Phylogenetic Relationships of the Bumblebee Subgenus *Pyrobombus* (Hymenoptera: Apidae) Inferred from Mitochondrial Cytochrome B and Cytochrome Oxidase I Sequences

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ABSTRACT Traditionally, the genus *Bombus* (Apidae: Apinae: Bombini) is divided into several subgenera. This study derives an approximation of the relationships between 10 species of the subgenus *Pyrobombus* (from both Europe and North America), *B. lapidarius* L. and *B. sichelii* Radoszkowski from the subgenus *Melanobombus,* and *B. terrestris* L. from the subgenus *Bombus s. str.,* by comparing the mitochondrial cytochrome b and cytochrome oxidase I (COI) genes. Although bootstrap values for deep branches are mostly low, the sequences show significant phylogenetic signal, low homoplasy, and all trees share some patterns that are consistent with those from other phylogenetic studies on bumblebees. These results show that the subgeneric names *Pyrobombus* and *Melanobombus* do not accurately reflect phylogeny, and therefore it would seem wise to revise the existing system into monophyletic species-groups or even ignore the subgeneric names altogether.

KEY WORDS *Pyrobombus,* bumblebees, cytochrome b, cytochrome oxidase I, mitochondrial DNA, phylogeny

BUMBLEBEES OF THE subgenus *Pyrobombus* (Apidae: Apinae: Bombini: *Bombus*) are Holarctic. *Pyrobombus* is the largest subgenus of *Bombus,* comprising of >40 species (Williams 1994), and accounts for almost half of the North American fauna. Like all bumblebees, species of this subgenus are morphologically very similar (Williams 1985, 1994), and this has resulted in much confusion about species definitions because they are also highly polymorphic with respect to coat color (Scholl et al. 1995). *Pyrobombus,* however, has been found to be heterogeneous with respect to enzymes (Pekkarinen et al. 1979, Pamilo et al. 1987, Scholl et al. 1995) and male marking pheromones (Svensson 1979).

The close relationship of *Pyrobombus* to the subgenus *Melanobombus* has been established by both morphological (Plowright and Stephen 1973) and molecular (Pamilo et al. 1987, Pedersen 1996) studies. *Pyrobombus* has been found to be paraphyletic by an extensive morphological study using wing venation by Plowright and Stephen (1973) and a molecular study, which only included 2 *Pyrobombus* species, by Pedersen (1996). These 2 studies show a close association between *B. lapidarius* L. (*Melanobombus*) and the *Pyrobombus* species *B. bimaculatus* Cresson and *B. pratorum* L. There is, however, evidence to the contrary (i.e., no association between *B. pratorum* and *B. lapidarius*) from the morphology of the penis valve (Williams 1985). Plowright and Stephens's (1973) study also separated 6 species from the main *Pyrobombus* cluster and grouped these with species belonging to *Bombus s. str.*

The current study provides a comparison of the mitochondrial cytochrome b and cytochrome oxidase I (COI) genes from 10 *Pyrobombus* species (from both Europe and North America), *B. lapidarius* and *B. sichelii* Radoszkowski from the subgenus *Melanobombus,* and *B. terrestris* L. from the subgenus *Bombus s. str.,* to derive an approximation of the relationships among these species. *Tetragona dorsalis* Friese (Apidae: Apinae: Meliponini), identified as the sister group to the Bombini by several independent studies (Cameron 1991, 1993; Sheppard and McPheron 1991; Koulianos et al. 1999), and *Apis mellifera* L. (Apidae: Apinae: Apini) (Crozier and Crozier 1993) were used as outgroups.

Materials and Methods

Total DNA was extracted from part of the thorax or from 2 legs of single individuals of 10 *Pyrobombus* species (Table 1), *B. lapidarius, B. sichelii,* and the outgroup *T. dorsalis* using a modification of the protocol of Edwards and Hoy (1993), as in Koulianos et al. (1999).

A 716-bp fragment of the cytochrome b gene was amplified from these bees (excluding *T. dorsalis,* for which the sequence was already available, see Table 1) using 2 oligonucleotide primers [CATTATT(G/A)TCC(T/A)AATATTGA(A/T)ATTGC and ATTA CACCTCCTAATTTATTAGGAAT] (5′ ends corresponding to positions 11168 and 11884, respectively, in Crozier and Crozier [1993]), and *Taq* polymerase (Promega, Wallisellen, Switzerland) in a Perkin Elmer (Rotkreuz, Switzerland) Cetus thermal cycler for 40

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Table 1. List of taxa used

Pyrobombus	*Bombus lapponicus* (F.)
	B. jonellus (Kirby)
	B. hypnorum (L.)
	B. pratorum (L.)
	B. monticola Smith (COI only)
	B. frigidus (Smith) (cyt b only)
	B. bifarius (Cresson)
	B. ternarius (Say)
	B. flavifrons (Cresson)
	B. huntii (Greene) (COI only)
Melanobombus	*B. lapidarius* (L.)
	B. sichelii Radoszkowski
Bombus s. str.	*B. terrestris* (L.)
Outgroups	*Tetragona dorsalis ziegleri* (Friese)
	Apis mellifera (L.)

Apis mellifera sequences are from Crozier and Crozier (1993) (GenBank accession #L06178); *B. hypnorum*, *B. terrestris*, and *B. lapidarius* COI sequences are from Pedersen (1996) (GenBank accession #L26569-70, 73); and *B. terrestris* and *T. dorsalis* cytochrome b sequences are from Koulianos et al. (in press) (GenBank accession #AF002721, 25).

cycles under the following conditions: denaturation at 92°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 1 min. A 521-bp fragment of the COI gene was also amplified from these bees (excluding those already sequenced by Pedersen [1996]; see Table 1 again) using primers AP-L-2176 and AP-H-2650, and conditions in Pedersen (1996). All products were purified using the Wizard polymerase chain reaction (PCR) Preps DNA Purification System (Promega).

One individual per species was sequenced in both directions from the purified double-stranded PCR product, by Microsynth GmbH (Bulgach, Switzerland).

Sequence information was aligned by eye. Divergence between bumblebee species (d) was estimated by the method of Tajima and Nei (1984). Parsimony analyses were carried out separately on the cytochrome b and COI sequences, as well as on the combined sequences using PAUP 4.0 d64 (Swofford, personal communication) and a branch and bound search. Support for the nodes was estimated by 1,000 bootstrap replications using Seqboot, Dnapars, and Consense in PHYLIP 3.57c (Felsenstein 1995). Maximum likelihood analyses were performed using PAUP 4.0 d64 and corrected with an estimated shape parameter of the gamma distribution.

For each of the data partitions, PAUP 4.0 d64 was used to evaluate the strength of the phylogenetic signal by performing skewness tests (Hillis and Huelsenbeck 1992) using 10,000 randomly selected trees, and to assess the impact of character homoplasy by reweighting characters based on their rescaled consistency indices and reanalyzing them with parsimony.

All voucher specimens are kept at Experimental Ecology, ETH Zürich, Switzerland.

Results and Discussion

Five hundred and eighty-six base pairs from the cytochrome b gene, 333 bp from the COI gene, and the combined sequences were compared with estimate the phylogenetic relationships among the species included in this study and to assess the phylogenetic informativeness of these sequences. All new sequences have been deposited in GenBank and are accessible by the numbers AF066969–72, 84–85, 89, 91, AF077918–19, and AF084908–17.

Table 2. Pairwise distances calculated according to Tajima and Nei (1984) for the combined cytochrome b and COI sequences

	2	3	4	5	6	7	8	9	10
1	0.14	0.12	0.11	0.12	0.11	0.14	0.12	0.12	0.13
2	—	0.11	0.12	0.11	0.10	0.11	0.11	0.12	0.13
3		—	0.07	0.08	0.08	0.10	0.09	0.09	0.12
4			—	0.07	0.07	0.09	0.07	0.09	0.11
5				—	0.07	0.11	0.08	0.09	0.12
6					—	0.09	0.06	0.08	0.10
7						—	0.09	0.10	0.13
8							—	0.09	0.12
9								—	0.12

1, *B. terrestris*; 2, *B. lapidarius*; 3, *B. sichelii*; 4, *B. jonellus*; 5, *B. hypnorum*; 6, *B. lapponicus*; 7, *B. pratorum*; 8, *B. ternarius*; 9, *B. flavifrons*; 10, *B. bifarius*.

Pairwise distances from the combined sequences ranged from 0.06 to 0.14 (Table 2). The distance between *B. sichelii* and the *Pyrobombus* species *B. jonellus* Kirby, *B. hypnorum* L., and *B. lapponicus* F. was as low or lower than comparisons within the *Pyrobombus* subgenus. In general, the largest distances were obtained by comparing *B. terrestris*, *B. lapidarius*, and *B. bifarius* Cresson to each other and any other species. The distance between *B. lapponicus* and *B. monticola* Smith calculated from the COI sequences alone was 0.006, >10-fold smaller than all other comparisons. This suggests that *B. monticola* and *B. lapponicus* are not 2 separate species, contrary to evidence from morphology, behavior, and male marking pheromones (Svensson 1979).

Parsimony analysis of the cytochrome b gene yielded 133 informative characters, 2 most-parsimonious trees, a skewness value of $g_1 = -1.26$ ($P < 0.01$), and a homoplasy index of 0.08. Analysis of the COI gene yielded 51 informative characters, 12 most-parsimonious trees, a skewness value of $g_1 = -0.44$ ($P < 0.01$), and a homoplasy index of 0.06. Analysis of the combined sequences yielded 175 informative characters, 1 most-parsimonious tree, a skewness value of $g_1 = -1.32$ ($P < 0.01$), and a homoplasy index of 0.05.

The skewness values show that the cytochrome b, COI, and combined sequences all contain highly significant nonrandom structure which is likely to reflect phylogenetic signal. The topology based on the analysis of the COI region showed the lowest average bootstrap values and the lowest homplasy index, which is consistent with the findings of Sanderson and Donoghue (1996) and Kuzoff et al. (1998) that homoplasy indices are not inversely proportional to average bootstrap values.

The parsimony trees obtained from the analyses are shown in Fig. 1. There are some differences in the topologies obtained from the 3 data partitions, however, the trees also share some patterns. For example,

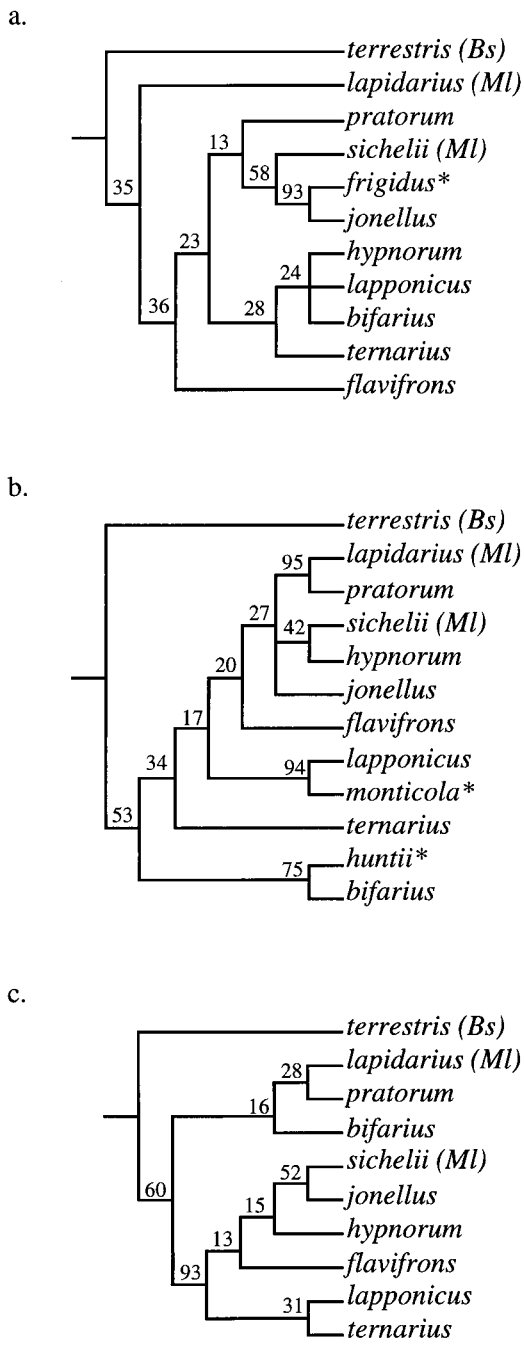

Fig. 1. Relationship of the 10 *Pyrobombus* species to each other. Trees a-c were obtained by parsimony analysis of the cytochrome b, COI and combined sequences, respectively. Trees a and b are consensus trees. Numbers at nodes are bootstrap percentages (1,000 replicates). Bs and Ml represent the subgenera *Bombus s. str.* and *Melanobombus*, respectively. All other taxa are from the subgenus *Pyrobombus.* *, Represents species for which only 1 gene region was sequenced and therefore are missing from tree c. Maximum likelihood trees did not differ significantly from the parsimony trees for the same data partition.

in all trees *B. terrestris* is basal to all other bumblebees included in the analysis. In 2 of the 3 trees (Fig. 1 b and c), *B. lapidarius* clusters with *B. pratorum.* Finally, in all trees *B. sichelii* clusters within the *Pyrobombus* species than with *B. lapidarius*, which is currently in the same subgenus.

Many patterns shown here are consistent with those from other phylogenetic studies on bumblebees (e.g., Plowright and Stephen 1973, Scholl et al. 1995, Pedersen 1996). Scholl et al. (1995) screened 18 enzymes and found a close association between *B. jonellus* from Europe and *B. frigidus* Smith from North America to the exclusion of *B. pratorum* also from Europe. Both parsimony and maximum likelihood analyses of the cytochrome b gene also cluster *B. jonellus* with *B. frigidus* to the exclusion of the other European *Pyrobombus* species present. The grouping of *B. bifarius*, *B. ternarius* Say, and *B. huntii* Greene, based on brood-rearing, colony-development characteristics, and wing venation (Plowright and Stephen 1973), is only partially supported by both analyses of the COI region, which includes all 3 species. In Fig. 1b, *B. bifarius* clusters with *B. huntii*, and *B. ternarius* is the sister group of all the other *Pyrobombus* species and both *Melanobombus* species. In Fig. 1c, *B. ternarius* clusters with *B. lapponicus* to the exclusion of *B. bifarius.* The close relationship between *B. lapidarius* and *B. pratorum* is well supported from Fig. 1b and is consistent with the results of Pedersen (1996).

The trees inferred from the cytochrome b and COI genes by both parsimony and maximum likelihood methods show that the subgeneric names *Pyrobombus* and *Melanobombus* do not accurately reflect phylogeny. One solution would be to include both *B. lapidarius* and *B. sichelii* within the subgenus *Pyrobombus.* However, it would be necessary also to reappraise the remaining species within *Melanobombus.* Because other subgenera (e.g., *Mendacibombus*, *Sibiricobombus*) (Williams 1985, 1994) and *Fervidobombus* (S.K., unpublished data) have also been found to be paraphyletic or even polyphyletic, it would seem wise to revise the existing system into monophyletic species-groups or even to ignore the subgeneric names altogether (Williams 1994).

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