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

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## Short Title

Species discrimination by molecular and chemical evidences

## 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 (EF-1 $\alpha$ ) and one mitochondrial gene (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 2-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 2-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*.

## Keyword

43 Bee taxonomy, Sexual marking pheromones, Phylogeny, *Bombus barbutellus*, *Bombus*  
44 *maxillosus*

## 45 **Introduction**

46 The species notion is one of the most confusing and controversial concepts in biology  
47 (Mayr, 1942; Templeton, 1989; Baum, 1992; De Queiroz, 2007; Gourbière & Mallet, 2009).  
48 However, the species is a fundamental biological unit and its definition is of major importance  
49 to biology. Numerous ways have been explored to determine the species status. In  
50 hymenopteran systematics (review for bees in Engel, 2011), authors have tried to define  
51 taxonomic status using morphology (i.e. Michener, 1951; Pekkarinen, 1982; Williams, 1998),  
52 geometric morphometry (i.e. Aytakin *et al.*, 2007), allozyme based models (i.e. Pamilo *et al.*,  
53 1997), genetic (i.e. Koulianos & Schmid-Hempel, 2000; Hines *et al.*, 2006; Bernasconi *et*  
54 *al.*, 2010), or chemoecology (i.e. Svensson, 1979; Bertsch *et al.*, 2005; Rasmont *et al.*, 2005;  
55 Martin *et al.*, 2008 ).

56 The morphological approach is the earliest, the easiest and the most common method used  
57 to define species (i.e. Løken, 1983; Bolton, 1995; Michez & Eardley, 2007). However,  
58 determining objective morphological characters which accurately reflect biological species is  
59 difficult (Bickford *et al.*, 2007). Results of morphological analyses have conflicted with other  
60 kind of evidences (i.e. Svensson, 1979; Batalha-Filho *et al.*, 2010).

61 Genetic characters are taken more and more into consideration in order to assess species  
62 status (i.e. Leaché & Fujita, 2010). Development of DNA barcoding studies (i.e. Hebert *et al.*,  
63 2003) to describe cryptic species (i.e. Yassin *et al.*, 2007), or included into integrative  
64 taxonomic framework (i.e. Gibbs, 2009) exemplify this trend. A phylogenetic species concept  
65 (Baum, 1992) has been developed and defined a species as a monophyletic group  
66 (Papadopoulou *et al.*, 2008), perhaps based on multiple gene genealogies (Baum & Shaw,  
67 1995). Nevertheless, the genetic approach remains controversial in defining species limits

68 because DNA sequences analyzed are chosen arbitrarily or because mating isolation can  
69 happen faster than differentiation of the genetic markers used (Avisé, 2000; Trewick, 2008;  
70 Symonds *et al.*, 2009; Bauer *et al.*, 2010).

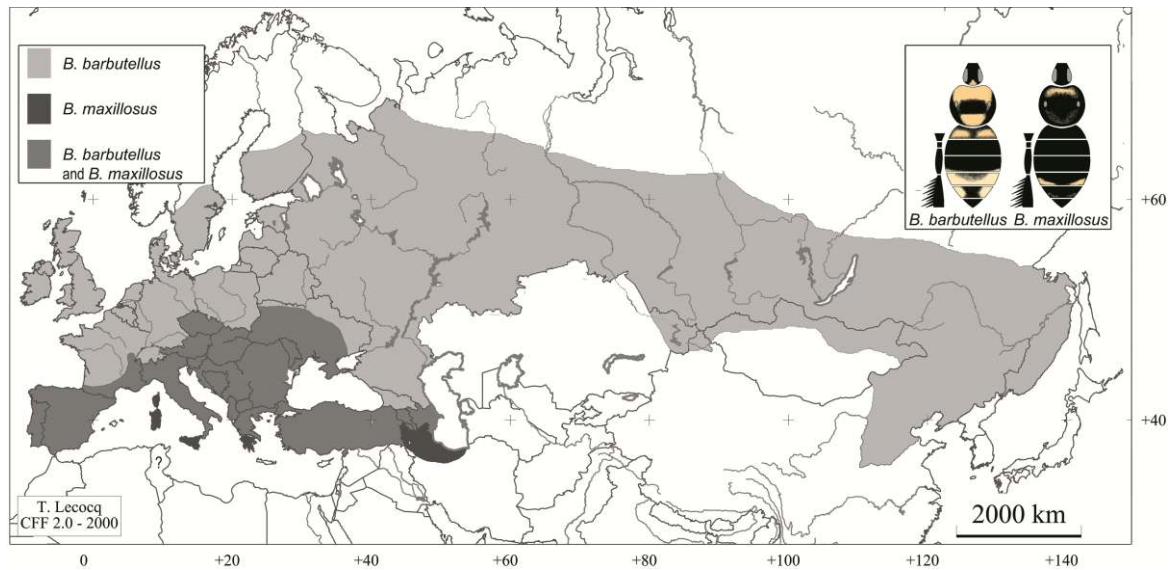
71 Chemical features, mainly species specific secretions (i.e. Lockety, 1991), can provide  
72 useful tools in separating species. Among bumblebees, Sexual Marking Pheromones (SMP),  
73 synthesized by male cephalic labial glands (Kullenberg *et al.*, 1973), play a major role in  
74 species specific pre-mating behavior (Bergman, 1997) and are highly species specific (Calam,  
75 1969; Valterova & Urbanova, 1997). Therefore, authors used these chemical markers in  
76 resolving species status (Bergström *et al.*, 1981; Svensson & Bergström, 1977; Rasmont *et*  
77 *al.*, 2005; Coppée *et al.*, 2008; Lecocq *et al.*, 2009a, 2009b) according to the species  
78 recognition concept (Paterson, 1993). In this concept, conspecific individuals sharing the  
79 same recognition signals are supposed to recognize each other as sexual partners (review in  
80 Terzo *et al.*, 2003). However it is difficult to determine a threshold of species level  
81 differentiation, even with ethological test (i.e. Ings *et al.*, 2010). Only *B. terrestris* has been  
82 studied enough to determine a well supported threshold thanks to numerous chemo-  
83 ethological tests (review in Coppée 2010).

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

87 The present study deals with taxonomic problems in the monophyletic subgenus *Psithyrus*  
88 included in the genus *Bombus* Latreille, 1802. 30 *Psithyrus* species described worldwide live  
89 in the Northern hemisphere (Williams, 1998 update at NHN). *Psithyrus* species are social  
90 parasites (cuckoo bumblebees) of other bumblebees. Cuckoo bumblebees lack pollen-  
91 collecting corbiculae and a worker caste. They are therefore completely dependent on hosts to  
92 rear their offspring. They parasitize hosts from across the bumblebee phylogeny with very

93 little cophylogenetic pattern (Williams, 2008). Typically, *Psithyrus* species parasitize just one  
94 host or a few host species of the same subgenus (review in: Alford 1975; Løken, 1984;  
95 Lhomme, 2009), but some species are considered to be generalists, with the extent of their  
96 host range often unclear. The ecology of host-parasite interactions of most of the cuckoo  
97 bumblebees is poorly known or unknown for almost half of *Psithyrus*.

98 This study focuses on two sister taxa, *Bombus (Psithyrus) barbutellus* (Kirby, 1802) and  
99 *Bombus (Psithyrus) maxillosus* Klug in Germar, 1817 of the former subgenus *Allopsithyrus*  
100 Popov, 1931, or *barbutellus*-group (Fig. 1) (i.e. Williams, 1998). The taxonomic status of  
101 these two taxa is doubtful (Grütte, 1940; Tkalců, 1969; Rasmont, 1988; Williams, 1998).  
102 Most authors consider these taxa as separate species (Popov, 1931; Pittioni, 1939; May, 1942,  
103 1943; Tkalců, 1969; Løken, 1984) while very few argue for their conspecificity (Grütte,  
104 1940). Most of these studies refer to morphology, highlighting slight differences between the  
105 two taxa (Table1), mainly from hair length and wing color (e.g. Tkalců, 1969). With such  
106 weak morphological criteria a large number of individuals remain unidentified (Rasmont,  
107 1988; Rasmont & Adamski, 1996; Urbanová *et al.*, 2004) especially in geographical areas  
108 shared by both taxa. *B. barbutellus* has a large palaeartic distribution while *B. maxillosus* is  
109 restricted around the Mediterranean Sea (Fig. 1) (Rasmont, 1988; Pagliano, 1993; Rasmont &  
110 Flagothier, 1996; Williams, 1998 update at NHM). Many overlapping areas and exceptions to  
111 these trends are known (Vogrin, 1955; Schwartz *et al.*, 1996; Schwartz *et al.*, 2005; Straka *et*  
112 *al.*, 2007; Pawlikowski, 2008; Iserbyt, 2009). The splitting into subspecies differs for many  
113 authors (Pittioni, 1939; Grütte, 1940; Rasmont 1988). In this study, as a preliminary approach,  
114 we use the subspecies classification of Rasmont (1988): *Bombus barbutellus* no subspecies, *B.*  
115 *maxillosus maxillosus* Klug, 1817 (France, S-E Europe, Anatolia, Transcaucassia, Caucasus,  
116 and N Iran) and *B. maxillosus italicus* (Grütte, 1940) (Iberian Peninsulas, Italian Peninsulas,  
117 Corsica, and Sardinia).



118

119 **Fig. 1.** Distribution area of *Bombus maxillosus* and *Bombus barbutellus* in the Palaearctic region according to the literature (Maidl, 1922;  
 120 Pittioni, 1939; Haas, 1949, 1967; Vogrin, 1955; M'ocz'ar, 1957; Dylewska, 1966; Elfving, 1968; Reinig, 1971; Ressel, 1974; Alford, 1975;  
 121 Tum'ss, 1975; Löken, 1984; Ornos, 1984; Intoppa *et al.*, 1995; Monsevicius, 1995; Rasmont *et al.*, 1995; Amiet, 1996; Baker, 1996;  
 122 Rasmont & Flagothier, 1996; Rasmont & Adamski, 1996; Peeters *et al.*, 1999; Dathe *et al.*, 2001; Yefremova, 2001; Anagnostopoulos, 2005;  
 123 Fitzpatrick *et al.*, 2006; Byvaltsev, 2008, 2009; Pawlikowski, 2008; W. F. Reinig, personal data).

124

125 The sparsity of *B. barbutellus* and *B. maxillosus* makes information about their ecology  
 126 very scarce. According to Pittioni & Schmidt (1942) and Rasmont (1988), *B. barbutellus* and  
 127 *B. maxillosus* have different habitats. *B. barbutellus* is a forest edge dweller. It is a plains  
 128 species in the north of its range and a mountain species in the south. *B. maxillosus* seems to be  
 129 more of an open field species (Pittioni & Schmidt, 1942) or a wood edge species (Rasmont,  
 130 1988). They also seem to have different hosts. The main host of *B. barbutellus* is *Bombus*  
 131 (*Megabombus*) *hortorum* (L., 1761) (Sladen, 1912; Ball, 1914; Müller, 1936; Cumber, 1949;  
 132 Tkalců, 1969; Löken, 1984) whereas the main hosts of *B. maxillosus* seem to be two sisters  
 133 species *Bombus* (*Megabombus*) *argillaceus* (Scopoli, 1805) and *Bombus* (*Megabombus*)  
 134 *runderatus* (Fabricius, 1793) (Reinig, 1935; Rasmont, 1988; Rasmont & Adamski, 1996) close  
 135 related to *B. hortorum* inside the *Megabombus* subgenus. However, other observations  
 136 (Reinig, 1935; Pittioni & Schmidt, 1942; May, 1943; Pouvreau, 1973; Ornos, 1984; Rasmont  
 137 & Adamski, 1996; Westrich, 1989; Peeters *et al.*, 1999) show that both taxa have no strict  
 138 host-parasite relationship (Table 2). In this case, there is no definitive evidence to separate  
 139 both taxa according to their host choice contrary to Tkalců's (1969) observations.

140 Chemical data are scarce too. The SMP of a few *B. barbutellus* specimens from Sweden  
141 have been described by Kullenberg *et al.* (1970) and Cederberg *et al.* (1984). The SMP of  
142 four specimens of *B. maxillosus* from Czech Republic have been described by Urbanová *et al.*  
143 (2004). These last authors compared their data with *B. barbutellus* of Kullenberg *et al.* (1970)  
144 and Cederberg *et al.* (1984). They found similarities in the blend of the SMP. However, due to  
145 the improvement in analysis techniques, data published before 1996 need to be re-examined  
146 (Terzo *et al.*, 2003). Comparison between such old data and new data is difficult. Very few  
147 genetic studies including both taxa have been undertaken and none have dealt with species  
148 status. In recent estimates of bumblebee phylogeny, the two taxa are treated as different  
149 species (Cameron *et al.*, 2007; Hines 2008).

150 Morphological, phylogenetic, ecological, and ethological data from literature about *B.*  
151 *barbutellus* and *B. maxillosus* are conflicting. A comparison of all available data fails to  
152 determine a strongly supported taxonomic hypothesis. We need more evidences in order to  
153 evaluate the status of both taxa (Williams, 1998 update at NHN). This paper aims to solve the  
154 taxonomic status of *B. barbutellus* and *B. maxillosus* using new molecular and chemical data  
155 from larger samples than previous studies.

## 156 **Material and Methods**

### 157 *Sampling and Morphological identification*

158 We compared morphological, molecular, and chemical (SMP) features from the same  
159 sampling of males (Table 3). Specimens were caught in the wild and killed by freezing.  
160 Identification keys which discriminate *B. barbutellus* and *B. maxillosus* as two species were  
161 used to determine individuals (Popov 1931; May 1943; Pittioni 1939; Rasmont & Adamski,  
162 1996). The SMP were sampled by dissection of cephalic labial glands which were placed in



163 200 µl hexane (Terzo *et al.*, 2005). The rest of the male bodies were conserved in ethanol  
164 (99%) for molecular analyses. All samples were stored at -40°C until analysis.

165 Scarcity of the species in the field restricts collection of a large quantity of specimens. We  
166 have analyzed 9 specimens of *B. barbutellus*, 10 of *B. maxillosus maxillosus*, and 3 of *B.*  
167 *maxillosus italicus* for pheromonal dataset. We added 4 specimens of *B. barbutellus* females  
168 to the molecular sampling. One male of *B. maxillosus maxillosus* from SMP sample was too  
169 deteriorated for DNA extraction. Sampled populations are mainly European, corresponding to  
170 the sympatric area of the two taxa (Table3; Fig.1).

171 In order to root phylogenetic trees, we choose basal *Psithyrus* species and one other  
172 bumblebee species as outgroup. We used *Bombus (Psithyrus) campestris* (Panzer, 1801) from  
173 the *Metapsithyrus* group (Pedersen, 1996; Pedersen, 2002; Cameron *et al.*, 2007), *Bombus*  
174 (*Psithyrus*) *rupestris* (Fabricius, 1793) from the *Psithyrus s.s.* group (Cameron *et al.*, 2007;  
175 Hines, 2008) and *Bombus (Kallobombus) soroensis* (Fabricius 1777) as outgroup (Table 3).

#### 176 *DNA extraction, PCR, sequencing and sequence analyses*

177 Total DNA was extracted using a QIAGEN DNeasy<sup>®</sup> Tissue Kit (Quiagen Inc., Valencia,  
178 CA). Legs were removed from the specimen and crushed using liquid nitrogen and digested  
179 (4h in proteinase K at 56°C). Voucher specimens and PCR products used in the molecular  
180 investigation were deposited at the University of Mons (Belgium). Genes were sequenced  
181 with ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). Both strands of  
182 each PCR product were sequenced. Consensus sequences were computed with CodonCode  
183 Aligner 3.0.1. (Table 3). The bumblebee origin of each sequence was checked with BLAST  
184 2.2.20 (Zhang *et al.*, 2000). The alignment was performed by MAFFT ver.6. (using FFT-NS-2  
185 algorithms, default parameters) (Katoh *et al.*, 2002). The translation in proteins (using the  
186 drosophila mt DNA genetic code) for verification and the data matrix was realized on

187 Mesquite 2.6. (build 486) (Maddison & Maddison, 2007). Sequences were deposited in  
188 Genbank (Table 3).

189 We generated sequences for two genes: Mitochondrial Cytochrome Oxidase 1 (COI) and  
190 nuclear protein-encoding gene Elongation Factor-1 alpha (EF-1 $\alpha$ ). COI show a high mutation  
191 rate and it is commonly used as a species and subspecies level marker for phylogenetic  
192 analyses (e.g. Boursot & Bonhomme, 1986; Estoup *et al.*, 1996; Danforth, 1999; Koulianos &  
193 Schmid-Hempel, 2000). EF-1 $\alpha$  has been used to infer relationships at multiple taxonomic  
194 levels in many insect taxa including bumblebees (Danforth, 1999; Carapelli, 2000; Kjer,  
195 2001; Jordal, 2002; Cameron *et al.*, 2007; Hines, 2008). Both genes have already been used to  
196 infer the phylogenetic relationship among European bumblebees (Pedersen, 2002). Primers  
197 used for all individuals were Jerry (5'-CCA CAT TTA TTT TGA TTT TTT GG-3') and Pat  
198 (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') (Danforth 1999) for COI and F2-ForH  
199 (5'-GGR CAY AGA GAT TTC ATC AAG AAC-3') and F2-RevH2 (5'-TTG CAA AGC  
200 TTC RKG ATG CAT TT-3') (Hines *et al.*, 2006) for EF-1 $\alpha$ . PCR amplification was carried  
201 out (initial denaturing for 4min at 94°C, 35 cycles of 1min denaturing at 94°C, 1min  
202 annealing at 54°C (EF-1 $\alpha$ ) -51°C (COI), 1min elongation at 72°C and a final extension for  
203 1min 15sec (EF-1 $\alpha$ ) -1min 30sec (COI) at 72°C). Gene fragments of the following sizes were  
204 amplified: 849 nucleotides of COI and 786 nucleotides of EF-1 $\alpha$  F2 copy, containing a ~200  
205 bp intron.

#### 206 *Phylogenetic analyses*

207 We analyzed each gene (COI and EF-1 $\alpha$ ) independently and in combination using  
208 Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Methods (MB). A test  
209 of saturation was applied to each fragment in PAUP\* 4.0b 10 (Swofford, 2001). The  
210 Incongruence Length Difference test (ILD test) (Farris *et al.*, 1994) was used to test for  
211 incongruence between the two genes. The test was implemented in PAUP\* (Swofford, 2001).

212 All trees were rooted with the basal taxon *B. soroensis* (Williams, 1985; Cameron *et al.*,  
213 2007).

214 Heuristic searches were performed in MP using 1000 random additions and TBR branch  
215 swapping, keeping the best trees only. Gaps were regarded as 5th state. Majority rule 50%  
216 consensus (MJ50) trees were constructed from analyses of individual genes and from all  
217 genes combined using parsimony criteria in Paup\* 4.0b 10 (Swofford, 2001) for equally-  
218 weighted MP analyses. Clades support values were estimated using nonparametric  
219 bootstrapping (Felsenstein, 1985) in PAUP\* (10000 replicates, 1000 random additions, 500  
220 trees saved per replicate).

221 ML analyses were conducted in GARLI-PART Version 0.97 (Zwickl, 2006). The dataset  
222 was partitioned as followed to explore the best submission model: (1) EF-1 $\alpha$  into two exons  
223 and one intron, (2) COI and each EF-1 $\alpha$  exon by base position. The best fitting substitution  
224 models were chosen with jModeltest (Posada, 2008) using Akaike information criteria  
225 (Akaike 1974) for each dataset. The models chosen are: GTR+G (COI 1<sup>st</sup> position), GTR  
226 (COI 2<sup>nd</sup> position), GTR+I+G (COI 3<sup>rd</sup> position), F81 (EF-1 $\alpha$  exon 1 1<sup>st</sup> position), F81 (EF-1 $\alpha$   
227 exon 1 2<sup>nd</sup> position), HKY (EF-1 $\alpha$  exon 1 3<sup>rd</sup> position), GTR+G (EF-1 $\alpha$  intron), HKY (EF-1 $\alpha$   
228 exon 2 1<sup>st</sup> position), JC (EF-1 $\alpha$  exon 2 2<sup>nd</sup> position) and GTR+G (EF-1 $\alpha$  exon 2 3<sup>rd</sup> position).  
229 A random starting tree and the automated stopping criteria (stop when the ln score remained  
230 constant for 20000 consecutive generations) were used. Ten independent runs in GARLI were  
231 carried out for each gene and for the combined data; the topology and  $-\ln L$  were nearly  
232 identical among replicates. The highest likelihood of those runs was retained. Statistical  
233 confidence in nodes was evaluated using 100 non-parametric bootstrap replicates (Felsenstein,  
234 1985) using the automated stopping criteria set at 10000 generations. More bootstrap  
235 replicates could not be performed because it would have required unpractical computing

236 times. Topology with  $\geq 70\%$  of the bootstrap trees were considered well supported (Hillis &  
237 Bull, 1993).

238 Bayesian analyses (MB) were carried out using Mr. Bayes 3.1.2 (Ronquist &  
239 Huelsenbeck, 2003). The model selection process was the same as that for ML analysis.  
240 Moreover, genes were analyzed individually and collectively. Five independent analyses were  
241 carried out for each gene and for the combined data (10 millions generations, four chains with  
242 mixed-models, default priors, saving trees every 100 generations). The analyses were stopped  
243 after checking convergence between runs using the average standard deviation of split  
244 frequencies and by plotting likelihood values across generations using Tracer 1.2 (Rambaut &  
245 Drummond, 2003). The first one million generations were discarded as burn-in. The  
246 phylogeny and posterior probabilities were then estimated from the remaining trees and a  
247 majority-rule 50% consensus tree was constructed. Topologies with posterior probabilities  $\geq$   
248 0.95 were considered well supported (Wilcox *et al.*, 2002).

#### 249 *Chemical Analyses*

250 The samples were analyzed using a gas chromatograph Shimadzu GC-2010 with a SLB-  
251 5ms non-polar capillary column (5% diphenyl/95% dimethyl siloxane; 30 m X 0.25 mm X  
252 0.25 $\mu$ m) and a flame ionization detector. A split less injector mode (220°C) and He carrier  
253 gas (50cm/s) were used. The temperature program of the column was 70°C for 2min;  
254 10°C/min to 320°C; 5min. Relative proportions in percentage of each compound were  
255 calculated by summing up the absolute amounts of all compounds using GCsolution Postrun.  
256 The data matrix was elaborated with the relative proportion of each compound for each  
257 individual. The composition of SMP was determined using a gas chromatograph - mass  
258 spectrometer (GC-MS) Finigan GCQ with a DB-5ms non-polar capillary column (5% phenyl  
259 (methyl) polysiloxane stationary phase; 30 m X 0.25 mm X 0.25 mm) and an ion trap in  
260 electron impact mode “full scan (300-600)”. A split less injector mode (220°C) and He carrier

261 gas (50cm/s) were used. The temperature program of the column was 70°C for 2min;  
262 10°C/min to 320°C; 5 min. Compounds were identified in Xcalibur™ using their mass spectra  
263 compared to those at National Institute of Standards and Technology library (NIST, U.S.A)  
264 using Nist MS Search 2.0.

265 Statistical analyses were performed in R (Quinn & Keough, 2006). Data were transformed  
266 and standardized. Clustering methods were used to detect divergence between taxa (Rasmont  
267 *et al.*, 2005). Four different association matrixes based on pheromonal data matrix (relative  
268 abundance of each compound) were computed: Euclidian, Pearson Phi Correlation, Chi-  
269 Square, and Manhattan. Three clustering methods were used for each association matrix:  
270 single, complete and UPGMA. Only one of these clusters is shown in the results (UPGMA  
271 cluster based on Phi Correlation matrix). In addition, a 2-group k-means method (MacQueen,  
272 1967) was performed (repeated 50.000 times) (De Meulemeester *et al.*, 2011) to test a  
273 putative split into two groups: *B. barbutellus* versus *B. maxillosus*.

#### 274 *Correlation analyses*

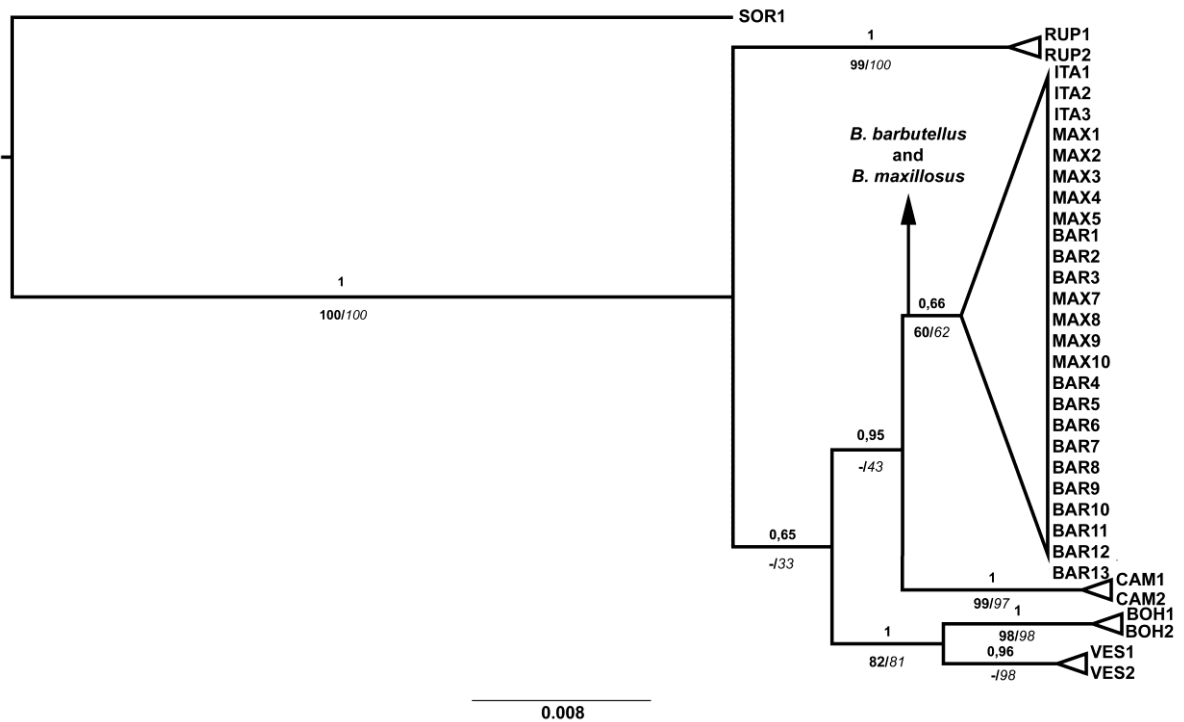
275 The spatial structuring of the chemical and genetic data set and a potential correlation  
276 between these two data sets were investigated by performing three Mantel tests (9999 random  
277 permutations) (Mantel, 1967) in R (Quinn & Keough, 2006): (1) geographical distance among  
278 population *versus* individual by individual correlation distance in relative amounts of SMP  
279 compounds, (2) geographical distance *versus* individual by individual genetic distance and (3)  
280 correlation distance of the SMP *versus* Genetic distance. The correlation distance matrix of  
281 SMP compounds was performed in R (Quinn & Keough, 2006). Genetic distances were  
282 calculated in Mega 4.0.1 (Tamura *et al.*, 2007) using the maximum composite likelihood  
283 (MCL). The MCL calculations used data for both transitions and transversions and assumed a  
284 heterogeneous pattern of sequence evolution with a gamma distribution of among-site rate  
285 variation (gamma parameter = 1.0). Gaps, missing data and individuals without all data set

286 were deleted from the analysis and standard errors were calculated with 1000 bootstrap  
287 replicates.

## 288 **Results**

### 289 *Phylogenetic analyses*

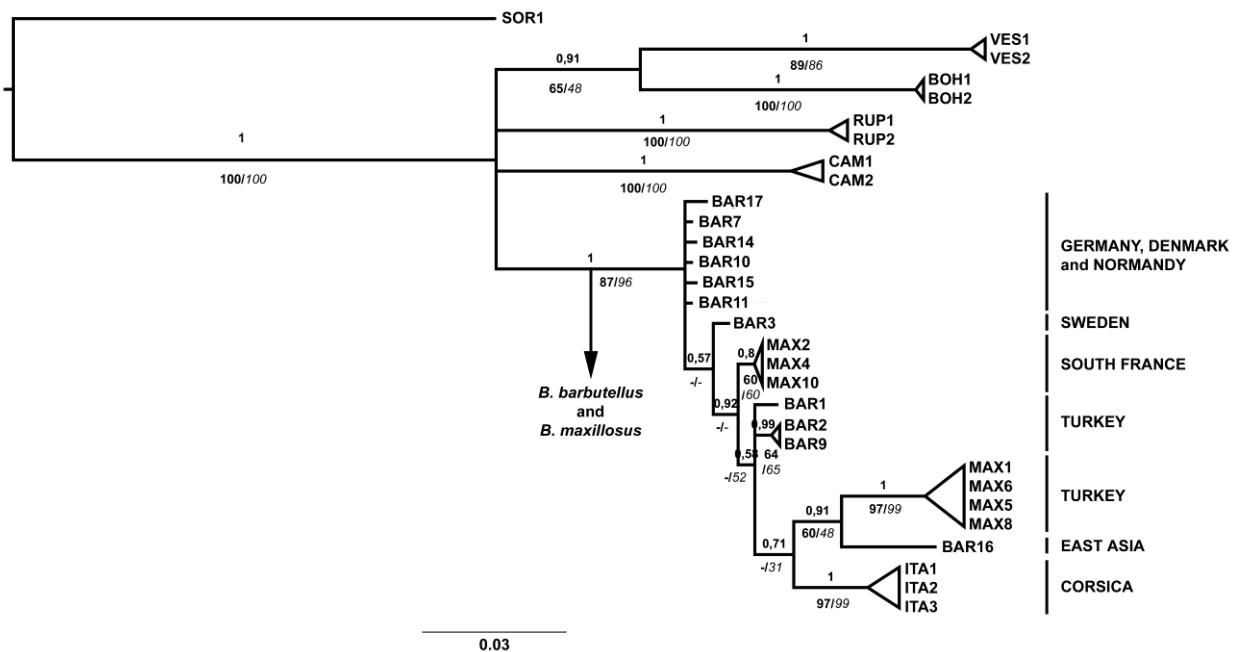
290 A total of 850 bp from the COI gene (145 parsimony informative sites), 773 nucleotides of  
291 EF-1 $\alpha$  F2 copy (24 parsimony informative sites), containing a ~200 bp intron and the  
292 combined sequences were compared to estimate the phylogenetic relationships. All analyses  
293 (MP, ML and MB) on each single gene and combined dataset present the same topology (Figs  
294 2, 3, 4) but higher branch support values were found in MB. Phylogenetic analyses based on  
295 EF-1 $\alpha$  partially recover all deep relationships among *Psithyrus* subgroups but failed to  
296 provide much sequence variation within and between *B. barbutellus* and *B. maxillosus*.  
297 Moreover there is not any divergence between *B. barbutellus* and *B. maxillosus* while a clear  
298 divergence appeared in the *Ashtonipsithyrus* species group (*B. bohemicus* and *B. vestalis*).  
299 COI tree topology failed to resolve deep relationships among *Psithyrus* subgroups but  
300 provided considerable sequence variation of *B. barbutellus* and *B. maxillosus*. As in the EF-1 $\alpha$   
301 analyses, *B. barbutellus* and *B. maxillosus* were not split into two groups. Nevertheless  
302 geographic groups appeared: A West-Central European group, a Southern French group, two  
303 Turkish groups and a Corsican group (Fig. 3). However branch support values are low in  
304 posterior probability but well supported in ML and MP. Combined genes analyses summarize  
305 the differing contributions of the two molecules at the two ends of the tree. These trees  
306 presented a similar topology and similar branch supports to EF-1 $\alpha$  for deep relationship  
307 between *Psithyrus* subgroups and to COI for the tips of the phylogeny (including COI  
308 geographic groups). In each of the analyses, therefore, there was an unambiguous support for  
309 monophyly of the *B. barbutellus* + *B. maxillosus* group.



310

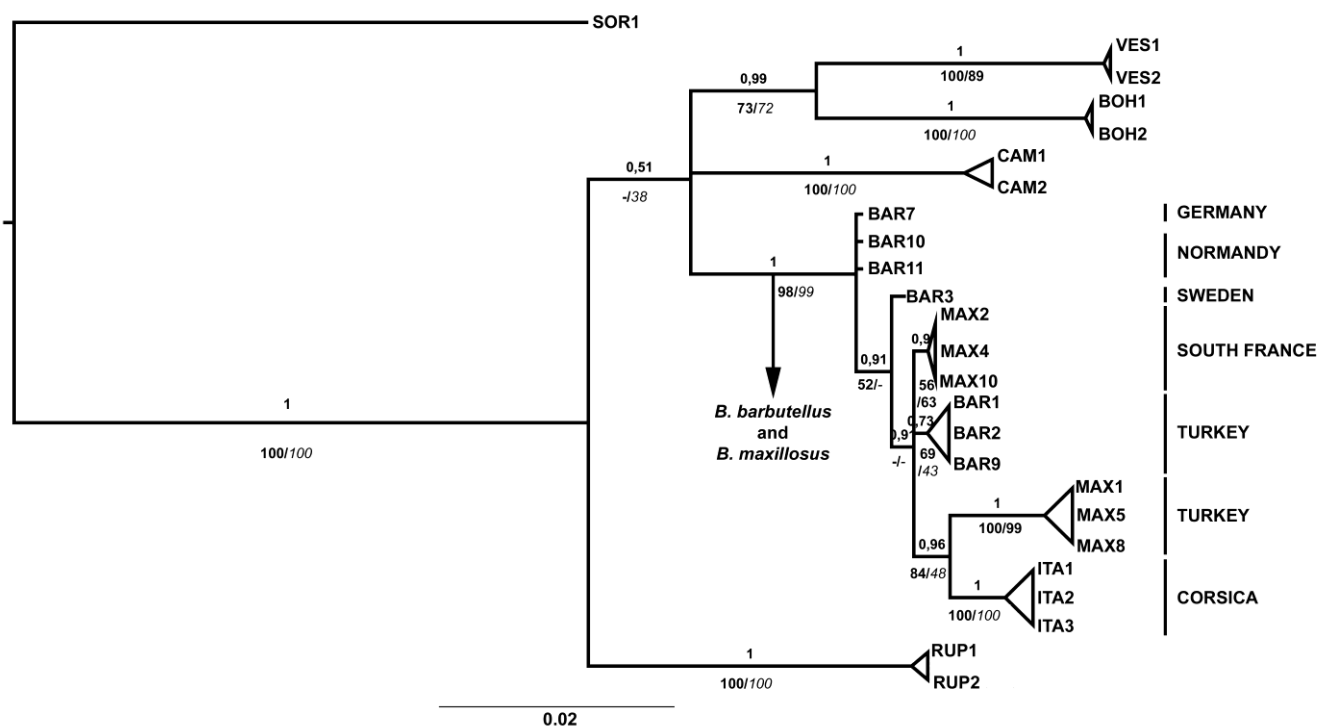
311 **Fig. 2.** Majority-rule (50%) consensus tree based on Bayesian analyses of elongation factor 1 $\alpha$  (*EF-1 $\alpha$* ) sequences; values above branches  
 312 are Bayesian posterior probabilities; values in bold below branches are maximum likelihood bootstrap values; values in *italic* below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

315



316

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



321

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

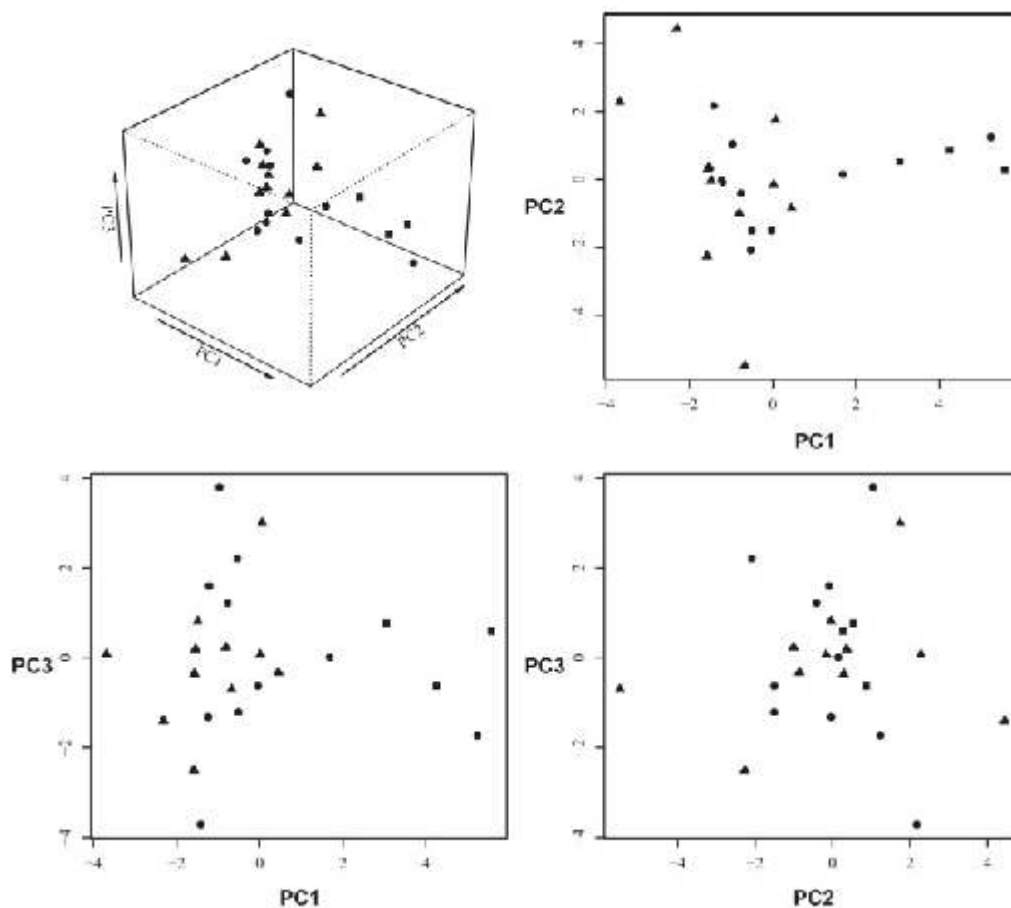
326

327 *Chemical analyses*

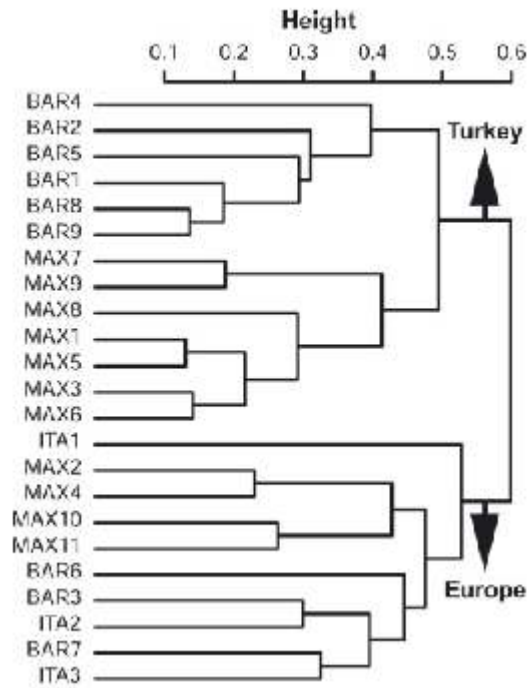
328 73 compounds were detected in SMP of *B. barbutellus* and *B. maxillosus* (Table 4). *B.*  
 329 *barbutellus* and *B. maxillosus* shared all compounds. No discrimination between *B.*  
 330 *barbutellus* and *B. maxillosus* was found in the statistical analyses (Figs 5, 6). PCA analysis  
 331 did not separate any group. However, all the clusters separated the samples into two main  
 332 groups; a Turkey cluster with nearly all the Turkish individuals and a European cluster with  
 333 all European samples and one Turkish specimen (Fig. 6). The two group k-means method  
 334 detected the same separation. Only slight quantitative and qualitative divergences in the minor  
 335 compounds separated the two groups. Examination of the wing condition (Tkalců, 1969) of  
 336 the Turkish individual grouped with the European group indicated that it is obviously an old  
 337 specimen. In some bumblebee species, old individuals, scarce in the wild, have a pheromonal  
 338 composition modification and are probably no longer attractive to females (Žaček *et al.*, 2009;



339 Coppée, 2010). In the Turkey group, *B. maxillosus* and *B. barbutellus* are separated (Fig. 6).  
340 A comparison between Turkish *B. barbutellus* and Turkish *B. maxillosus* compounds showed  
341 only slight differences in relative abundance.

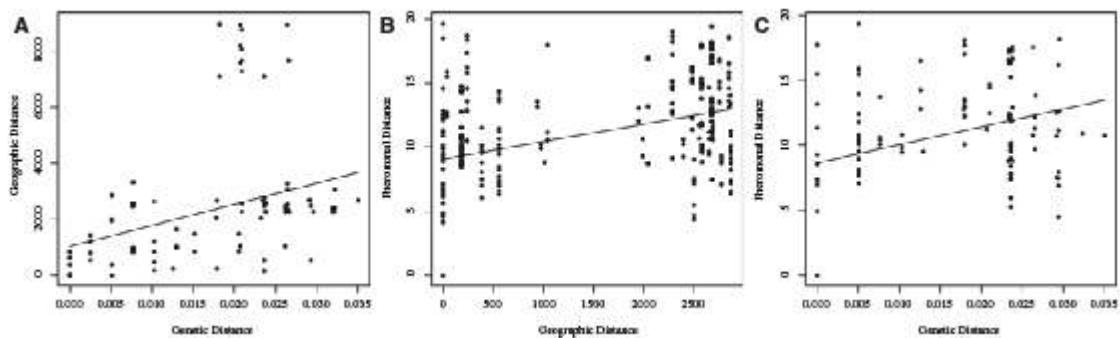


342  
343 **Fig. 5.** Three first axes of the principle components analysis (PCA) based on 20 compounds in 21 specimens of *Bombus barbutellus*,  
344 *Bombus maxillosus maxillosus* and *Bombus maxillosus italicus*; , *Bombus barbutellus*; , *Bombus maxillosus italicus*; , *Bombus maxillosus*  
345 *maxillosus*.  
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**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.



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**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  $r = 0.089$ ;  $P > 0.05$ ).

### Correlation analyses

358 The Mantel tests show a significant positive correlation between SMP dissimilarity and  
359 geographical distance (Mantel's  $r=0.298$ ,  $p\text{-value}<0.05$ ) and between genetic distance and  
360 geographical distance (Mantel's  $r=0.293$ ;  $p\text{-value}<0.05$ ) (Figs 7A, B). There is no correlation  
361 between SMP dissimilarity and genetic distance (Mantel's  $r=0.089$ ;  $p\text{-value}>0.05$ ) (Fig. 7C).

## Discussion

### Species status

364 The phylogenetic trees showed conflict with Cameron *et al.* (2007) and Hines (2008).  
365 Though these studies were not designed to deal with species problems, they showed genetic  
366 differences in EF-1 $\alpha$  sequence between *B. barbutellus* and *B. maxillosus*. However, these  
367 studies used only one Swedish *B. barbutellus* specimen and one Turkish *B. maxillosus*  
368 specimen. By performing a wider specimen sampling taking into consideration the  
369 intraspecific variability, we show that *B. barbutellus* or *B. maxillosus* respectively do not  
370 constitute monophyletic groups. Therefore they are not different species according to the  
371 Phylogenetic Species Concept (Baum, 1992).

372 Our results in SMP composition are similar to Urbanová *et al.* (2004). 58 compounds  
373 detected in GC-MS analysis were identical to the former study; 15 components with a relative  
374 abundance below 0.1% and four components with a relative abundance higher than 0.1%  
375 differed. Among these compounds only three were present in all individuals. However the  
376 differences observed with Urbanová *et al.* (2004) are of the same order of magnitude as the  
377 inter-population variation observed in our data set for *B. barbutellus*. Pheromonal analyses  
378 also support a lack of divergence between *B. barbutellus* and *B. maxillosus*. The comparison  
379 of SMP between the two taxa showed only some slight quantitative modifications and no  
380 qualitative modifications and supports their conspecificity according to the species  
381 recognition concept (Paterson, 1993). Indeed, the variation observed between *B. barbutellus*  
382 and *B. maxillosus* is lower than the intraspecific variation observed in *Bombus terrestris*  
383 (Coppée *et al.*, 2008) and even more so the variation between other bumblebees species  
384 (Bertch, 1997; Bertsch *et al.*, 2005; Rasmont *et al.*, 2005; Terzo *et al.*, 2005).

385 Our chemical and molecular analyses point towards conspecific status for *B. barbutellus*  
386 and *B. maxillosus* whatever the species concept considered. Moreover, these analyses do not  
387 support any separation of alternative species inside the *B. barbutellus* + *maxillosus* group.  
388 This result agrees with Grütte (1940). The taxonomic status proposed by Grütte (1940) was

389 criticized by Tkalčú (1969) according to wing darkness variations and host choice. Tkalčú  
390 (1969) considered wing darkness of all bees as a constant specific criterion unrelated to  
391 individual or geographic variations. However, according to our results, wing darkness is not a  
392 diagnostic species character of *B. barbutellus*. The relevance of the wing darkness as a species  
393 diagnostic character among bees, never demonstrated statistically from a large sample,  
394 therefore still needs to be proven. To summarize, according to the conspecificity shown in  
395 our results, the main morphological criteria (the hair length and the wings darkness) used to  
396 distinguish both taxa (Popov, 1931; Pittioni, 1939; May, 1942; May, 1943) is probably due to  
397 intraspecific variability. This could explain the numerous intermediate forms observed by  
398 Grütte (1940) and the large number of undeterminable individuals (Rasmont, 1988).

#### 399 *Intraspecific variability*

400 We found three colour patterns corresponding to the following subspecies (*sensus*  
401 Rasmont, 1988): *barbutellus*, *italicus*, and *maxillosus*. However colour patterns do not fit with  
402 any particular pheromonal clustering or phylogenetic tree. This could show that there is no  
403 clear subspecific subdivision with the Rasmont (1988) subspecies status but only two extreme  
404 forms: (1) a form with long hairs and hyaline wings (*barbutellus*) living in the North of  
405 Europe and in the Mediterranean mountains; (2) another form with short darker hairs and  
406 darkened wings (*maxillosus*) living mainly in the South of Europe and (3) between these two,  
407 there are intermediate forms; sometimes described as subspecies (Grütte, 1940). Similar  
408 patterns of variations have been observed in other bumblebees. On the one hand, hairs  
409 insulate the bumblebee bodies and decrease heat loss (Heinrich, 1975). Peat *et al.* (2005)  
410 showed that bumblebee species from hot regions exhibit shorter hairs than species from cold  
411 regions. They found the same geographic pattern at an intraspecific level for *B. terrestris*. On  
412 the other hand, melanism is also due to thermoregulation (Gloger, 1833). Hot region  
413 bumblebees are darker and temperate region bumblebees are paler (Williams, 2007). In

414 Scandinavia, Løken (1984) observed a slight tendency to a more pronounced melanism in the  
415 south of Sweden than in the north among *B. barbutellus*.

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

421 The slight SMP geographic variations mainly between Turkey and Europe are explained by  
422 the geographic distance. Mantel test shows that SMP distance is correlated to geographic  
423 distance like in other bees (Vereecken *et al.*, 2007; Coppée *et al.*, 2008; Lecocq *et al.*, 2009b).  
424 Among the Turkish group, the splitting into two forms (*barbutellus* forms and *maxillosus*  
425 forms) may be due to an interpopulational variation as observed in *B. terrestris* (Coppée  
426 2010) or more likely reflects sampling bias. Indeed, Turkish *B. barbutellus* samples were  
427 collected at the same time and from the same place. The likelihood that these individuals were  
428 related and thus have a nearly identical SMP composition is high. In this case, the clustering  
429 method separated them from other samples with no other biological reason than the kin  
430 relationship.

431 Even if we observed variations between *B. barbutellus* populations, currently, we do not  
432 know if these variations have an impact on the intraspecific sexual mating recognition among  
433 *B. barbutellus*. Ethological studies, difficult to perform among scarce species, would resolve  
434 this problem.

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

438 *Host relationship*

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

#### 447 *Impact on zoological nomenclature*

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

451 ? *Apis brachyptera* Villers in L., 1789: 331, *nomen oblitum*

452 ? *Apis monarcha* Christ, 1791: 131, *nomen oblitum*

453 ? *Apis cincta* Preysslner, 1793: 232, nec Fabricius, 1781, *nomen oblitum*

454 ? *Apis saltuum* Panzer, 1800-1801, part 75, Table 21, *nomen oblitum*.

455 *Apis barbutella* Kirby, 1802: 343, *nomen protectum*; locus typicus: England E.Suffolk  
456 Barham; lectotype ♀ Natural History Museum, London, designated by Yarrow, 1968. Revised  
457 by P. Rasmont 2010. Labels 1) handwritten "93♀"; 2) round blue pale label with glued right  
458 protarsus; 3) round with a black circle printed "Lectotype"; 4) handwritten "Apis barbutella  
459 Kirby 1802 LECTOTYPE I.H.H. Yarrow 1968; 5) printed and handwritten "B.M. TYPE  
460 HYM 17a 2996". The right fore tarsis is broken and glued to the second label. The 3 distal  
461 segments of the hind tarsus are missing. The coat colour pattern is typical to the northern form  
462 of *Bombus barbutellus*, but the wings are more infuscated than usual.

463 *Bombus maxillosus* Klug, 1817:269; locus typicus: "Süd Europa"; Holotype ♀ Zoologisches  
464 Museum, Berlin, see Tkalcù, 1969. Syn. N.

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

469 The oldest available name is *Apis barbutellus* Kirby; *Bombus maxillosus* Klug is therefore  
470 a subjective junior synonym. The valid name should be *Bombus (Psithyrus) barbutellus*  
471 (Kirby, 1802). Many names have been used for infraspecific colour forms, mainly by  
472 Kriechbaumer (1870), Popov (1931), Quilis-Pérez (1927), Müller (1936), Pittioni (1939) and  
473 May (1944). They are all considered here as synonyms of *Bombus barbutellus* (Kirby).

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

Female	<i>B. maxillosus</i>	<i>B. barbutellus</i>
<b>Morphology</b>		
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
<b>Colour pattern</b>		
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
<b>Male</b>		
<b>Morphology</b>		
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
<b>Colour pattern</b>		
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

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**Table 2.** Host/parasite relations in the *Allopsithyrus* group. Main hosts are in bold.(\*): Colonies in which *Psithyrus* offspring have been observed

<i>Allopsithyrus</i>	<i>Bombus</i> Host species	Main references
<i>B. barbutellus</i>	<b><i>B. (Megabombus) hortorum</i></b> *	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> *	Voveikov 1953; Pouvreau 1973
	<i>B. (Pyrobombus) jonellus</i>	Schenck 1859; Schmiedekneht 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>	<b><i>B. (Megabombus) ruderatus</i></b>	Vogt 1909 ; Popov 1931; Pittioni & Schmidt 1942; Rasmont 1988
	<i>B. (Megabombus) argillaceus</i>	Popov 1931; Skorikov 1922; Pittioni & Schmidt 1942; Pouvreau 1973
	<i>B. (Subterraneobombus) subterraneus</i>	Tkalčú 1969; Pouvreau 1973

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**Table 3.** *Allopsithyrus* and outgroup taxa examined, their collection locality, collector, vouchers numbers and GenBank accession numbers. # = number of samples; V.# = voucher numbers and sample codes used in trees and clustering; Ef-1 $\alpha$  = GenBank accession numbers for EF-1 $\alpha$  sequences; COI = GenBank accession numbers for COI sequences.

	Species	Subspecies	#	Locality	Lat	Long	Recorder	V.#	EF-1 $\alpha$	COI
<b>Ingroup</b>	<i>barbutellus</i>	-	7	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>	-	1	Sweden, Uppsala	59°51'15''N	17°39'14''E	P. Rasmont	BAR3	JF699171	JF699185
	<i>barbutellus</i>	-	1	Germany, Rottleberode	51°31'43''N	10°57'08''E	E. Stolle	BAR7	JF699172	JF699186
	<i>barbutellus</i>	-	4	France, Ablon	49°23'38''N	0°17'43''E	P. Lhomme	BAR10, BAR11, BAR12, BAR13	JF699170	JF699184
	<i>barbutellus</i>	-	1	Denmark, Ekkodalen	-	-	Pedersen (2002)	BAR14	-	AY181177.1
	<i>barbutellus</i>	-	1	Germany, Tüchersfeld	-	-	Pedersen (2002)	BAR15	-	AY181176.1
	<i>barbutellus</i>	-	1	Denmark	-	-	Pedersen (1996)	BAR17	-	L26574.1
	<i>barbutellus</i>	-	1	Russia, Primorsky	-	-	Tanaka <i>et al.</i> (unpublished)	BAR16	-	AF279576.1
	<i>maxillosus</i>	<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>	<i>maxillosus</i>	3	Turkey, Erzincan	39°52'06''N	39°33'56''E	T. De Meulemeester & P. Rasmont	MAX7, MAX8, MAX9	JF699179	JF699193
	<i>maxillosus</i>	<i>maxillosus</i>	2	France, Gonfaron	43°18'28''N	06°18'33''E	P. Rasmont	MAX2, MAX4	JF699178	JF699192
	<i>maxillosus</i>	<i>maxillosus</i>	1	France, Bargemon	43°38'22''N	06°34'07''E	P. Rasmont	MAX10, MAX11	JF699177	JF699191
	<i>maxillosus</i>	<i>italicus</i>	3	France, Evisa	42°17'25''N	08°52'40''E	T. Lecocq	ITA1, ITA2, IT3	JF699175	JF699189
<b>Outgroup</b>	<i>campestris</i>	<i>campestris</i>	2	France, Saint-Lyphard	47°23'52''N	02°18'24''W	G. Mahé	CAM1, CAM2	JF699174	JF699188
	<i>bohemicus</i>	<i>bohemicus</i>	2	France, Nyer	42°30'06''N	02°19'00''E	T. Lecocq	BOH1, BOH2	JF699173	JF699187
	<i>rupestris</i>	<i>rupestris</i>	2	Germany, Geiseltal	51°18'00''N	11°52'00''E	E. Stolle	RUP1, RUP2	JF699180	JF699194
	<i>vestalis</i>	<i>vestalis</i>	2	France, Paris	48°50'37''N	02°21'35''E	P. Rasmont	VES1, VES2	JF699182	JF699196
	<i>soroensis</i>	<i>lectitatus</i>	1	France, Nyer	42°30'09''N	02°19'12''E	T. Lecocq	SOR1	JF699181	JF699195

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**Table 4.** List of the identified compounds. 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 *B. barbutellus*, *B. maxillosus maxillosus* and *B. maxillosus italicus*. The undetermined compounds are indicated as Ux.

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 tetradecanoate	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
<b>Farnesyl acetate</b>	<b>264</b>	<b>10,21</b>	<b>11,35</b>	<b>16,58</b>	<b>20,98</b>	<b>37,89</b>	<b>0,19</b>	<b>20,84</b>	<b>25,53</b>	<b>28,37</b>	<b>45,4</b>
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
<b>Octadecenol</b>	<b>268</b>	<b>11,81</b>	<b>13,23</b>	<b>16,17</b>	<b>20,68</b>	<b>24,28</b>	<b>0,02</b>	<b>2,25</b>	<b>9,58</b>	<b>13,94</b>	<b>24,28</b>
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 I	350	0,02	0,04	0,07	0,1	0,23	0,01	0,03	0,05	0,09	3,62
Pentacosene 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
<b>1,3-Diacetyl-2-dodecanoylglycerol</b>	<b>358</b>	<b>8,72</b>	<b>16,83</b>	<b>25,11</b>	<b>26,86</b>	<b>31,11</b>	<b>0,96</b>	<b>10,85</b>	<b>19,02</b>	<b>24,08</b>	<b>31,11</b>



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
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