Eur. J. Entomol. **95**: 27–33, 1998
ISSN 1210-5759

Genetic variation among European populations of *Bombus pascuorum* (Hymenoptera: Apidae) from mitochondrial DNA sequence data

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***Bombus pascuorum*, bumble bee, genetic variation, Europe, mitochondrial DNA**

Abstract. Taxonomists have described chromatic and morphological variation for a number of European bumble bee species, although molecular studies have so far failed to find significant differentiation. We investigated the genetic variation of *Bombus pascuorum* from 13 locations by sequencing a fragment of the mitochondrial cytochrome b gene. We find considerably more variation than in published COII sequences for *B. terrestris*. The DNA sequences from these bees suggest that a difference exists between areas north and south of the Alps, and that the bees within these two areas are very similar over large areas. Therefore, mitochondrial cytochrome b sequences may be able to reveal geographic subdivision in *B. pascuorum* across the Alps.

INTRODUCTION

Scholl et al. (1990) and Estoup et al. (1996) did not find significant differentiation for the bumble bee *Bombus terrestris* (L.) on continental Europe with allozymes, mitochondrial DNA and microsatellite markers. Similarly, Pamilo et al. (1984) and Owen et al. (1992) did not find differentiation for other *Bombus* species with allozymes and concluded that *Bombus* is much less variable than other hymenopteran genera and other insects. However, taxonomists have described coat colour variation for *B. terrestris* and a number of other bumble bee species (Loken, 1973; Alford, 1975).

Apart from the chromatic differences, there is evidence for population subdivision among European bumble bee species. For example, British and Swedish specimens of *B. monticola* (Smith) differ from each other in metric characters, the Swedish specimens being closer to those from the Alps and the Pyrenees (Svensson, 1979). Differences between these populations indicate long and continued isolation (Pekkarinen & Teräs, 1993).

The bumble bee *B. pascuorum* (Scopoli), like *B. terrestris*, is widespread over much of Europe. Again, there is considerable coat colour variation (Krüger, 1928, 1931; Reinig, 1970; De Rijter & Wiebes, 1975; Rasmont, 1983). There is also differentiation between northern European populations in morphometric characters (Loken, 1973; Pekkarinen, 1979), but not allozymes (Pamilo et al., 1984). We investigated the genetic variation in *B. pascuorum* from 13 locations by sequencing a 400 base pair fragment of the mitochondrial cytochrome b gene. We use this approach because most species exhibit high levels of diversity in mtDNA (Wilson et al., 1985; Avise, 1986, 1989; Avise et al., 1987b; Moritz et al., 1987) and geographically separated haplotypes, given enough time, will sort into

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monophyletic lineages that occupy different branches of an intraspecific tree (Avise et al., 1987a; Riddle & Honeycutt, 1990; Bernatchez & Dobson, 1991).

MATERIAL AND METHODS

Queens and workers of the bumblebee *B. pascuorum* were sampled from 13 locations in Europe in the summer of 1994. These sites were in marginal areas of Europe (Tydal, Silwood Park and Jambon sur Lac), at both sides of the Alps (Viktorsberg, Oberplanken, Rothenfluh, Braunwald, Calpiogna, Lostallo and Monte Caslano), and in some alpine valleys (Mörel, Engadin and Aostatal). *B. humilis* (Illiger) from France, Austria, and northern Switzerland was used as an outgroup. The exact sampling localities are shown in Fig. 3. The caught bumble bees were frozen and kept at –80°C, or preserved in absolute ethanol, and kept at –20°C until used.

Individual DNA extractions were performed from the head according to the modified protocol of Garnery et al. (1991) as follows. The head of each individual was frozen in liquid nitrogen and macerated. To this, we added 600 µl of extraction buffer (Garnery et al., 1991) and 14 µl 10 mg/ml proteinase k. The samples were digested for 2 h at 50°C, and then centrifuged for 15 min at 10,000 g at 4°C. The supernatant was purified by phenol/chloroform extraction. The DNA was precipitated with the addition of 20 µl of 3 M NaAc and 400 µl ethanol. The DNA was pelleted, dried, and then resuspended in 50 µl H_2O.

A 433 base pair fragment of the cytochrome b gene was amplified from these bees using primers designed from the known *Apis* sequence (co-ordinates 11,400–11,425 and 11,859–11,884) in Crozier & Crozier (1993) and Taq polymerase (Stehelin and Cie AG) in a Perkin Elmer Cetus thermal cycler for 40 cycles under the following conditions: denaturation at 92°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. All products were purified with Ultrafree-MC 30,000 NMWL filter units (Millipore).

One individual per site was sequenced from single-stranded PCR product by modification of standard procedures (Kessing et al., 1989; Koulianos & Crozier, 1991) using the Sequenase version 2.0 kit (United States Biochemical Corporation).

Sequence information was entered using DNA Strider TM 1.2 (Marck, 1988). Nucleotide diversity (π), which can be considered as a measure of heterozygosity at the nucleotide level (Nei, 1987), was estimated using the index of Nei & Tajima (1981). Distances (d) were estimated using Kimura's (1980) method. Phylogenetic analyses were carried out using the maximum likelihood method implemented using the program DNAML in PHYLIP 3.57c (Felsenstein, 1995) and maximum parsimony using PAUP 3.1.1 (Swofford, 1993). Maximum likelihood trees were obtained by searching for the best tree with global rearrangements. Parsimony trees were obtained by the branch and bound method. Support for the nodes was estimated using 10,000 bootstrap replications. *B. humilis* was used as the outgroup. The transition/transversion ratio used for both methods was 2.1, as obtained by maximising the DNAML results.

RESULTS

Diversity and phylogenetic differentiation

A total of 400 base pairs were sequenced for *B. pascuorum* and *B. humilis* (Fig. 1). From the 13 locations, 6 different haplotypes were found for *B. pascuorum* (GenBank accession numbers AF017511-AF017516). The pairwise sequence differences among these haplotypes were between 0.24 and 1.46% [mean = 0.80% ± 0.44 (SD)]. Haplotype 1 was the most common and found at 6 of the 13 sites sampled, haplotype 2 at 3 sites, and haplotypes 3 to 6 each at one site. The nucleotide diversity (Nei & Tajima, 1981) for haplotypes (weighted by their relative frequencies) was equal to 0.006. This is considerably higher than that for haplotypes found north and south of the Alps (0.001 in both cases). In addition, only one haplotype was found for *B. humilis* (GenBank accession number AF017517).

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Haplotype 1 (6)  AATAGTAATTACAAATTTAATTTCAGCATTACCTTATATTGGTCAATTTACAGTTG
Haplotype 2 (3)  ........................................................
Haplotype 3 (1)  ........................................................
Haplotype 4 (1)  ........................................................
Haplotype 5 (1)  ........................................................
Haplotype 6 (1)  ........................................................
B. humilis  (3)  .........C..............................................

Haplotype 1  AATGAATTTGAGGAGGATTTTCTATTAATAATGATACATTAAATCGATTTTATTCATTTC
Haplotype 2  .................................................C..........
Haplotype 3  .................................................C..........
Haplotype 4  .............G..............................................
Haplotype 5  ............................................................
Haplotype 6  ............................................................
B. humilis   ............................................................

Haplotype 1  ATTTTATTTTACCATTTATTATTCTTATATTAGTATTTATTCATTTAATAATTTTACATA
Haplotype 2  ..................................................G.........
Haplotype 3  ..................................................G.........
Haplotype 4  ............................................................
Haplotype 5  ............................................................
Haplotype 6  ............................................................
B. humilis   ..................................T..............G..........

Haplotype 1  TTACAGGTTCTTCTAATCCTATACATTCAAAAATAAATATTTATAAAATTAATTTTCATC
Haplotype 2  ...................................G........................
Haplotype 3  ...................................G........................
Haplotype 4  ............................................................
Haplotype 5  ...................................G........................
Haplotype 6  ............................................................
B. humilis   .A...........A........T.....................................

Haplotype 1  CTTATTTTACAATTAAAGATTTAATTACAATAATTTTAACATTTTCAATATTTATAATTA
Haplotype 2  ............................................................
Haplotype 3  ...........................................................G
Haplotype 4  ............................................................
Haplotype 5  ............................................................
Haplotype 6  ............................................................
B. humilis   ...............................T....T.......................

Haplotype 1  TTAATTTACAGTTTCCGTATGTTTTAGGAGATCCAGATAATTTTAAAATAGCAAATTCAA
Haplotype 2  ....................A.......................................
Haplotype 3  ....................A.......................................
Haplotype 4  ...................................................?........
Haplotype 5  ....................A.......................................
Haplotype 6  ............................................................
B. humilis   .....C.T..A.....A...A.....................................T.

Haplotype 1  TAATTACTCCAATTCATATTAAACCAGAATGATATTTTTTATTT
Haplotype 2  ........................................?...
Haplotype 3  ............................................
Haplotype 4  ........................?...................
Haplotype 5  ............................................
Haplotype 6  .............................A..............
B. humilis   ............................................
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Fig. 1. Nucleotide sequences (400 base pairs) from the cytochrome b gene for the six *B. pascuorum* haplotypes and *B. humilis*. Common bases represented as dots, missing bases as question marks, and numbers in brackets are the number of bees sequenced that have this haplotype.

Both maximum likelihood and maximum parsimony analyses reveal 3 groups (Fig. 2). Groups A and B are separated by an average sequence divergence of 1.3%, compared to 0.33% within A and 0.25% within B (Table 1). Haplotype 5 belongs to a third group (C) which is equally divergent from group A (0.67%) and group B (0.62%) (see Table 1).

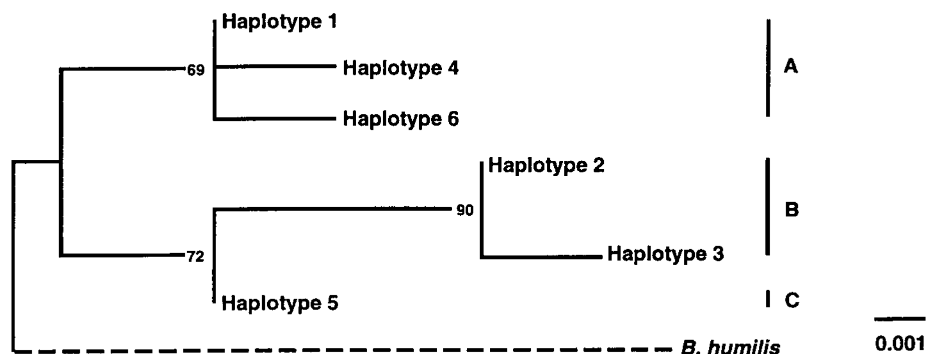

Fig. 2. The relationship of the six haplotypes to each other. The tree shown is that obtained using both maximum likelihood and parsimony, and is rooted with *B. humilis*. The branch lengths were obtained by maximum likelihood. The numbers at nodes are bootstrap percentages (10,000 replicates). A consists of haplotypes 1, 4 and 6, B of haplotypes 2 and 3, and C of haplotype 5.

TABLE 1. Pairwise distances calculated using Kimura's (1980) two parameter method.

	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 6
Haplotype 1	0.0101	0.0126	0.0025	0.0050	0.0025
Haplotype 2	–	0.0025	0.0127	0.0050	0.0126
Haplotype 3		–	0.0152	0.0075	0.0151
Haplotype 4			–	0.0076	0.0050
Haplotype 5				–	0.0075

Geographic distribution of mtDNA haplotypes

Group A contains haplotypes 1, 4, and 6. These are found in bees from sites north of the Alps, that is from central France (Jambon sur Lac), northern Switzerland (Braunwald and Oberplanken), Austria (Viktorsberg), England (Silwood Park), southern Sweden (Tydal), and two central alpine valleys with connections to the north and northeast (Mörel and the Engadin, respectively). Group B contains haplotypes 2 and 3 found in sites south of the Alps. Haplotype 2 is found in bees from three sites in the Ticino (Calpiogna, Lostallo and Monte Caslano) in southern Switzerland. Haplotype 3 is found in the Aostatal, another alpine valley that opens to the South. Group C (haplotype 5) is found at Rothenfluh in northern Switzerland. The geographic distribution of mtDNA haplotypes is illustrated in Fig. 3.

DISCUSSION

We have examined bumble bees of the species *B. pascuorum* from several parts of Europe, mainly from different sites close to the Alps. We found considerably more cytochrome b sequence variation than did Estoup et al. (1996) in COII sequences for 13 *B. terrestris* individuals (only 3 *B. terrestris* haplotypes were found with a nucleotide diversity equal to 0.002). This is interesting, as so far insect cytochrome b and COII genes have not been shown to differ significantly in evolutionary rate (Crozier & Crozier, 1993). Further, the nucleotide diversity we found for *B. pascuorum* is even higher than that found for

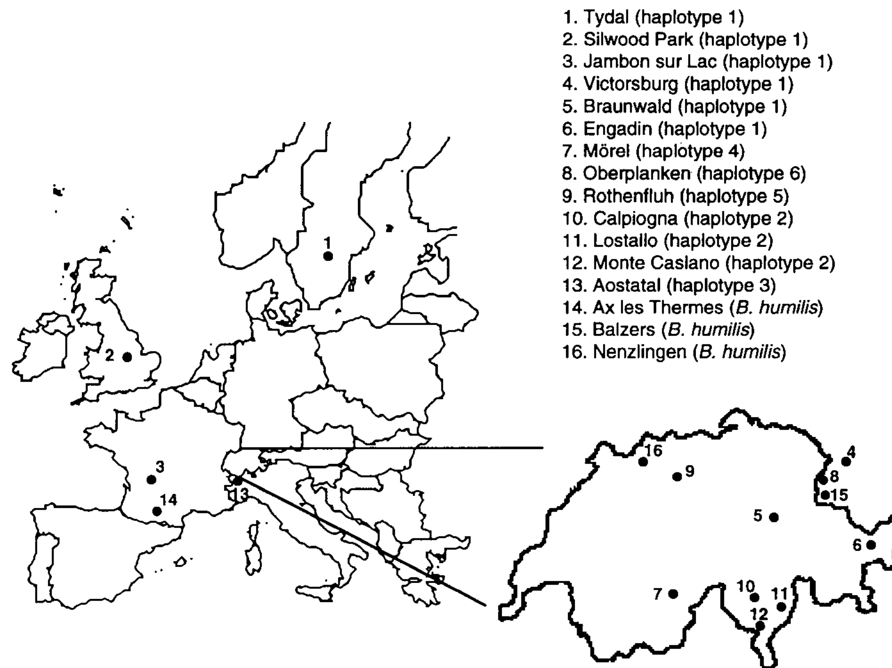

Fig. 3. Map showing the locations from where *B. pascuorum* and *B. humilis* were sampled and the corresponding haplotype designation.

honey bee mitochondrial haplotypes originating from different subspecies (Koulianos & Crozier, 1997).

The DNA sequences suggest that a difference exists in *B. pascuorum* from areas north and south of the Alps, and that the bees within these two areas are very similar over large areas. The sample size is not very large, but nevertheless the results suggest that mitochondrial cytochrome b sequences can reveal geographic subdivision in these bees across the Alps and so are an appropriate tool for possible later investigations. Therefore, there could be a difference in the population structure of *B. terrestris* and *B. pascuorum* which both are sympatric, widespread, and locally common. One explanation for this could be that the queens of *B. terrestris* are much larger and capable of long-distance flights (Estoup et al., 1996) and so it may be possible that they cross or circumvent the Alps. The Alps have before proven to be a significant barrier to gene flow to honey bees (Garnery et al., 1992) and other flying insects (Cooper et al., 1995).

It has always been suspected that the glaciation events of the Pleistocene played a role in the evolution of the *B. pascuorum* colour morphs (Rasmont, 1983; Reinig & Rasmont, 1983). Theoretically, time since divergence (t) can be calculated from t = d/r, where r is the rate of divergence between 2 sequences (Nei, 1987). Using a conservative rate of 2% per Myr. which has been estimated for mammals (Brown et al., 1979) and *Drosophila* (Desalle et al., 1987; Monnerot et al., 1990), the time of divergence of groups A and B is 500,000–760,000 years ago. This period correlates with the first major glaciation event of

the Middle Pleistocene, the Günz glaciation. During the height of the glaciation it was only possible for this species to survive in refugia on the Iberian, Italian and Balkan peninsulas. Then the most likely origin of the *B. pascuorum* haplotypes in northern Europe (group A) is from refugia on the Balkan peninsula and of the southern alpine haplotypes (group B) from refugia on the Italian peninsula. Retreat to, and expansion from, these refugia may well have been repeated in the subsequent glaciation events.

ACKNOWLEDGEMENTS. We thank A. Widmer for help with collecting bees, E. Benelli for the identification of these bees, H. Schwarz for comments on an earlier version of the manuscript, and the Swiss National Science Foundation for grant support to PSH.

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Received February 6, 1997; accepted August 18, 1997