	534	[*280*, 255]
Eicosanol hexadecanoate	36	34:53	536	[*280*, 257]
Geranylcitronellyl 7-octadecenoate	38	35:20	556	[69, 136]
Geranylcitronellyl 9-octadecenoate	38	35:26	556	[69, 136]
Geranylcitronellyl octadecanoate	38	35:40	558	[69, 136]
Docosanol 7-hexadecenoate	38	38:01	562	[??, 255]
Docosanol 9-hexadecenoate	38	38:06	562	[??, 255]
Docosanol hexadecanoate	38	38:22	564	[308, 257]
Docosanol octadecenoate	40	42:18	590	[308, 264, 283]
Tetracosanol hexadecenoate	40	42:18	590	[336, 236, 255]
Docosanol octadecanoate	40	42:45	592	[308, 285]
Tetracosanol hexadecanoate	40	42:45	592	[*336*, 257]

[a] RT: Retention time.
[b] CN: Carbon number = chain length.
[c] Mixture: Δ^{7-17}: A: 145, 325; B: 187, 283; Δ^{8-18} : A: 159; B: 173, 297.
[d] One DMDS adduct only.

I. The major compound was 3,7,11,15-tetramethyl-2,6,10-hexadecatrien-1-ol (geranylcitronellol) (Fig. 1, peak 14), and considerable amounts of *Z*-9-hexadecen-1-ol (peak 3) and hexadecan-1-ol (peak 4) and minor amounts of the corresponding aldehydes (9-hexadecenal (peak 1), hexadecanal (peak 2) and geranylcitronellal (peak 10) were also found. Substantial amounts of 9-hexadecenoic acid (peak 6a), 7-hexadecenoic acid (peak 6b) and hexadecanoic acid (peak 8) were identified besides minor quantities of 9-hexadecenoic, 7-hexadecenoic, hexadecanoic, 9-octadecenoic, 7-octadecenoic and octadecanoic acid (see Fig. 1, Table I).

A complex mixture of 26 wax type esters was found in the chromatogram (see Table I). The characteristic MS fragment ions of the alcohol and the acid part of these esters (Pepe *et al.*, 1993) are good tools to detect small amounts of alcohols or acids, respectively, which are normally difficult to detect and identify in gas chromatograms.

Characteristic for the GCs was also a rich mixture of mono- and di-unsaturated straight chain hydrocarbons. Most of the double bond positions could be established by the characteristic MS fragments in DMDS adducts (Table I).

Furthermore small amounts of campesterol, sitosterol and fucosterol were found (Fig. 2).

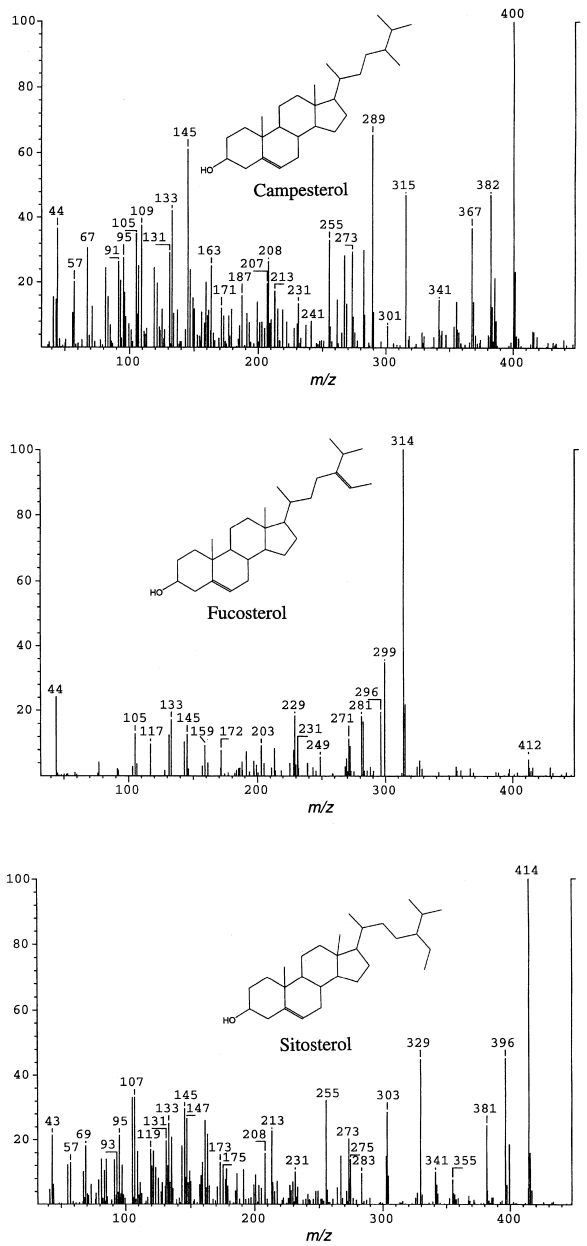

Fig. 2. EI-mass spectra of campesterol, fucosterol and sitosterol.

Discussion

Alkenols, alkenals and acetates

The main components in the male labial gland secretions of many bumble bee species are 1-alcohols, therefore it is likely that they play a major role in communication. Often mixtures of alcohol and the corresponding aldehyde are found in pairs in pheromone bouquets. The classical example in bumble bees is *B. (Megabombus) pascuorum* Scopoli, where about equal amounts of *Z*-7-hexadecenal and *Z*-7-hexadecenol have been identified as main components (Descoins *et al.*, 1983). It is therefore very probable, that the small amounts of aldehydes in the labial gland secretions of male *B. (Pyrobombus) perplexus* are also signal modulators in chemical communication.

The alcohols in the labial gland secretions are not very stable; if specimen are stored at – 25 °C the alcohols converted into carboxylates, the decrease of the alcohol peaks and the increase of the carboxylate peaks is quantitative and time dependent.

Fatty acids

7-hexadecenoic, 9-hexadecenoic, hexadecanoic, 7-octadecenoic, 9-octadecenoic and octadecanoic acid have been identified as compounds in the labial gland secretions of *B. (Pyrobombus) perplexus*. Fatty acids are normally not reported as compounds in labial gland secretions of bumble bees. They are not included in the reviews (Bergström *et al.*, 1981; Valterová and Urbanová, 1997). With the argument "they are most probably precursors of the components in the secretion" they are often excluded from further discussions. As the pattern of fatty acids, alcohols and corresponding esters is coherent (see wax type esters below), the argument of Francke *et al.* (1983), that esterases split esters in acid and alcohol, which may be the active components of these secretions, seems plausible. As long as we do not know exactly which compounds of the complex mixtures are active compounds and whether they are male-male or male-female signals all compounds should be treated equally.

Wax type esters

Various ethyl- and wax type esters have been found in the labial glands of bumble bee males. In the secretions of *B. (Bombus) cryptarum* it could

be shown (Bertsch, 1997), that all detected alkenols (C_{20}–C_{26}:enol) are also present as esters of lauric acid (C_{12}:acid), which results in a characteristic pattern of wax type esters. In the secretions of *B. (Pyrobombus) perplexus* all alcohols (9-C_{16}:enol, C_{16}:anol, C_{20}:anol, C_{22}:anol, C_{24}:anol and tetramethyl-C_{16}:trienol) are present as esters of the detected acids (7-C_{16}:enoic acid, 9-C_{16}:enoic acid, C_{16}:anoic acid, 7-C_{18}:enoic acid, 9-C_{18}:enoic acid and C_{18}:anoic acid) and a complex, but characteristic pattern of wax type esters results. As the esters of eicosanol, docosanol and tetracosanol are also present, it is most probable that these alcohols are also constituents of the secretion. In the EI-spectrum they are masked by the peaks of the alkadienes, but the CI-spectrum revealed their presence. Both the acids and the alcohols sometimes mysteriously disappear fromcould not be detected in the GC (most probably by column defects); therefore the possibility to check the detected acid-alcohol pattern by interpreting the wax type ester peaks is very instructive.

Hydrocarbons

In most labial gland secretions of male bumble bees saturated uneven numbered straight chain hydrocarbons ranging from heneicosane (C_{21}:ane) to nonacosane (C_{29}:ane) are characteristic, the C_{23}–C_{29} alkanes are normally accompanied by mono-unsaturated alkenes. These hydrocarbons are generally not considered to belong to the biologically active compounds of the species, but are often treated as contamination from the cuticular hydrocarbons (Oldham *et al.*, 1994). In the GC of *B. perplexus* the pattern of hydrocarbons is extended to hentriacontene (C_{31}:ene), and small amounts of even numbered hydrocarbons (C_{20}:ane–C_{30}:ane) are also present. These saturated hydrocarbons are accompanied by a pattern of three to four mono-unsaturated hydrocarbons with double-bond positions 7, 9, 11 and 13, and a mixture of C_{27}–C_{31} alkadienes with double-bond positions 7,17, 8,18, 7,19 and 9,19.

Comparable complex mixtures of aliphatic hydrocarbons have been reported from *B. (Rhodobombus) pomorum* (Valterová *et al.*, 2001). The composition of the secretion of *B. (Rhodobombus) pomorum* is exceptional among bumble bee species studied so far, because compounds other than hydrocarbons could only be detected in very small amounts (< 0.1%). The unusually large relative amounts of these alkadienes (up to 12%) and the absence of any other compounds make it very probable, that these mono- and di-unsaturated hydrocarbons play an essential role in the chemical communication in *B. pomorum*.

It is well established that complex mixtures of species-specific (Howard, 1993) cuticular hydrocarbons are the primary chemical cue involved in species and kin recognition systems (Howard and Blomquist, 1982; Bonavita-Cougourdan *et al.*, 1987). Cuticular hydrocarbons are also implicated in chemical camouflage or chemical mimicry systems in inquilines and parasitoids (Van der Meer and Wojcik, 1982; Howard *et al.*, 1990, 2001; Dettner and Lippert, 1994; Bonavita-Cougourdan *et al.*, 1996). In the sexually deceptive flowers of the orchid *Ophrys sphegodes* mimicking female bees complex mixtures of hydrocarbons have been identified which are biologically active for flower visiting males of the solitary bee *Andrena nigroaenea* and elicit copulation attempts in the males (Schiestl *et al.*, 1999). Therefore it could be useful to include a detailed analysis of the hydrocarbons of the male labial gland secretions of bumble bees in future research.

Steroids

The labial glands of *B. (Pyrobombus) perplexus* contain three sterols, campesterol (24-methyl-5-cholesten-3β-ol), sitosterol (24-ethyl-5-cholesten-3β-ol) and fucosterol (24-ethylidene-5-cholesten-3β-ol) (Fig. 2). Sterols are normally not reported as compounds in labial glands of male bumble bees. In the labial glands of *B. (Bombus) cryptarum* Fab., campesterol and sitosterol have previously been identified (Bertsch, 1997).

B. perplexus vs. B. hypnorum

The characteristics found in the male genital structures of *B. (Pyrobombus) hypnorum* by Krüger (1943), "well defined inner basal notch of the gonostylus" and "broad head of the penis valve narrowed to a rounded point", are also characteristic for the North American *B. (Pyrobombus) perplexus*. Williams (1991) states the close similarity between the two species. Alignment gaps within intron sequences of arginin kinase (Kawakita *et al.*, 2003) from *B. (Pyrobombus) hypnorum* (Italy) and *B. (Pyrobombus) perplexus* (Canada) confirm their close relationship and their distinct

separation from all other species of the subgenus *Pyrobombus*.

The composition of the labial glands of male *B. (Pyrobombus) hypnorum* is well documented (Kullenberg *et al.*, 1970; Svensson and Bergström, 1977; Genin *et al.*, 1984); in qualitative and quantitative respects the labial gland secretions of *B. (Pyrobombus) hypnorum* and *B. (Pyrobombus) perplexus* are identical. Morphological and molecular evidence prove the "closeness" of the two species and similarity of the species recognition signals is strong evidence that they are conspecific.

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