

Molecular and chemical characters to evaluate species status of two cuckoo bumblebees: *Bombus barbutellus* and *Bombus maxillosus* (Hymenoptera, Apidae, Bombini)

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Abstract. Many methods, based on morphological, molecular or chemical characters, have been used to address the question of species taxonomic status. Integrative taxonomy aims to define stronger supported taxonomic hypotheses by considering complementary datasets from different characters. By following an integrative approach, the present study includes molecular, chemical and morphological criteria to establish the taxonomic status of two rare and doubtful cuckoo bumblebee taxa: *Bombus (Psithyrus) barbutellus* and *Bombus (Psithyrus) maxillosus*. These two sympatric taxa are discriminated by few morphological criteria (mainly wing darkness and hair length). We used these morphological character diagnoses to establish an *a priori* status of our samples (23 specimens). We developed a combined molecular dataset from one nuclear gene, elongation factor 1 α (*EF-1 α*), and one mitochondrial gene, cytochrome *c* oxidase subunit I (*COI*), spanning 1623 bp, and a chemical dataset of sexual marking pheromones (73 compounds). The molecular data were subjected to maximum-likelihood and Bayesian phylogenetic inference under partitioned model and maximum parsimony. The chemical data were analysed by clustering and the two-group *k*-means method to test divergences between the two species. The resulting phylogenetic trees show no consistent divergence between the two taxa. Moreover, we found no divergence in the sexual marking pheromones in the clustering and two-group *k*-means analyses. These converging results support the conspecificity of both taxa. Nonetheless, our determinations using the traditional morphological criteria separated our samples into two taxa. We conclude that the morphological criteria seem to relate to intraspecific variations: *B. maxillosus* is regarded as a **syn.n.** of *B. barbutellus*.

Introduction

The notion of a species is one of the most confusing and controversial concepts in biology (Mayr, 1942; Templeton,

1989; Baum, 1992; De Queiroz, 2007; Gourbière & Mallet, 2009). However, the species is a fundamental biological unit, and its definition is of major importance to biology. Numerous ways have been explored to determine the species status. In hymenopteran systematics (reviewed for bees in Engel, 2011), authors have tried to define taxonomic status using morphology (i.e. Michener, 1951; Pekkarinen, 1982; Williams, 1998), geometric morphometry (i.e. Aytakin *et al.*, 2007), allozyme-based models (i.e. Pamilo *et al.*, 1997), DNA (i.e. Koulianos & Schmid-Hempel, 2000; Hines *et al.*, 2006; Bernasconi *et al.*,

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2010) or chemoecology (i.e. Svensson, 1979; Bertsch *et al.*, 2005; Rasmont *et al.*, 2005; Martin *et al.*, 2008).

The morphological approach was the earliest, the easiest and has been the most common method used to define species (i.e. Løken, 1984; Bolton, 1995; Michez & Eardley, 2007). However, determining objective morphological characters that accurately reflect biological species is difficult (Bickford *et al.*, 2007). The results of morphological analyses have conflicted with other types of evidence (i.e. Svensson, 1979; Batalha-Filho *et al.*, 2010).

Genetic characters are more and more often taken into consideration in order to assess species status (i.e. Leaché & Fujita, 2011). The development of DNA barcoding studies (i.e. Hebert *et al.*, 2003) to describe cryptic species (i.e. Yassin *et al.*, 2007), or being included into an integrative taxonomic framework (i.e. Gibbs, 2009), exemplify this trend. A phylogenetic species concept (Baum, 1992) has been developed, and defined a species as a monophyletic group (Papadopoulou *et al.*, 2008), perhaps based on multiple gene genealogies (Baum & Shaw, 1995). Nevertheless, the genetic approach remains controversial in defining species limits because the DNA sequences analysed are chosen arbitrarily or because mating isolation can happen faster than differentiation of the genetic markers used (Avice, 2000; Trewick, 2008; Symonds *et al.*, 2009; Bauer *et al.*, 2011).

Chemical features, mainly species-specific secretions (i.e. Lockey, 1991), can provide useful tools in separating species. Among bumblebees, sexual marking pheromones (SMPs), synthesized by male cephalic labial glands (Kullenberg *et al.*, 1973), play a major role in species-specific pre-mating behaviour (Bergman, 1997), and are highly species specific (Calam, 1969; Valterová & Urbanová, 1997). Therefore, authors have used these chemical markers in resolving species status (Svensson & Bergström, 1977; Bergström *et al.*, 1981; Rasmont *et al.*, 2005; Coppée *et al.*, 2008; Lecocq *et al.*, 2009a, b), according to the species recognition concept (Paterson, 1993). In this concept, conspecific individuals sharing the same recognition signals are supposed to recognize each other as sexual partners (reviewed in Terzo *et al.*, 2003). However, it is difficult to determine a threshold of species-level differentiation, even with ethological testing (i.e. Ings *et al.*, 2010). Only *Bombus terrestris* (L., 1758) has been studied enough to determine a well-supported threshold, thanks to numerous chemo-ethological tests (reviewed in Coppée, 2010).

The current trend in species limitation studies is to follow a multiple evidence approach (Burns *et al.*, 2008; Fisher & Smith, 2008; Smith *et al.*, 2008) to differentiate species (i.e. morphological, chemical, molecular and ecological).

The present study deals with taxonomic problems in the monophyletic subgenus *Psithyrus* included in the genus *Bombus* Latreille. Thirty *Psithyrus* species described worldwide live in the Northern hemisphere (Williams, 1998 update at

NHN). *Psithyrus* species are social parasites (cuckoo bumblebees) of other bumblebees. Cuckoo bumblebees lack pollen-collecting corbiculae and a worker caste. They are therefore completely dependent on hosts to rear their offspring. They parasitize hosts from across the bumblebee phylogeny with very little cophylogenetic pattern (Williams, 2008). Typically, *Psithyrus* species parasitize just one host or a few host species of the same subgenus (reviewed in Alford, 1975; Løken, 1984; Lhomme, 2009), but some species are considered to be generalists, with the extent of their host range often unclear. The ecology of host–parasite interactions of most of the cuckoo bumblebees is poorly known or unknown for almost half of *Psithyrus*.

This study focuses on two sister taxa, *Bombus (Psithyrus) barbutellus* (Kirby) and *Bombus (Psithyrus) maxillosus* Klug, of the former subgenus *Allopsithyrus* Popov or *barbutellus* group (Fig. 1) (Williams, 1998). The taxonomic status of these two taxa is doubtful (Grütte, 1940; Tkalců, 1969; Rasmont, 1988; Williams, 1998). Most authors consider these taxa as separate species (Popov, 1931; Pittioni, 1939; May, 1942, 1943; Tkalců, 1969; Løken, 1984), although a very few argue for their conspecificity (Grütte, 1940). Most of these studies refer to morphology, highlighting slight differences between the two taxa (Table 1), mainly in hair length and wing colour (e.g. Tkalců, 1969). With such weak morphological criteria a large number of individuals remain unidentified (Rasmont, 1988; Rasmont & Adamski, 1996; Urbanová *et al.*, 2004), especially in geographical areas shared by both taxa. *Bombus barbutellus* has a large palaearctic distribution, whereas *B. maxillosus* is restricted around the Mediterranean Sea (Fig. 1) (Rasmont, 1988; Pagliano, 1993; Rasmont & Flagotherier, 1996; Williams, 1998; update at Natural History Museum, London). Many overlapping areas and exceptions to these trends are known (Vogrin, 1955; Schwarz *et al.*, 1996, 2005; Straka *et al.*, 2007; Pawlikowski, 2008; Iserbyt, 2009). The splitting into subspecies differs for many authors (Pittioni, 1939; Grütte, 1940; Rasmont, 1988). In this study, as a preliminary approach, we use the subspecies classification of Rasmont (1988): *B. barbutellus* no subspecies, *B. maxillosus maxillosus* Klug (France, south-east Europe, Anatolia, Transcaucasia, Caucasus and northern Iran) and *B. maxillosus italicus* (Grütte, 1940) (Iberian Peninsula, Italian Peninsula, Corsica and Sardinia).

The sparsity of *B. barbutellus* and *B. maxillosus* makes information about their ecology very scarce. According to Pittioni & Schmidt (1942) and Rasmont (1988), *B. barbutellus* and *B. maxillosus* have different habitats. *Bombus barbutellus* is a forest edge dweller. It is a plains species in the north of its range, and a mountain species in the south. *Bombus maxillosus* seems to be more of an open field species (Pittioni & Schmidt, 1942) or a wood edge species (Rasmont, 1988). They also seem to have different hosts. The main host of *B. barbutellus* is *Bombus (Megabombus) hortorum* (L.) (Sladen, 1912; Ball, 1914; Müller, 1936; Cumber, 1949; Tkalců, 1969; Løken, 1984), whereas the main hosts of *B. maxillosus* seem to be two sister species *Bombus (Megabombus) argillaceus* (Scopoli)

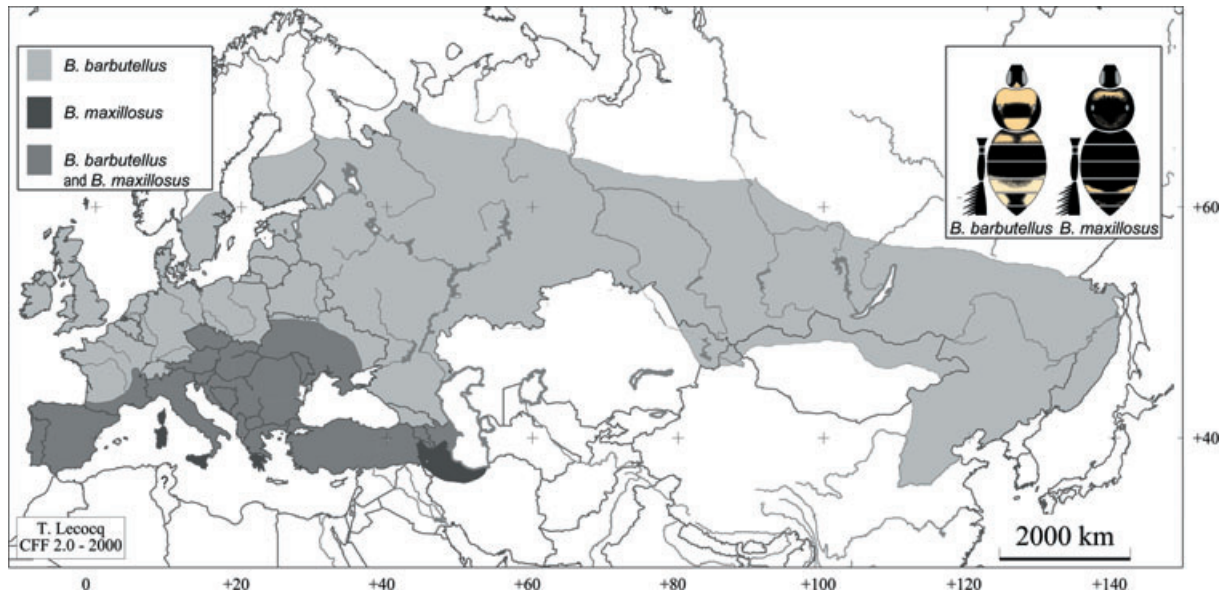


Fig. 1. Distribution area of *Bombus maxillosus* and *Bombus barbutellus* in the Palearctic region according to the literature (Maidl, 1922; Pittioni, 1939; Haas, 1949, 1967; Vogrin, 1955; Móczár, 1957; Dylewska, 1966; Elfving, 1968; Reinig, 1971; Ressel, 1974; Alford, 1975; Tumšs, 1975; Løken, 1984; Ornos, 1984; Intoppa *et al.*, 1995; Monsevičius, 1995; Rasmont *et al.*, 1995; Amiet, 1996; Baker, 1996; Rasmont & Flagothier, 1996; Rasmont & Adamski, 1996; Peeters *et al.*, 1999; Dathe *et al.*, 2001; Yefremova, 2001; Anagnostopoulos, 2005; Fitzpatrick *et al.*, 2006; Byvaltsev, 2008, 2009; Pawlikowski, 2008; W. F. Reinig, personal data).

and *Bombus (Megabombus) ruderatus* (Fabricius) (Reinig, 1935; Rasmont, 1988; Rasmont & Adamski, 1996), which are closely related to *B. hortorum* inside the *Megabombus* subgenus. However, other observations (Reinig, 1935; Pittioni & Schmidt, 1942; May, 1943; Pouvreau, 1973; Ornos, 1984; Westrich, 1989; Rasmont & Adamski, 1996; Peeters *et al.*, 1999) show that both taxa have no strict host–parasite relationship (Table 2). In this case, there is no definitive evidence to separate both taxa according to their host choice, contrary to Tkalců's (1969) observations.

Chemical data are scarce too. The SMP of a few *B. barbutellus* specimens from Sweden have been described by Kullenberg *et al.* (1970) and Cederberg *et al.* (1984). The SMP of four specimens of *B. maxillosus* from the Czech Republic have been described by Urbanová *et al.* (2004). These last authors compared their data with *B. barbutellus* of Kullenberg *et al.* (1970) and Cederberg *et al.* (1984). They found similarities in the blend of the SMP. However, as a result of the improvement in analysis techniques, data published before 1996 need to be re-examined (Terzo *et al.*, 2003). Comparison between such old data and new data is difficult. Very few genetic studies including both taxa have been undertaken, and none have dealt with species status. In recent estimates of bumblebee phylogeny, the two taxa are treated as different species (Cameron *et al.*, 2007; Hines, 2008).

Morphological, phylogenetic, ecological and ethological data from the literature about *B. barbutellus* and *B. maxillosus* are conflicting. A comparison of all available data fails to determine a strongly supported taxonomic hypothesis. We need more evidence in order to evaluate the status of both taxa

(Williams, 1998: update at Natural History Museum, London). This paper aims to solve the taxonomic status of *B. barbutellus* and *B. maxillosus* using new molecular and chemical data from larger samples than previous studies.

Material and methods

Sampling and morphological identification

We compared morphological, molecular, and chemical (SMP) features from the same sampling of males (Table 3). Specimens were caught in the wild and killed by freezing. Identification keys that discriminate *B. barbutellus* and *B. maxillosus* as two species were used to determine individuals (Popov, 1931; Pittioni, 1939; May, 1943; Rasmont & Adamski, 1996). The SMPs were sampled by dissection of cephalic labial glands, which were placed in 200 μ L of hexane (Terzo *et al.*, 2005). The rest of the male bodies were conserved in ethanol (99%) for molecular analyses. All samples were stored at -40°C until analysis.

The scarcity of the species in the field restricts collection of a large number of specimens. We have analysed nine specimens of *B. barbutellus*, ten of *B. maxillosus maxillosus* and three of *B. maxillosus italicus* for the pheromonal dataset. We added four specimens of *B. barbutellus* females to the molecular sampling. One male of *B. maxillosus maxillosus* from our SMP sample was too deteriorated for DNA extraction. The populations sampled are mainly European, corresponding to the sympatric area of the two taxa (Fig. 1; Table 3).

Table 1. Main morphological and colour pattern differences between *Bombus maxillosus* and *Bombus barbutellus* males and females according to Popov (1931), Pittioni (1939), May (1943) and Løken (1984).

Female	<i>B. maxillosus</i>	<i>B. barbutellus</i>
Morphology		
Longest hairs of the posterior fringe of the hind tibia	Shorter than half the largest width of the segment	Longer than half the largest width of the segment
Longest hairs of the hind basitarsus	Shorter than the width of the segment; clearly narrower at the apex than at the base	Longer than the width of the segment; its anterior and posterior edges almost parallel
Coat	Short	Long and shaggy
Colour pattern		
Wings	Darkened, almost opaque	Hyaline
Collare and scutellare	Narrower, mixed with black hairs	Very wide, yellow
T1	Black	Yellow mixed with black
T4	Off-white	With white sides
T5	With off-white sides	White
Male		
Morphology		
Longest hairs of the anterior fringe on the hind basitarsus	Shorter than the width of the segment	As long as, or slightly longer than, the width of the segment
Coat	Short	Shaggy
Colour pattern		
Wings	Darkened	Hyaline
Vertex	With a few yellowish hairs	With a few yellow hairs
Collare and scutellare	Narrower, yellowish, often mixed with many black hairs	Wide, yellow
T1	Either black or yellowish, mixed with black hairs	Yellow
T3	With off-white sides	With white posterior margins
T5	With off-white sides	With white posterior margins
T6	With small off-white tuft on its side	With white sides

Table 2. Host–parasite relations in the *Allopsithyrus* group.

<i>Allopsithyrus</i>	<i>Bombus</i> host species	Main references	
<i>B. barbutellus</i>	<i>B. (Megabombus) hortorum</i> ^a	Sladen (1912), Ball (1914), Müller (1936), Cumber (1949), Postner (1952)	
	<i>B. (Megabombus) ruderatus</i>	Westrich (1989), Peeters <i>et al.</i> (1999)	
	<i>B. (Megabombus) portschinsky</i>	Rasmont (unpublished data)	
	<i>B. (Pyrobombus) hypnorum</i> ^a	Voveikov (1953), Pouvreau (1973)	
	<i>B. (Pyrobombus) jonellus</i>	Schenck (1859), Schmiedeknecht (1883), Skorikov (1922)	
	<i>B. (Pyrobombus) pratorum</i>	Hoffer (1889), Skorikov (1922)	
	<i>B. (Thoracobombus) pascuorum</i>	Hoffer (1889), Skorikov (1922), Popov (1931)	
	<i>B. (Thoracobombus) humilis</i>	Hoffer (1889), Skorikov (1922)	
	<i>B. (Thoracobombus) ruderarius</i>	Skorikov (1922)	
	<i>B. (Subterraneobombus) distinguendus</i>	Popov (1931)	
	<i>B. (Subterraneobombus) subterraneus</i>	Knechtel (1955)	
	<i>B. maxillosus</i>	<i>B. (Megabombus) ruderatus</i>	Rasmont (1988), Vogt (1909), Popov (1931), Pittioni & Schmidt (1942)
		<i>B. (Megabombus) argillaceus</i>	Skorikov (1922), Popov (1931), Pittioni & Schmidt (1942), Pouvreau (1973)
<i>B. (Subterraneobombus) subterraneus</i>		Tkalcù (1969), Pouvreau (1973)	

^aMain hosts are in bold. Colonies in which *Psithyrus* offspring have been observed.

In order to root the phylogenetic trees, we chose basal *Psithyrus* species and one other bumblebee species for the out-group. We used *Bombus (Psithyrus) campestris* (Panzer) from the *Metapsithyrus* group (Pedersen, 1996, 2002; Cameron *et al.*, 2007), *Bombus (Psithyrus) rupestris* (Fabricius) from the *Psithyrus* s.s. group (Cameron *et al.*, 2007; Hines, 2008) and *Bombus (Kallobombus) soroeensis* (Fabricius) as our out-group (Table 3).

DNA extraction, polymerase chain reaction, sequencing and sequence analyses

Total DNA was extracted using a QIAGEN DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA). Legs were removed from the specimen and crushed using liquid nitrogen and then digested (4 h in proteinase K at 56°C). Voucher specimens and polymerase chain reaction (PCR) products used in the

Table 3. *Allopsithyrus* and out-group taxa examined, their collection locality, collector, voucher numbers and GenBank accession numbers.

Species	Subspecies	#	Locality	Lat	Long	Recorder	V.#	EF-1 α	COI
In-group	<i>barbutellus</i>	—	Turkey, Rize	40°56'01"N	41°08'26"E	M. Terzo & P. Rasmont	BAR1, BAR2, BAR4, BAR5, BAR6, BAR8, BAR9	JF699169	JF699183
	<i>barbutellus</i>	—	Sweden, Uppsala	59°51'15"N	17°39'14"E	P. Rasmont	BAR3	JF699171	JF699185
	<i>barbutellus</i>	—	Germany, Rottlieberode	51°31'43"N	10°57'08"E	E. Stolle	BAR7	JF699172	JF699186
	<i>barbutellus</i>	—	France, Ablon	49°23'38"N	0°17'43"E	P. Lhomme	BAR10, BAR11, BAR12, BAR13	JF699170	JF699184
	<i>barbutellus</i>	—	Denmark, Ekkodalen	—	—	Pedersen (2002)	BAR14	—	AY181177.1
	<i>barbutellus</i>	—	Germany, Tücherfeld	—	—	Pedersen (2002)	BAR15	—	AY181176.1
	<i>barbutellus</i>	—	Denmark	—	—	Pedersen (1996)	BAR17	—	L26574.1
	<i>barbutellus</i>	—	Russia, Primorsky	—	—	Tanaka <i>et al.</i> (unpublished)	BAR16	—	AF279576.1
	<i>maxillosus</i>	4	Turkey, Kayseri	38°28'36"N	35°30'06"E	M. Terzo	MAX1, MAX3, MAX5, MAX6	JF699176	JF699190
	<i>maxillosus</i>	3	Turkey, Erzurum	39°52'06"N	39°33'56"E	T. De Meulemeester & P. Rasmont	MAX7, MAX8, MAX9	JF699179	JF699193
Out-group	<i>maxillosus</i>	2	France, Gouffon	43°18'28"N	06°18'33"E	P. Rasmont	MAX2, MAX4	JF699178	JF699192
	<i>maxillosus</i>	1	France, Bargemon	43°38'22"N	06°34'07"E	P. Rasmont	MAX10, MAX11	JF699177	JF699191
	<i>maxillosus italicus</i>	3	France, Evisa	42°17'25"N	08°52'40"E	T. Lecocq	ITAL1, ITA2, IT3	JF699175	JF699189
	<i>campestris campestris</i>	2	France, Saint-Lyphard	47°23'52"N	02°18'24"W	G. Mahé	CAM1, CAM2	JF699174	JF699188
	<i>bohemicus bohemicus</i>	2	France, Nyer	42°30'06"N	02°19'00"E	T. Lecocq	BOH1, BOH2	JF699173	JF699187
	<i>rupestris rupestris</i>	2	Germany, Geiseltal	51°18'00"N	11°52'00"E	E. Stolle	RUP1, RUP2	JF699180	JF699194
	<i>vestalis vestalis</i>	2	France, Paris	48°50'37"N	02°21'35"E	P. Rasmont	VES1, VES2	JF699182	JF699196
	<i>soroensis lectitatus</i>	1	France, Nyer	42°30'09"N	02°19'12"E	T. Lecocq	SOR1	JF699181	JF699195

#, number of samples; V.#, voucher numbers and sample codes used in trees and clustering; EF-1 α , GenBank accession numbers for elongation factor 1 α sequences; COI, GenBank accession numbers for cytochrome *c* oxidase subunit I sequences.

molecular investigation were deposited at the University of Mons (Belgium). Genes were sequenced with an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA). Both strands of each PCR product were sequenced. Consensus sequences were computed with CODONCODE ALIGNER 3.0.1 (Table 3). The bumblebee origin of each sequence was checked with BLAST 2.2.20 (Zhang *et al.*, 2000). The alignment was performed by MAFFT 6 (using FFT-NS-2 algorithms, default parameters; Katoh *et al.*, 2002). The translation in proteins (using the *Drosophila* mitochondrial DNA genetic code) for verification and the data matrix was realized on MESQUITE 2.6 (build 486) (Maddison & Maddison, 2007). Sequences were deposited in GenBank (Table 3).

We generated sequences for two genes: mitochondrial cytochrome *c* oxidase subunit I (*COI*) and nuclear protein-coding gene elongation factor 1 α (*EF-1 α*). *COI* shows a high mutation rate, and is commonly used as a species and subspecies level marker for phylogenetic analyses (e.g. Boursot & Bonhomme, 1986; Estoup *et al.*, 1996; Danforth, 1999; Koulianos & Schmid-Hempel, 2000). *EF-1 α* has been used to infer relationships at multiple taxonomic levels in many insect taxa, including bumblebees (Danforth, 1999; Carapelli *et al.*, 2000; Kjer *et al.*, 2001; Jordal, 2002; Cameron *et al.*, 2007; Hines, 2008). Both genes have already been used to infer the phylogenetic relationship among European bumblebees (Pedersen, 2002). Primers used for all individuals were Jerry (5'-CCACATTTATTTTGATTTTTTGG-3') and Pat (5'-TCCAATGCACTAATCTGCCATATTA-3') (Danforth, 1999) for *COI*, and F2-ForH (5'-GGRCAYAGAGATTCATCAAGA AC-3') and F2-RevH2 (5'-TTGCAAAGCTTCRKGATGCAT TT-3') (Hines *et al.*, 2006) for *EF-1 α* . PCR amplification was carried out [initial denaturing for 4 min at 94°C, 35 cycles of 1-min denaturing at 94°C, 1-min annealing at 54°C (*EF-1 α*) or 51°C (*COI*), 1-min elongation at 72°C and a final extension for 1 min 15 s (*EF-1 α*) or 1 min 30 s (*COI*) at 72°C]. Gene fragments of the following sizes were amplified: 849 nucleotides of *COI* and 786 nucleotides of *EF-1 α* F2 copy, containing a ~200-bp intron.

Phylogenetic analyses

We analysed each gene (*COI* and *EF-1 α*) independently and in combination using maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods (MB). A saturation test was applied to each fragment in PAUP* 4.0b 10 (Swofford, 2001). The incongruence length difference test (ILD test) (Farris *et al.*, 1994) was used to test for incongruence between the two genes. The test was implemented in PAUP* (Swofford, 2001). All trees were rooted with the basal taxon *B. soroeensis* (Williams, 1985; Cameron *et al.*, 2007).

Heuristic searches were performed in MP using 1000 random additions and tree bisection reconnection (TBR) branch swapping, keeping the best trees only. Gaps were regarded as a fifth state. Majority rule 50% consensus (MJ50) trees were constructed from analyses of individual

genes and from all genes combined using parsimony criteria in PAUP* 4.0b 10 (Swofford, 2001) for equally-weighted MP analyses. Clades support values were estimated using nonparametric bootstrapping (Felsenstein, 1985) in PAUP* (10 000 replicates, 1000 random additions, 500 trees saved per replicate).

The ML analyses were conducted in GARLI-PART 0.97 (Zwickl, 2006). The dataset was partitioned as follows to explore the best submission model: (i) *EF-1 α* into two exons and one intron; (ii) *COI* and each *EF-1 α* exon by base position. The best fitting substitution models were chosen with JMODELTEST (Posada, 2008) using Akaike's information criteria (Akaike, 1974) for each dataset. The models chosen are: GTR + G (*COI* first position), GTR (*COI* second position), GTR + I + G (*COI* third position), F81 (*EF-1 α* exon 1 first position), F81 (*EF-1 α* exon 1 second position), HKY (*EF-1 α* exon 1 third position), GTR + G (*EF-1 α* intron), HKY (*EF-1 α* exon 2 first position), JC (*EF-1 α* exon 2 second position) and GTR + G (*EF-1 α* exon 2 third position). A random starting tree and the automated stopping criteria (stop when the ln score remained constant for 20 000 consecutive generations) were used. Ten independent runs in GARLI were carried out for each gene and for the combined data; the topology and -lnL were nearly identical among replicates. The highest likelihood of those runs was retained. Statistical confidence in nodes was evaluated using 100 non-parametric bootstrap replicates (Felsenstein, 1985), using the automated stopping criteria set at 10 000 generations. More bootstrap replicates could not be performed because it would have required unpractical computing times. Topologies with $\geq 70\%$ of the bootstrap trees were considered as well supported (Hillis & Bull, 1993).

Bayesian analyses (MB) were carried out using MR BAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). The model selection process was the same as that for ML analysis. Moreover, genes were analysed individually and collectively. Five independent analyses were carried out for each gene and for the combined data (10 million generations, four chains with mixed models, default priors, and saving trees every 100 generations). The analyses were stopped after checking convergence between runs using the average standard deviation of split frequencies and by plotting likelihood values across generations using TRACER 1.2 (Rambaut & Drummond, 2003). The first one million generations were discarded as burn-in. The phylogeny and posterior probabilities were then estimated from the remaining trees and a majority rule 50% consensus tree was constructed. Topologies with posterior probabilities ≥ 0.95 were considered to be well supported (Wilcox *et al.*, 2002).

Chemical analyses

The samples were analysed using a gas chromatograph Shimadzu GC-2010 with an SLB-5 ms non-polar capillary column (5% diphenyl/95% dimethyl siloxane; 30 m \times 0.25 mm \times 0.25 μ m) and a flame ionization detector. A splitless injector mode (220°C) and He carrier gas (50 cm s⁻¹) were used. The temperature programme of the column was 70°C for

2 min, 10°C per min to 320°C and 5 min. The relative proportions in percentage of each compound were calculated by summing up the absolute quantities of all compounds using GCSOLUTION POSTRUN. The data matrix was elaborated with the relative proportion of each compound for each individual. The composition of SMP was determined using a gas chromatograph–mass spectrometer (GC–MS) Finigan GCQ with a DB-5 ms non-polar capillary column [5% phenyl (methyl) polysiloxane stationary phase; 30 m × 0.25 mm × 0.25 mm] and an ion trap in electron impact mode ‘full scan (300–600)’. A splitless injector mode (220°C) and He carrier gas (50 cm s⁻¹) were used. The temperature programme of the column was 70°C for 2 min; 10°C per min to 320°C and 5 min. Compounds were identified in XCALIBUR™ using their mass spectra compared with those at the National Institute of Standards and Technology library (NIST, U.S.A.) using NIST MS SEARCH 2.0.

Statistical analyses were performed in R (Quinn & Keough, 2006). Data were transformed and standardized. Clustering methods were used to detect the divergence between taxa (Rasmont *et al.*, 2005). Four different association matrices based on a pheromonal data matrix (relative abundance of each compound) were computed: Euclidian, Pearson phi correlation, chi-square and Manhattan. Three clustering methods were used for each association matrix: single, complete and unweighted pair group method with arithmetic mean (UPGMA). Only one of these clusters is shown in the results (UPGMA cluster based on phi correlation matrix). In addition, a two-group *k*-means method (MacQueen, 1967) was performed (repeated 50 000 times) (De Meulemeester *et al.*, 2011) to test a putative split into two groups: *B. barbutellus* versus *B. maxillosus*.

Correlation analyses

The spatial structuring of the chemical and genetic dataset and a potential correlation between these two datasets were investigated by performing three Mantel tests (9999 random permutations) (Mantel, 1967) in R (Quinn & Keough, 2006): (i) geographical distance among population versus individual-by-individual correlation distance in relative levels of SMP compounds; (ii) geographical distance versus individual-by-individual genetic distance; and (iii) correlation distance of the SMP versus genetic distance. The correlation distance matrix of SMP compounds was performed in R (Quinn & Keough, 2006). Genetic distances were calculated in MEGA 4.0.1 (Tamura *et al.*, 2007) using maximum composite likelihood (MCL). The MCL calculations used data for both transitions and transversions, and assumed a heterogeneous pattern of sequence evolution with a gamma distribution of among-site rate variation (gamma parameter = 1.0). Gaps, missing data and individuals without all of the dataset were deleted from the analysis, and standard errors were calculated with 1000 bootstrap replicates.

Results

Phylogenetic analyses

A total of 850 bp from the *COI* gene (145 parsimony informative sites), 773 nucleotides of *EF-1α* F2 copy (24 parsimony informative sites), containing a ~200-bp intron, and the combined sequences were compared with an estimate of the

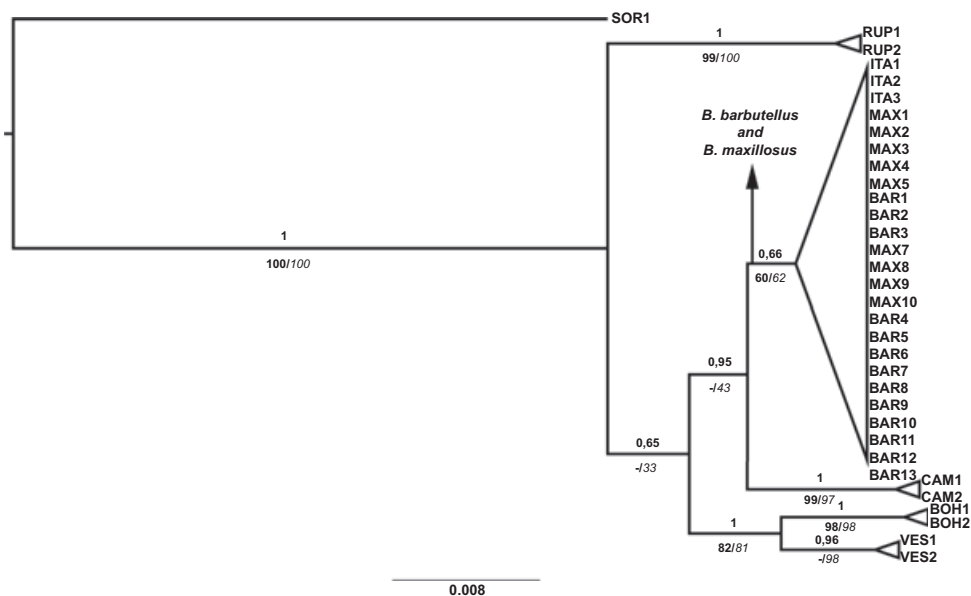


Fig. 2. Majority-rule (50%) consensus tree based on Bayesian analyses of elongation factor 1 α (*EF-1 α*) sequences; values above branches are Bayesian posterior probabilities; values in bold below branches are maximum likelihood bootstrap values; values in italic below branches are maximum parsimony bootstrap values. *Bombus soroeensis* is used as the out-group. The triangles represent clades with shared haplotypes. The sample codes are defined in Table 3.

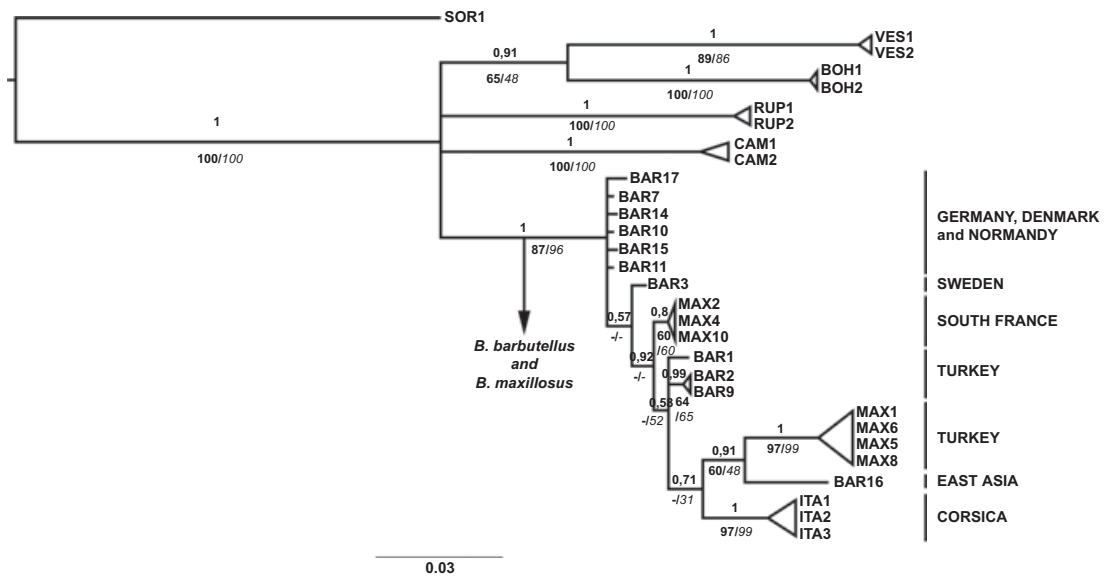


Fig. 3. Majority rule (50%) consensus tree based on Bayesian analyses of cytochrome *c* oxidase subunit I (*COI*) sequences; values above branches are Bayesian posterior probabilities; values in bold below branches are maximum likelihood bootstrap values; values in italics below branches are maximum parsimony bootstrap values. *Bombus soroeensis* is used as the out-group. The triangles represent clades with shared haplotypes. The sample codes are defined in Table 3.

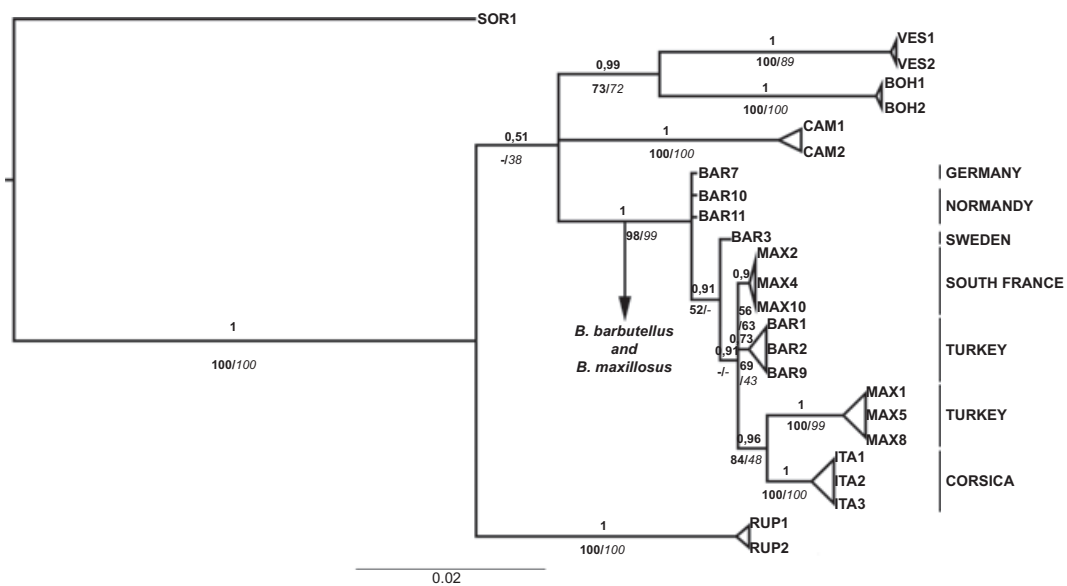


Fig. 4. Majority rule (50%) consensus tree based on Bayesian analyses of the combined molecular data matrix (*EF-1α* and *COI*); values above branches are Bayesian posterior probabilities; values in bold below branches are maximum likelihood bootstrap values; values in italic below branches are maximum parsimony bootstrap values. *Bombus soroeensis* is used as the out-group. The triangles represent clades with shared haplotypes. The sample codes are defined in Table 3.

phylogenetic relationships. All analyses (MP, ML and MB) on each single gene and combined dataset present the same topology (Figs 2–4), but higher branch support values were found in MB. Phylogenetic analyses based on *EF-1α* partially recover all deep relationships among *Psithyrus* subgroups, but failed to provide much sequence variation within and between *B. barbutellus* and *B. maxillosus*. Moreover, there is

not any divergence between *B. barbutellus* and *B. maxillosus*, while a clear divergence appeared in the *Ashtonipsithyrus* species group (*B. bohemicus* and *B. vestalis*). The *COI* tree topology failed to resolve deep relationships among *Psithyrus* subgroups, but provided considerable sequence variation between *B. barbutellus* and *B. maxillosus*. As in the *EF-1α* analyses, *B. barbutellus* and *B. maxillosus* were not split

into two groups. Nevertheless, geographic groups appeared: a west–central European group; a southern French group; two Turkish groups; and a Corsican group (Fig. 3). However branch support values are low in posterior probability, but are well supported in ML and MP. Combined genes analyses summarize the differing contributions of the two molecules at the two ends of the tree. These trees presented a similar topology and similar branch support to *EF-1 α* for a deep relationship between *Psithyrus* subgroups, and to *COI* for the tips of the phylogeny (including *COI* geographic groups). In each of the analyses, therefore, there was an unambiguous support for the monophyly of the *B. barbutellus* + *B. maxillosus* group.

Chemical analyses

Seventy-three compounds were detected in the SMP of *B. barbutellus* and *B. maxillosus* (Table 4). *Bombus barbutellus* and *B. maxillosus* shared all compounds. No discrimination between *B. barbutellus* and *B. maxillosus* was found in the statistical analyses (Figs 5, 6). Principle components analysis (PCA) analysis did not separate any group. However, all the clusters separated the samples into two main groups: a Turkey cluster with nearly all of the Turkish individuals; and a European cluster with all European samples and one Turkish specimen (Fig. 6). The two-group *k*-means method detected the same separation. Only slight quantitative and qualitative divergences in the minor compounds separated the two groups. Examination of the wing condition (Tkalců, 1969) of the Turkish individual grouped with the European group indicated that it is obviously an old specimen. In some bumblebee species, old individuals, scarce in the wild, have a pheromonal composition modification, and are probably no longer attractive to females (Žáček *et al.*, 2009; Coppée, 2010). In the Turkey group, *B. maxillosus* and *B. barbutellus* are separate (Fig. 6). A comparison between Turkish *B. barbutellus* and Turkish *B. maxillosus* compounds showed only slight differences in relative abundances.

Correlation analyses

The Mantel tests show a significant positive correlation between SMP dissimilarity and geographical distance (Mantel's $r = 0.298$; $P < 0.05$), and between genetic distance and geographical distance (Mantel's $r = 0.293$; $P < 0.05$) (Fig. 7A, B). There is no correlation between SMP dissimilarity and genetic distance (Mantel's $r = 0.089$; $P > 0.05$) (Fig. 7C).

Discussion

Species status

The phylogenetic trees showed conflict with Cameron *et al.* (2007) and Hines (2008). Although these studies were not

designed to deal with species problems, they showed genetic differences in the *EF-1 α* sequence between *B. barbutellus* and *B. maxillosus*. However, these studies used only one Swedish *B. barbutellus* specimen and one Turkish *B. maxillosus* specimen. By performing a wider specimen sampling, taking into consideration the intraspecific variability, we show that *B. barbutellus* or *B. maxillosus*, respectively, do not constitute monophyletic groups. Therefore, they are not different species according to the phylogenetic species concept (Baum, 1992).

Our results in SMP composition are similar to those of Urbanová *et al.* (2004). Fifty-eight compounds detected in GC–MS analysis were identical to those from the former study; 15 components with a relative abundance below 0.1% and four components with a relative abundance higher than 0.1% differed. Among these compounds only three were present in all individuals. However, the differences observed with the data from Urbanová *et al.* (2004) are of the same order of magnitude as the interpopulation variation observed in our dataset for *B. barbutellus*. Pheromonal analyses also support a lack of divergence between *B. barbutellus* and *B. maxillosus*. The comparison of SMPs between the two taxa showed only some slight quantitative modifications and no qualitative modifications, and supports their conspecificity according to the species recognition concept (Paterson, 1993). Indeed, the variation observed between *B. barbutellus* and *B. maxillosus* is lower than the intraspecific variation observed in *B. terrestris* (Coppée *et al.*, 2008), and even lower than the variation between other bumblebees species (Bertsch, 1997; Bertsch *et al.*, 2005; Rasmont *et al.*, 2005; Terzo *et al.*, 2005).

Our chemical and molecular analyses point towards a conspecific status for *B. barbutellus* and *B. maxillosus*, whichever species concept is considered. Moreover, these analyses do not support any separation of alternative species inside the *B. barbutellus* + *maxillosus* group. This result agrees with Grütte (1940). The taxonomic status proposed by Grütte (1940) was criticized by Tkalců (1969) regarding variations in wing darkness and host choice. Tkalců (1969) considered the wing darkness of all bees as a constant specific criterion unrelated to individual or geographic variations. However, according to our results, wing darkness is not a diagnostic species character of *B. barbutellus*. The relevance of the wing darkness as a species diagnostic character among bees, never demonstrated statistically from a large sample, therefore still needs to be proven. To summarize, according to the conspecificity shown in our results, the main morphological criteria (hair length and wing darkness) used to distinguish both taxa (Popov, 1931; Pittioni, 1939; May, 1942, 1943) is probably the result of intraspecific variability. This could explain the numerous intermediate forms observed by Grütte (1940) and the large number of undeterminable individuals (Rasmont, 1988).

Intraspecific variability

We found three colour patterns corresponding to the following subspecies (*sensu* Rasmont, 1988): *barbutellus*, *italicus* and *maxillosus*. However, colour patterns do not fit

Table 4. List of the identified compounds in two *Bombus* species.

Compounds	MW	<i>B. barbutellus</i>					<i>B. maxillosus</i>				
		Min	Q1	Med	Q3	Max	Min	Q1	Med	Q3	Max
Decanoic acid	172	0.01	0.02	0.02	0.02	0.15	0	0.02	0.02	0.05	0.15
Geranyl acetone	194	0.01	0.02	0.02	0.15	0.2	0	0.02	0.03	0.05	0.2
Tetradecane	198	0.27	0.36	0.75	1.58	3.49	0	1.43	2.3	2.85	4.12
Dodecanoic acid	200	0.01	0.02	0.03	0.16	2.72	0.01	0.05	0.3	1.83	5.15
Farnesal, isomer I	220	0.04	0.12	0.18	0.21	0.31	0	0.05	0.16	0.33	0.61
Farnesal, isomer II	220	0.14	0.29	0.36	0.45	0.59	0	0.1	0.23	0.63	0.92
Farnesol	222	0.03	0.05	0.1	0.2	0.37	0	0	0.21	0.47	1.54
Dihydrofarnesol	224	0.13	0.2	0.38	0.42	0.53	0	0.08	0.15	0.24	0.53
Ethyl dodecanoate	228	0	0.02	0.02	0.07	0.15	0	0	0.01	0.02	0.15
Tetradecenoic acid	228	0	0	0.02	0.03	0.04	0	0.01	0.04	0.1	0.16
Heptadecane	240	0	0.01	0.02	0.02	0.06	0	0.01	0.02	0.05	0.15
Ethyl tetradecenoate	254	0	0	0	0.01	0.07	0	0.02	0.1	0.25	19
Hexadecenoic acid	254	0	0.03	0.16	0.21	0.76	0	0.2	0.35	0.44	1.69
Ethyl tetradecanoate	256	0	0	0	0.01	0.01	0	0	0.02	0.11	0.79
Farnesyl acetate	264	10.21	11.35	16.58	20.98	37.89	0.19	20.84	25.53	28.37	45.4
Octadecenal	266	0.02	0.03	0.04	0.06	0.6	0	0.08	0.32	0.55	1.93
Dihydrofarnesyl acetate	266	0.09	0.29	0.49	0.64	0.82	0	0.1	0.31	0.89	1.7
Octadecanal	268	0	0	0.01	0.01	0.13	0	0.02	0.07	0.13	0.32
Octadecenol	268	11.81	13.23	16.17	20.68	24.28	0.02	2.25	9.58	13.94	24.28
Ethyl hexadecenoate	282	0	0	0	0.01	0.02	0	0.03	0.04	0.18	0.47
Octadecenoic acid	282	0	0.02	0.07	0.15	0.61	0	0.01	0.13	0.22	1.95
Ethyl hexadecanoate	284	0.01	0.03	0.06	0.08	0.22	0	0.04	0.15	0.36	0.79
Geranylgeranial	288	0.14	0.33	0.46	0.48	1.04	0	0.12	0.19	0.23	1.04
Farnesyl butyrate	292	0	0	0.18	0.5	0.9	0	0.26	0.63	0.83	2.33
Geranylcitronellol	292	1.54	2.6	3.25	3.69	6.34	0.12	1.49	2.04	2.4	6.61
Heneicosene	294	0	0	0.03	0.66	4.85	0	0.12	0.33	0.81	4.72
Heneicosane	296	0.3	0.77	0.97	1.54	1.68	0.02	0.11	0.15	0.17	1.68
Eicosenol	296	0	0	0	0	9.03	0	0	4.22	4.73	9.03
Octadecenoic acid ethyl ester	310	0	0	0	0.08	0.08	0	0	0.03	0.09	0.44
Octadecenyl acetate	310	0.75	0.85	0.9	1.05	2.12	0.4	0.93	1.04	1.1	2.04
Docosane	310	0	0.14	0.16	0.18	0.22	0	0.11	0.15	0.21	0.25
Tricosene	322	0.08	0.1	0.19	0.21	0.33	0	0.02	0.18	0.23	0.54
Tricosane	324	0.04	0.18	6.19	7.51	9.91	0.02	0.07	0.11	0.14	10.91
1,3-Diacetyl-2-decanoylglycerol	330	0	0.17	0.68	7.72	14.32	0	0.3	0.47	1.14	15.6
Geranylcitronellyl acetate	334	0.12	0.46	0.51	0.98	1.47	0.2	0.57	0.67	0.82	2.87
Eicosenyl acetate	338	0.03	0.17	0.31	0.35	0.8	0.04	0.07	0.1	0.19	2.61
Tetracosane	338	0.04	0.04	0.07	0.08	0.49	0	0.04	0.06	0.08	0.49
Eicosyl acetate	340	0	0	0	0.01	0.18	0	0.01	0.02	0.03	0.22
Pentacosene, isomer I	350	0.02	0.04	0.07	0.1	0.23	0.01	0.03	0.05	0.09	3.62
Pentacosene, isomer II	350	0.04	0.08	0.11	0.17	0.28	0.02	0.05	0.08	0.11	0.28
Pentacosane	352	1.17	1.7	1.96	2.15	4.66	0.76	1.18	1.6	1.85	4.66
1,3-Diacetyl-2-dodecanoylglycerol	358	8.72	16.83	25.11	26.86	31.11	0.96	10.85	19.02	24.08	31.11
Hexacosene	364	0.02	0.02	0.04	0.08	0.12	0.01	0.01	0.02	0.04	0.12
Farnesyl decanoate	376	0.04	0.07	0.08	0.13	0.2	0.05	0.06	0.08	0.22	0.38
Heptacosene, isomer I	378	0	0.03	0.04	0.1	0.22	0.01	0.03	0.05	0.09	3.01
Heptacosene, isomer II	378	0.05	0.27	0.37	0.59	1.08	0.07	0.13	0.27	0.45	1.08
Heptacosane	380	0.52	0.83	1.06	1.63	3.34	0.61	0.8	0.94	1.49	4.11
Hexacosenol	380	0.04	0.09	0.12	0.17	0.35	0	0.04	0.12	0.23	0.35
Hexacosanol	382	0.02	0.05	0.05	0.13	0.32	0.01	0.02	0.06	0.12	1.55
1,3-Diacetyl-2-tetradecanoylglycerol	384	0	0.23	0.37	0.45	0.53	0.04	0.22	0.91	1.74	2.58
1,3-Diacetyl-2-tetradecanoylglycerol	386	0.04	0.08	0.13	0.15	0.19	0	0.05	0.18	0.35	1.12
Octadecenyl octanoate	394	0.02	0.03	0.05	0.07	0.09	0.01	0.03	0.05	0.08	0.19
Hexadecyl decanoate	396	0.03	0.04	0.05	0.07	0.33	0	0.01	0.02	0.07	0.33
Farnesyl dodecanoate	404	0.07	0.08	0.1	0.24	0.69	0.02	0.15	0.38	1.36	4.41
Nonacosene	406	0.04	0.07	0.12	0.16	0.21	0.03	0.04	0.08	0.14	0.27
Nonacosane	408	0.1	0.16	0.2	0.25	0.72	0.11	0.15	0.18	0.26	0.76
Squalene	410	0	0	0.01	0.01	0.02	0	0.01	0.01	0.03	0.09
1,3-Diacetyl-2-hexadecanoylglycerol	412	0	0	0	0	0.13	0	0	0	0	0.13
Octadecenyl decanoate	422	0.09	0.19	0.28	0.38	1.79	0.05	0.13	0.42	0.95	3.35
Hexadecyl dodecanoate	424	0.01	0.03	0.04	0.09	0.16	0	0.02	0.08	0.13	0.24
Hentriacontene	434	0.02	0.02	0.03	0.06	0.08	0.01	0.02	0.04	0.07	0.16
Geranylcitronellyl decanoate	446	0	0.01	0.02	0.04	0.25	0	0.01	0.02	0.07	0.61

Table 4. Continued

Compounds	MW	<i>B. barbutellus</i>					<i>B. maxillosus</i>				
		Min	Q1	Med	Q3	Max	Min	Q1	Med	Q3	Max
Octadecenyl dodecanoate	450	1.53	2.61	3.64	7.04	10.08	0.58	2.51	7.4	13.93	20.94
Farnesyl hexadecanoate	460	0	0.01	0.01	0.12	0.46	0	0	0.09	0.3	0.93
Geranylcitronellyl dodecanoate	474	0.14	0.16	0.19	1.21	1.76	0.02	0.14	0.33	1.75	3.93
Eicosenyl dodecanoate	478	0.74	0.88	1.3	2.03	3.64	0.3	0.72	1.61	4.72	12.06
Farnesyl octadecenoate	486	0	0	0	0.06	0.12	0	0.04	0.06	0.08	0.5
U1	MW?	0.26	0.4	3.51	7.37	9.87	0	2.67	3.61	3.88	9.87
U2	MW?	0.01	0.07	0.09	0.18	0.35	0	0.09	0.15	0.21	2.68
U3	MW?	0	0.02	0.03	0.04	2.89	0	0	0.01	0.03	2.89
U4	MW?	0.03	0.05	0.06	0.1	0.18	0	0.03	0.08	0.1	0.21
U5	MW?	0.01	0.02	0.02	0.03	0.04	0.01	0.03	0.07	0.14	0.59
U6	MW?	0.01	0.01	0.01	0.02	0.11	0	0.01	0.03	0.05	0.41

Molecular weight [MW (m/z)], median [Med (%)], first and fourth quartiles [Q1 (%) and Q2 (%)], minimum and maximum [Min (%) and Max (%)] of the 73 identified compounds in *Bombus barbutellus*, *Bombus maxillosus maxillosus* and *Bombus maxillosus italicus*. Ux, undetermined compounds.

The compounds in bold are main compounds of the sexual marking pheromone (SMP) for the two taxa.

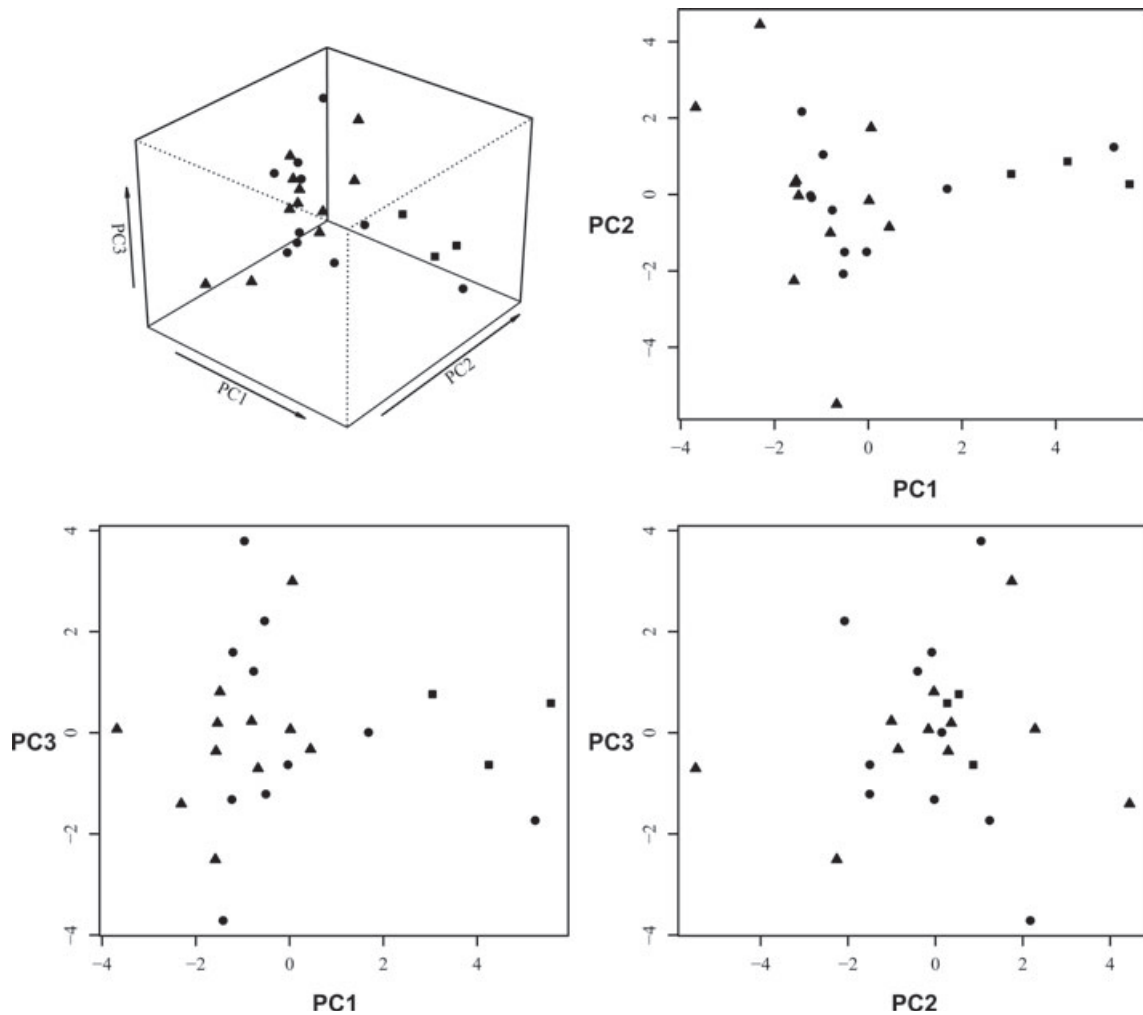


Fig. 5. Three first axes of the principle components analysis (PCA) based on 20 compounds in 21 specimens of *Bombus barbutellus*, *Bombus maxillosus maxillosus* and *Bombus maxillosus italicus*; ●, *Bombus barbutellus*; ■, *Bombus maxillosus italicus*; ▲, *Bombus maxillosus maxillosus*.

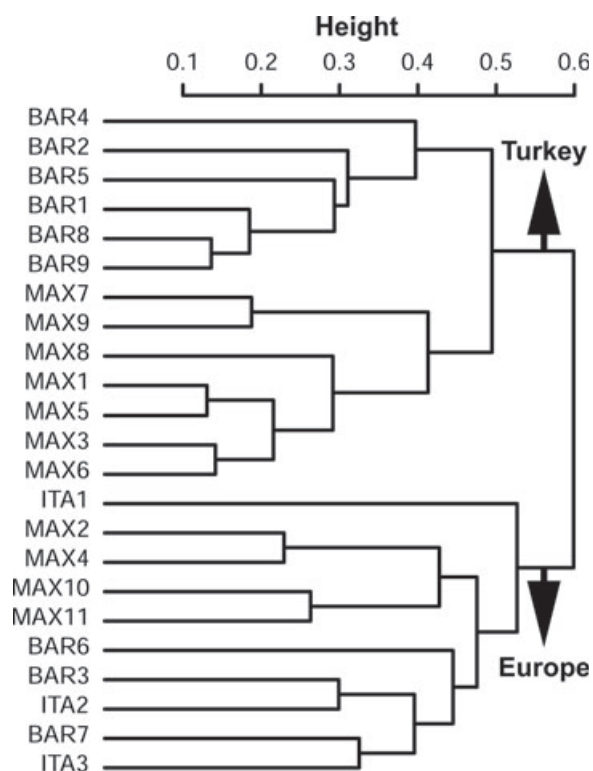


Fig. 6. Unweighted pair group method with arithmetic mean (UPGMA) cluster based on a correlation matrix calculated from the sexual marking pheromone (SMP) matrix of *Bombus barbutellus*, *Bombus maxillosus maxillosus* and *Bombus maxillosus italicus* (73 compounds in 21 specimens). The sample codes are defined in Table 3.

with any particular pheromonal clustering or phylogenetic tree. This could show that there is no clear subspecific subdivision with the Rasmont (1988) subspecies status, but only two extreme forms: (i) a form with long hairs and hyaline wings (*barbutellus*) living in the north of Europe and in the Mediterranean mountains; (ii) another form with short, darker hairs and darkened wings (*maxillosus*) living mainly

in the south of Europe; and (iii) between these two, there are intermediate forms, sometimes described as subspecies (Grütte, 1940). Similar patterns of variations have been observed in other bumblebees. On the one hand, hairs insulate the bumblebee bodies and decrease heat loss (Heinrich, 1975). Peat *et al.* (2005) showed that bumblebee species from hot regions exhibit shorter hairs than species from cold regions. They found the same geographic pattern at an intraspecific level for *B. terrestris*. On the other hand, melanism is also caused by thermoregulation (Gloger, 1833). Bumblebees from hot regions are darker, and bumblebees from temperate regions are paler (Williams, 2007). In Scandinavia, Løken (1984) observed a slight tendency to a more pronounced melanism in the south of Sweden than in the north among *B. barbutellus*.

The genetic distance observed in *B. barbutellus* is correlated with geographic distances, according to our Mantel test, as amongst other bumblebees (Estoup *et al.*, 1996; Widmer *et al.*, 1998a, b). However, any groups inside the *barbutellus* phylogenetic tree match with any distinct coloration group. It could support population variations, but not a clear subspecific taxonomy.

The slight SMP geographic variations, mainly between Turkey and Europe, are explained by geographic distance. The Mantel test shows that SMP distance is correlated with geographic distance, as in other bees (Vereecken *et al.*, 2007; Coppée *et al.*, 2008; Lecocq *et al.*, 2009b). Among the Turkish group, the splitting into two forms (*barbutellus* and *maxillosus*) may result from an interpopulational variation, as observed in *B. terrestris* (Coppée, 2010), but is more likely to reflect sampling bias. Indeed, Turkish *B. barbutellus* samples were collected at the same time and from the same place. The likelihood that these individuals were related and thus have a nearly identical SMP composition is high. In this case, the clustering method separated them from other samples with no other biological reason than the kin relationship.

Even if we observed variations between *B. barbutellus* populations, we currently do not know if these variations have an impact on the intraspecific sexual mating recognition among

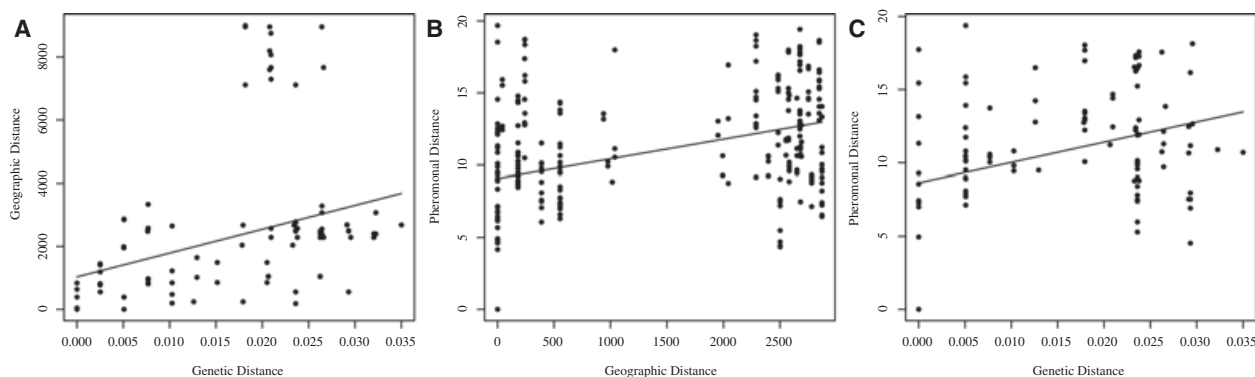


Fig. 7. Mantel's correlogram of (A) genetic distance among genetic samples plotted by spatial distance among samples sites (Mantel's $r = 0.293$; $P < 0.05$); (B) correlation distance among chemical samples plotted by spatial distance among samples sites (Mantel's $r = 0.298$; $P < 0.05$); (C) genetic distance among genetic samples plotted by correlation distance among chemical samples (Mantel's $r = 0.089$; $P > 0.05$).

B. barbutellus. Ethological studies, difficult to perform among scarce species, would resolve this problem.

Moreover, phylogenetic and pheromonal geographic groups never match in our analysis, and are not correlated according to the Mantel test. Therefore, we were unable to discover any subspecies differentiation.

Host relationship

As a consequence of our present taxonomic definition, *B. barbutellus* seems to be an opportunistic cuckoo bumblebee with preferences for host species belonging to the subgenus *Megabombus*. The differences in the main host choice of the *barbutellus* form (*B. hortorum*) and the *maxillosus* form (*B. argillaceus* and *B. ruderatus*) do not reflect a species differentiation. The definition of the species status in inquiline bumblebees should not be based on differences in host choice. Indeed, in the few cases where information is available (reviewed in Williams, 2008), socially parasitic bumblebee lineages tend not to be strict specialists: several *Psithyrus* species are known to parasitize multiple species in their communities.

Impact on zoological nomenclature

Bombus barbutellus (Kirby) is the only species belonging to the group formerly known as the subgenus *Allopsithyrus* Popov. *Bombus maxillosus* should be regarded as a simple synonym. The oldest available names for the species are the following:

- ? *Apis brachyptera* Villers in Linnaeus, 1789: 331, *nomen oblitum*
- ? *Apis monarcha* Christ, 1791: 131, *nomen oblitum*
- ? *Apis cincta* Preyßler, 1793: 232, *nec* Fabricius, 1781, *nomen oblitum*
- ? *Apis saltuum* Panzer, 1800–1801: part 75, table 21, *nomen oblitum*

Apis barbutella Kirby, 1802: 343, *nomen protectum*; locus typicus, England, East Suffolk, Barham; lectotype ♀, Natural History Museum, London, designated by Yarrow, 1968; revised by P. Rasmont 2010. Labels (i) handwritten '93♀'; (ii) round pale-blue label with glued right protarsus; (iii) round with a black circle printed 'Lectotype'; (iv) handwritten 'Apis barbutella Kirby 1802 LECTOTYPE I.H.H. Yarrow 1968; (v) printed and handwritten 'B.M. TYPE HYM 17a 2996'. The right fore tarsus is broken and glued to the second label. The three distal segments of the hind tarsus are missing. The coat colour pattern is typical of the northern form of *B. barbutellus*, but the wings are more infuscated than usual.

Bombus maxillosus Klug, 1817: 269; locus typicus, 'Süd Europa'; holotype ♀, Zoologisches Museum, Berlin, see Tkalců, 1969. **syn.n.**

The oldest names, *Apis brachyptera* Villers, *Apis monarcha* Christ, *Apis cincta* Preyßler and *Apis saltuum* Panzer have a description that does not allow us to identify the taxa with certainty, whereas no types are presently known. These names have not been used since their initial description. They are therefore all *nomina oblita*.

The oldest available name is *Apis barbutellus* Kirby; *Bombus maxillosus* Klug is therefore a subjective junior synonym. The valid name should be *Bombus (Psithyrus) barbutellus* (Kirby, 1802). Many names have been used for infraspecific colour forms, mainly by Kriechbaumer (1870), Popov (1931), Quilis-Pérez (1932), Müller (1936), Pittioni (1939) and May (1943). They are all considered here as synonyms of *Bombus barbutellus* (Kirby).

Acknowledgements

We are very grateful to B.N. Danforth and M.M. López-Urbe for their hospitality at Cornell University (Ithaca, NY), and to P. Mardulyn for hospitality in the Université Libre de Bruxelles (Belgium) for the molecular analysis. We gratefully acknowledge M. Terzo (Mons, Belgium), A. Roelandts (Mons, Belgium), E. Stolle (Halle, Germany) and G. Mahé (Saint-Nazaire, France) for providing *Psithyrus* samples. Thanks also to P.H. Williams (London, U.K.) for help and comments on the taxonomic nomenclature. We are also grateful to T. De Meulemeester (Mons, Belgium) for statistical analysis. The authors acknowledge the Fonds pour la recherche dans l'industrie et l'agriculture (FRIA), Fonds pour la formation à la recherche fondamentale et collective (FNRS), the Office de l'Environnement de la Corse and the Direction Régionale de l'Environnement de Corse for financial support. Special thanks are also due to Dr A. Coppée (Mons, Belgium), Prof. A.M. Aytekin (Ankara, Turkey), Dr S. Patiny (Gembloux, Belgium), Dr D. Lanterberg (Mons, Belgium) and S. Lambert for helpful comments and discussions on earlier versions of this article, and B. Lhomme for help with the English.

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Accepted 24 March 2011