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Phylogeny of bumble bees in the New World subgenus *Fervidobombus* (Hymenoptera: Apidae): congruence of molecular and morphological data

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Abstract

We present new DNA sequence data (12S, 16S, and opsin gene fragments) and morphological characters of the male genitalia for a phylogenetic analysis of the bumble bee subgenus *Fervidobombus*. There is no significant incongruence between the three molecular data sets, and little incongruence between the DNA and morphology. Simultaneous analysis of all the data partitions resulted in a tree that was entirely congruent with the All-DNA tree. Optimization of the geographic locations of the taxa onto the tree topology using dispersal/vicariance analysis suggests a complex picture of spread and diversification of *Fervidobombus* from the Old World into the southern New World. There is a phylogenetic component to their spread into tropical rain forest, as the two primary rain forest species (*Bombus transversalis* and *Bombus pullatus*) comprise a monophyletic clade, along with a third species, *Bombus atratus*, which is widely distributed in South America, including lowland subtropical habitats.

Keywords: Bombini; Molecular systematics; Morphology; Social insects; Neotropical; Biogeography; Dispersal/vicariance; DIVA

1. Introduction

Bumble bees (genus *Bombus*) are large, colorful, ubiquitous pollinators found throughout the Holarctic, Oriental, and Neotropical regions of the world, especially in alpine and arctic zones. Their greatest species diversity occurs in the mountainous regions bordering Tibet and in the mountains of Central Asia (Williams, 1998). However, bumble bees of the subgenus *Fervidobombus* are unusual in their widespread occurrence in lowland grasslands and tropical regions of South America (Milliron, 1973). They are the only early-diverging (Williams, 1994) large subgenus of bumble bees restricted to the New World. Four putative species occur in North America (Franklin, 1912; Mitchell, 1962; Thorp et al., 1983), but the majority of taxa are found to the south, from central Mexico through South America as far as the southern tip of Chile (Franklin, 1913; Labougle, 1990; Milliron, 1973; map in Williams, 1998). Of the 22 described species of *Fervidobombus*, none are exclusively alpine, although three taxa can be found at higher altitudes (*Bombus excellens*, *Bombus weisi*, and *Bombus digressus*). In fact, many species are of lowland Neotropical distribution (Milliron, 1973), including one (*Bombus transversalis*) found in lowland tropical rain forest of the Amazon Basin (Cameron et al., 1999; Milliron, 1973; Moure and Sakagami, 1962; Taylor and Cameron, 2003).

While all bumble bees are eusocial (or inquilines), with overlapping generations in a single nest and reproductive division of labor (Cameron, 1989; Michener, 1974), some *Fervidobombus* exhibit additional social characteristics (not usually found in bumble bees) more typical of the highly eusocial honey bees and stingless bees. These include large colonies of up to several thousand individuals with unusually well-developed defense systems (Cameron et al., 1999; Dias, 1958; Michener and LaBerge, 1954), and perennial colonies

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that can persist for many years (Sakagami and Zucchi, 1965; Taylor and Cameron, 2003; Zucchi, 1973). Some species even show behavioral novelties unseen in other bees, such as the fluctuating cycles of polygyny and monogyny found in *Bombus atratus* (Cameron and Jost, 1998; Zucchi, 1973) and the clearing of trails by means of cutting and removing leaves from the forest floor, as reported in the Amazonian bumble bee (Cameron and Whitfield, 1996; Cameron et al., 1999; Taylor and Cameron, 2003).

The closest subgeneric relatives of *Fervidobombus* (*Thoracobombus* and *Mucidobombus*) are entirely Old World (Williams, 1994, 1998). Under a simple model of colonization across the Bering Straits into North America, with subsequent divergence and dispersal in the New World from North America into South America, the cladistic structure of *Fervidobombus* should reflect this biogeographic pattern. A perfect fit to this model would reveal the basal clades to be North American, the intermediately positioned clades to inhabit Central America, and the most derived clades to be South American.

Tracing the above-mentioned behavioral novelties and biogeographic origins of this New World *Bombus* group is contingent upon a well-supported estimate of phylogeny. None has been available. Although the monophyly of *Fervidobombus* is supported from a morphological study (Williams, 1985), this has been questioned in a preliminary molecular study of several *Bombus* subgenera (Koulianos and Schmid-Hempel, 2000). More importantly, we know little about species relationships within *Fervidobombus*. Species designations of the four Nearctic (mostly North American) taxa have been controversial because of different opinions regarding putative color intergradation between species pairs in areas of sympatry. Specifically, *Bombus pennsylvanicus* and *Bombus sonorus* have been considered conspecific (Handlirsch, 1888; Labougle, 1990; Milliron, 1962, 1973; Peters, 1968) or distinct species (Franklin, 1912; discussed in Thorp et al., 1983; Williams, 1998). Likewise, *B. californicus* and *B. fervidus* have been assigned both subspecific (Labougle, 1990; Milliron, 1973; Williams, 1998) and specific status (Thorp et al., 1983, based on the ecological observations of Hobbs, 1966). Morphological and behavioral characters (many related to nest architecture) were treated in a combined cladistic analysis of South American species of bumble bees (G. Chavarría, unpublished data), including *Fervidobombus*. However, the near complete absence of resolution among all the *Fervidobombus* taxa suggests that other characters are required.

This study used DNA sequences from two mitochondrial genes (12S and 16S), a nuclear gene (opsin, LW *Rh*) and morphology to reconstruct a phylogeny for *Fervidobombus*, and then to explore the evolution of the novel behavior and biogeographic distribution of species.

2. Materials and methods

2.1. Bees examined

We obtained DNA sequences from 18 of the 22 described species of *Fervidobombus* listed in Table 1. Of the four that were unavailable for molecular analyses, *B. rubriventris* is thought to be extinct (Williams, 1998). Two outgroup taxa were selected from the Old World subgenus *Thoracobombus*), considered by Williams (1994) to be among the closest relatives of *Fervidobombus*. Male genitalic characters, used in the morphological study, were obtained for all of the named taxa, except *B. rubriventris*, the males and workers of which remain unknown. Collection localities and GenBank accession numbers for all examined species are presented in Table 1. Voucher specimens and unused remnants of specimens from the molecular study are deposited at the Illinois Natural History Survey, University of Illinois at Urbana-Champaign. Morphological vouchers are retained in the personal collection of Paul Williams.

2.2. Sequencing protocols

Genomic DNA was extracted from fresh, frozen (−80 °C) and ethanol-preserved thoracic, abdominal or leg tissue using the methods of Cameron (1993). PCR amplification of the 12S gene fragment made use of the following primers designed for *Bombus* (modified from Kocher et al., 1989): 12Sa-5′ TGGGATTAGATACCC CACTAT and 12SLR-5′ YYTACTATGTTACGACT TAT. Primers used to amplify the 16S and opsin fragments are reported in Cameron et al. (1992) and Mardulyn and Cameron (1999), respectively. PCR amplification of the 16S and 12S rRNA subunits (35 cycles of denaturation at 94 °C for 60 s, annealing at 50 °C for 60 s, extension at 70 °C for 60 s) resulted in fragments of 564 and 406 bp, respectively. Amplification of the nuclear-encoding major opsin (35 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, with a final extension step of 140 s) produced a 648 bp fragment. Negative controls (DNA replaced by water) were used to safeguard against contamination. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega). Sequencing was carried out with an ABI 377 automated sequencer, using the PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit FS (Perkin–Elmer) according to manufacturer's specifications. Both strands were sequenced for all taxa using the primer pairs employed in amplification.

2.3. DNA sequences and alignment

Sequences were edited in the computer program SeqPup version 0.6 (Gilbert, 1996). Length variation in

Table 1
Taxa examined

Subgenus	Species	Collection locality	Collector	GenBank Accession Nos.
Fervidobombus	*Bombus atratus* Franklin	Brasil, São Paulo	SAC	12S: AF529426 16S AY268398 opsin AY268376
	B. bellicosus Smith	Brasil, Paraná	SL	12S: AF529427 16S AY268399 opsin AY268377
	B. brasiliensis Lepeletier	Brasil, Paraná	SL	12S: AF529428 16S AY268400 opsin AY268378
	B. brevivillus Franklin*	Brasil, Belem	TCH	—
	B. californicus Smith	Canada, Alberta	RO	12S: AF529429 16S AY268401 opsin AY268379
	B. dahlbomii Guérin-Méneville	Chile, Concepción	AA	12S: AF529430 16S AY268402 opsin AY268380
	B. digressus (Milliron)	Mex, R. Grande de Orosí	RD	12S: AF529431 16S AY268403 opsin AY268381
	B. diligens Smith	Mexico, Jalisco	RA	12S: AF529432 16S AY268404 opsin AY268382
	B. excellens Smith	Venezuela, Aragua	JLG	12S: AF529433 16S AY268405 opsin AY268383
	B. fervidus (Fabricius)	USA, Missouri	SAC	12S: AF529434 16S AY268406 opsin AY268384
	B. medius Cresson	Mexico, Chiapas	RB, RA	— 16S AY268407 opsin AY268385
	B. mexicanus Cresson	Mexico, Chiapas	RB, RA	— 16S AY268408 opsin AY268386
	B. morio	Argentina, Porto Alegro; Brasil, Paraná	DW SL	12S: AF529435 16S AY268409 opsin AY268387
	B. opifex Smith*	Bolivia, Cochabamba	MC	—
	B. pennsylvanicus (DeGeer)	USA, Missouri	SAC	12S: AF529436 16S AY268410 opsin AY268388
	B. pullatus Franklin	Costa Rica, Guanacaste	SAC	12S: AF529437 16S AY268411 opsin AY268389
	B. rubriventris	Extinct		—
	B. sonorus Say	Mexico, San Luis Potosí	SAC	12S: AF529438 16S AY268412 opsin AY268390
	B. steindachneri Handlirsch	Mexico, Morelos	RA	12S: AF529439 16S AY268413 opsin AY268391
	B. transversalis (Olivier)	Peru, Madre de Dios	SAC	12S: AF529440 16S AY268414 opsin AY268392
	B. trinominatus Dalla Torre*	Fig. from Milliron (1973)	HEM	—
	B. weisi Friese	Mexico, Jalisco	FAN	12S: AF529441 16S AY268415 opsin AY268393
Thoracobombus	*B. muscorum* (Linnaeus)	UK, Kent	PW	12S: AF529442 16S AY268417 opsin AY268395
	B. pascuorum (Scopoli)	UK, Kent	PW	12S: AF529443 16S AY268418 opsin AY268396

Note. Asterisked taxa were available only for the morphological analysis. *Collector abbreviations*: SAC, Sydney Cameron; SL, Sebastião Laroca; TCH, unknown from museum label; RO, Robin Owen; AA, Arturo Angulo; RD, R. Delgado; RA, Ricardo Ayala; JLG, José Luiz García; RB, Robert Brooks; DW, Dieter Wittman; MC, M. Cooper; HEM, Henry E. Milliron; FAN, F.A. Nogaera; PW, Paul Williams.

both the 16S and 12S sequences required that they be aligned by computer. Computer alignments were implemented in CLUSTAL X (Thompson et al., 1997) (gap opening of 10, gap extension of 0.05). The ingroup sequences were first aligned separately, then the outgroup sequences were added and aligned to the ingroup sequences (profile alignment in CLUSTAL). There was no length variation in the opsin sequences, thus alignment was straightforward. Because visual pigment genes are part of a multigene family (Chang et al., 1996), we verified the homology of LW *Rh* sequences by comparison with known Hymenoptera LW *Rh* sequences retrieved from GenBank (method of Mardulyn and Cameron, 1999). Introns were identified by comparing known LW *Rh* sequences (Chang et al., 1996).

Base frequencies and uncorrected pairwise nucleotide sequence divergences were calculated for each gene fragment using the computer program PAUP*4.0b8 (Swofford, 1998). A χ^2 test of homogeneity of base frequencies across taxa was performed with PAUP*4.0b8. MacClade 4.0 (Maddison and Maddison, 2000) was used to estimate the frequency distribution of observed number of substitutional changes per character for each gene and for each codon position of the protein coding genes.

2.4. Morphology

All morphological characters and character states used in this study are listed in Appendix A. For discussion of morphological homologies and terminology for parts of the male genitalia of bumble bees, see Williams (1985, 1991, 1994). Morphological characters were coded as multistate, unordered, with the exception of four characters that were treated as ordered (Appendix A).

2.5. Phylogenetic analyses

Fervidobombus species relationships were inferred from unweighted maximum parsimony analyses, implemented in PAUP*4.0b8. All parsimony analyses utilized branch and bound searches, unless otherwise indicated. Maximum parsimony (MP) trees were estimated separately for each of the three nucleotide data sets and the morphological data set. Gaps were coded as single characters, regardless of their length, and appended to the 12S and 16S nucleotide data matrices (see Swofford, 1993). This method assigns different character states to inferred homologous gap regions that differ in length among taxa (Swofford, 1993). Introns were included in phylogenetic analyses. Maximum likelihood (ML) analyses were performed to test for effects of nucleotide frequency bias in the mtDNA genes (GTR model; Yang, 1994) and of site to site rate variation in opsin (HKY model; Hasegawa et al., 1985). As the ML procedures (described in detail in Cameron and Mardulyn, 2001) led to identical tree topologies as those estimated using the parsimony criterion, the results will not be discussed further. Additional analyses included linking data sets into the following partitions: (1) all DNA data and (2) DNA and morphology data. As a measure of homogeneity among the original and new partitions, incongruence length difference (ILD) tests (Farris et al., 1994) were implemented in PAUP* with invariant characters removed (Cunningham, 1997). Multiple pairwise tests were performed on each of the original molecular partitions (12S, 16S, and opsin) and on new partitions (all DNA) vs. (morphology). Bootstrap analyses implemented in PAUP* (heuristic search, 400 replicates, TBR swapping, and simple addition sequence) were performed to provide measures of relative support for each node estimated in the maximum parsimony trees. Partion Bremer support values (Baker and DeSalle, 1997; Baker et al., 1998) implemented in TreeRot.v2 (Sorenson, 1999) were obtained as a measure of the contribution of branch support from each data partition for each node of the combined (DNA + Morph) tree.

Saturation of transitions (TIs) or transversions (TVs) may require differential weighting in phylogenetic analyses (Swofford et al., 1996). Although this is unlikely to be a problem among closely related taxa, such as the *Fervidobombus* species, we tested for differential saturation by plotting the number of TIs against the number of TVs for all possible pairs of taxa. This was also done for each codon position of the opsin sequences, as described in detail in Mardulyn and Cameron (1999).

2.6. Dispersal–vicariance analysis

To infer the history of biogeographic distributions in *Fervidobombus*, we entered the known distributions of each extant species, as coded in Appendix B. These distributions were then optimized onto the combined-data MP tree using dispersal–vicariance analysis (DIVA 1.1: Ronquist, 1996, 1997), which infers ancestral distributions based on a three-dimensional cost-matrix derived from a simple biogeographical model. The advantage of this approach is that it does not require a general a priori hypothesis of area relationships.

3. Results

3.1. Data characteristics

With the introduction of gaps, the 12S and 16S data sets used for analyses contain 428 and 573 aligned sites, respectively. Of these sites, 53 were parsimony informative (342 constant) for 12S and 72 were informative (462 constant) for16S. Several small indels (from 1–5

bases) for 12S and 16S corresponded to highly AT-rich regions, but because these were small there were no ambiguous regions of alignment.

The opsin fragment contains 648 aligned sites, 16 of which were parsimony informative (609 constant), including two introns (Mardulyn and Cameron, 1999). The *Fervidobombus* opsin sequence begins 125 codon positions (375 bp) downstream of the cDNA opsin (LW *Rh* gene) sequence reported by Chang et al. (1996) for *Apis mellifera*. The first intron inserts after the first 201 bases of our amplified fragment and comprises 95 nucleotides; the second intron inserts 258 bases downstream of the end of the first intron and comprises 80 bases. The remainder of the opsin fragment contains only 14 bases. The introns do not vary in length among any of the taxa, and together contain 50% of the informative sites (Table 2) and were therefore retained in the phylogenetic analyses. Aligned sequences are available from TreeBase.

There are significant differences among and within genes in the degree of substitutional change that has occurred. The protein-coding opsin gene exhibits relatively few substitutions (39 bp; 6% variable sites), and most of these are at the third position and within the introns (Table 2). Third position transitions do not appear saturated after plotting TIs against TVs (data not shown; see Methods). There is no length variation among sequences, including outgroups, even in the two introns. This suggests a high degree of conservation in the LW *Rh* gene among closely related species of bumble bees. The rRNA genes, as expected, are much more variable, with each fragment showing approximately 20% variable sites overall (Table 2).

Mean uncorrected pairwise divergences for the ingroup and outgroup taxa are given for each gene in Table 3. Between ingroup taxa these range from 0 to 2.6% for opsin, 0.75–8.6% for 12S and 0.36–8.5% for 16S. Variation in base composition differs among genes and codon positions.The two mitochondrial rRNA genes are highly AT rich (16S:78%; 12S:83%), a pattern that has been seen repeatedly in the mtDNA of insects, particularly in Hymenoptera (Whitfield and Cameron, 1998). The protein-encoding LW *Rh* gene exhibits no overall base composition bias, except at the third position, which is slightly AT biased due to a significant under representation of cytosine bases (Mardulyn and Cameron, 1999).

Fifteen phylogenetically informative morphological characters were identified from the male genitalia (Appendices A and B). All morphological characters were informative for the ingroup.

3.2. Fervidobombus relationships

Unweighted parsimony analysis of the 648 bp LW *Rh* sequences resulted in 124 trees, with the bootstrap tree shown in Fig. 1a. The taxa are well resolved, although bootstrap support is relatively low for some clades, which is expected given the small number of informative sites relative to the number of taxa.

Unweighted parsimony analysis of the 428 bp 12S sequences produced 129 equally parsimonious trees; the bootstrap consensus tree is shown in Fig. 1b. Sequences were unavailable for two of the ingroup taxa, *B. medius* and *B. mexicanus*. Although the species relationships are not well supported, the estimated tree topology is quite similar to that of opsin (Table 4).

Unweighted parsimony analysis of the 16S sequences (26 equally parsimonious trees; Fig. 1c) resulted in the resolution of many of the same major clades as those from opsin and 12S (Figs. 1a–b; Table 4), though with highly uneven branch support across the tree. In general, 16S provides considerably higher bootstrap support than 12S.

Pairwise ILD tests for all gene comparisons were strongly non-significant (opsin:12S $p = 0.999$; opsin:16S $p = 1.0$; 12S:16S $p = 0.891$), indicating that combining the data for all genes would be meaningful. Unweighted parsimony analysis of the combined molecular data resulted in 39 trees, with the bootstrap tree shown in Fig. 1d. Clearly, the combined analysis resulted in improved support for many of the deeper branches as well as some of the tip clades.

Table 3
Mean uncorrected pairwise nucleotide distances for each gene

	Ingroup mean *p*-distance	Ingroup range (%)	Outgroup mean (%)
Opsin	1.0	0.00–2.6	2.10
12S	5.0%	0.75–8.6	7.25
16S	3.9%	0.36–8.5	10.40

Table 2
Number of variable and phylogenetically informative sites in the coding and intron regions of the opsin (LW *Rh*) fragment, and among the mitochondrial rRNA genes

	LW *Rh* coding region			LW *Rh* introns		12S rRNA	16S rRNA
	1st	2nd	3rd pos	#1	#2		
No. of variable sites (%)	4	1	15	9	10	86	111
No. of informative sites (%)	0	0	8	4	4	53	72
Total sites	158	158	157	95	80	428	573

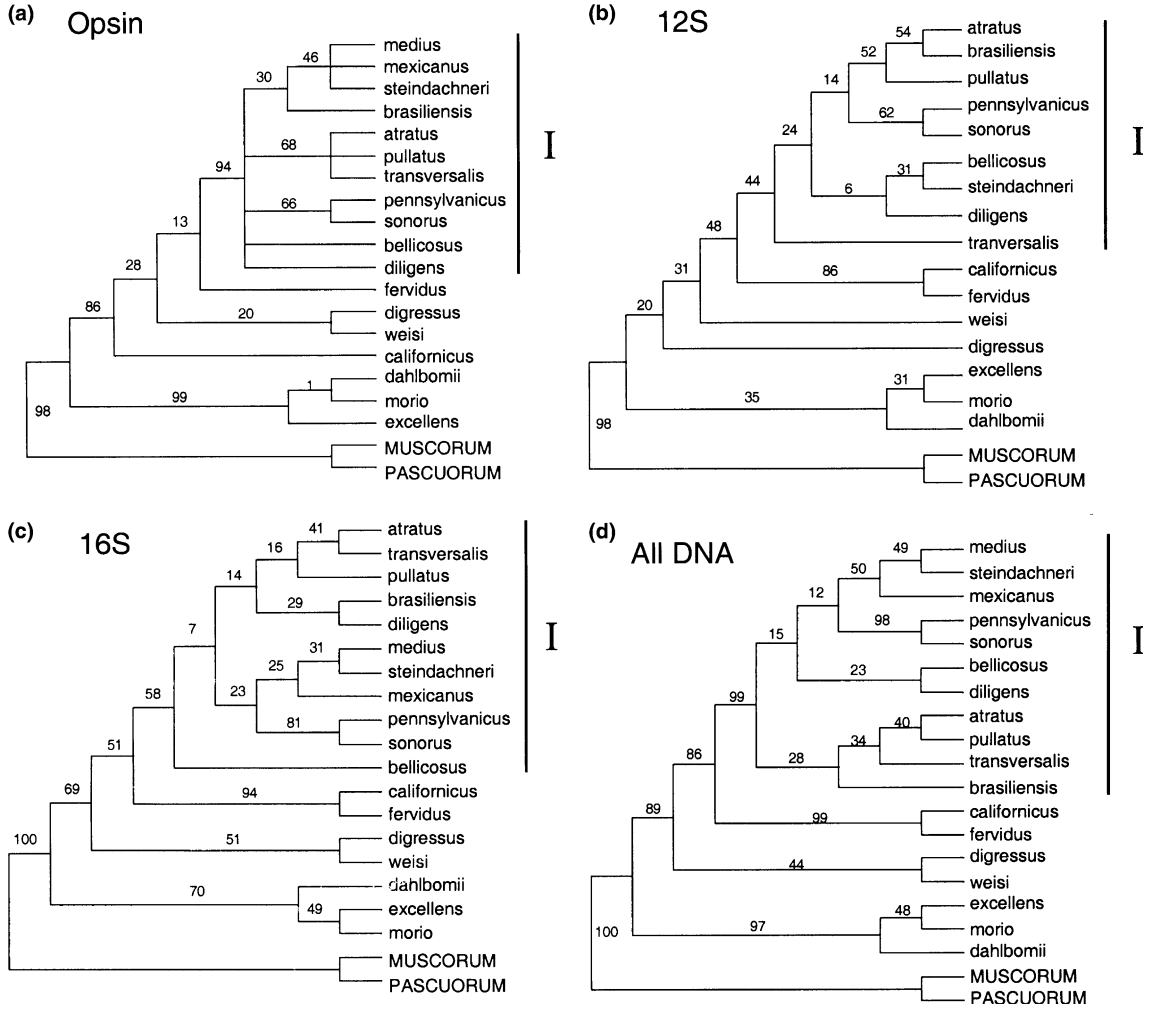

Fig. 1. Bootstrap consensus trees for (a) opsin (124 trees; TL 44, CI 0.87, and RI 0.95), (b) 12S (179 trees; TL 172, CI 0.49, and RI 0.54), (c) 16S (26 trees; TL 208, CI 0.49, and RI 0.59) and (d) All DNA (39 trees; TL 431, CI 0.51, and RI 0.61). Numbers above nodal branches are the bootstrap values. The largely unresolved clade I comprises the same taxa in each tree (a–d), except that the 12S tree is missing *B. medius* and *B. mexicanus* because sequences are unavailable.

Table 4
Congruent clades supported by the different genes and by morphology

Clade		12S	16S	Opsin	All DNA	Morph	Total data
1.	*atratus + pullatus + transversalis*		+	+	+		+
2.	*medius + mexicanus + steindachneri*		+	+	+		+
3.	*bellicosus + diligens*				+	+	+
4.	*pennsylvanicus + sonorus*	+	+	+	+	+	+
5.	*californicus + fervidus*	+	+		+		+
6.	*digressus + weisi*		+	+	+		+
7.	*dahlbomii + excellens + morio*	+	+	+	+	+	+
8.	Clades 1 + 2 + 3 + 4 + *brasiliensis*	+	+		+		+
9.	Clades 8 + 5	+	+		+		+
10.	Clades 9 + 6		+	+	+		+
11.	Monophyly of *Fervidobombus*	+	+	+	+	+	+

Analysis of the 15 male genitalic characters (plus the additional three ingroup taxa that were not available for sequencing) resulted in 3 equally parsimonious trees (strict consensus tree, Fig. 2), partially congruent with the DNA trees (Table 4). Note in particular the recovery of the (*dahlbomii + morio + excellens*) clade and (*pennsylvanicus + sonorus*), which are recovered by each of the genes independently; *Fervidobombus* also appears monophyletic relative to *Thoracobombus*. ILD test comparison of the morphology versus the combined

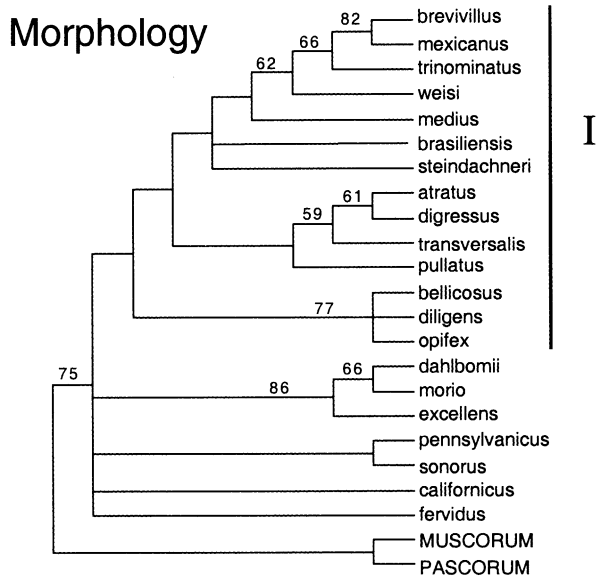

Fig. 2. Strict consensus tree for morphology (3 trees; TL 33, CI 0.67, and RI 0.84) with bootstrap values above 50% placed on branches. The tip clade I includes the same taxa as in Fig. 1, except that three additional species are included in the morphology analysis that are not included in the DNA analyses: *B. brevivillus*, *B. trinominatus*, and *B. opifex*.

molecular partitions indicated these two data partitions to be significantly incongruent, but just barely so ($p = 0.009$, Cunningham, 1997). Due to the partial congruence of morphology with DNA trees, we combined all data partitions into a single analysis, deleting the three taxa not shared between the morphology and DNA partitions. The unweighted parsimony analysis resulted in 35 MP trees, with the bootstrap tree shown in Fig. 3. The striking result is that cladistic support increased for all but one clade, and all clades are congruent (with relatively strong support) with the All DNA tree (Table 4).

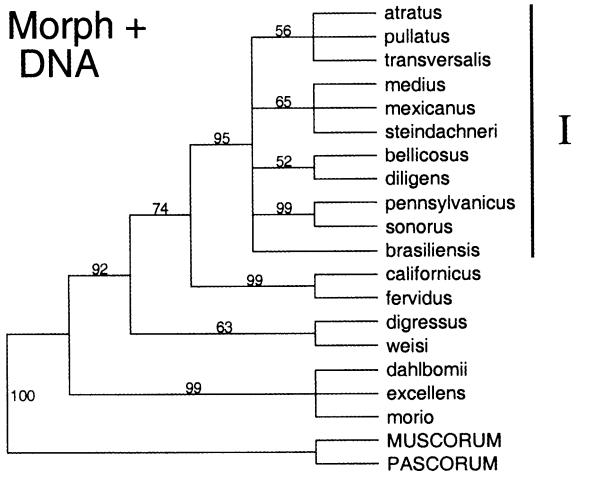

Fig. 3. Bootstrap consensus tree for morphology + DNA (35 trees; TL 477, CI 0.60, RI 0.60). Clade I as described in Fig. 1.

Partitioned Bremer support (PBS) values for the DNA + Morph tree are shown in Table 5. Note that most of the values represent averages (non-integer values), which result when more than one maximum parsimony tree is found during a constrained search for trees lacking a particular node. The averaging process can conceal variation (Lambkin et al., 2002) so additional investigation may be warranted. Overall, the 16S sequences offer the strongest support for the clades, followed by opsin. The majority of conflict is coming from the 12S and morphology partitions. The most strongly supported of the clades is the basal group (*dahlbomii*, *excellens*, and *morio*, which is strongly supported by three of the four data partitions, including morphology. Most of the remaining clades are variably supported by two or three genes.

3.3. DIVA analysis

Optimization of the geographic locations of the taxa Appendix B onto the tree topology using dispersal/vicariance analysis (Fig. 4) revealed the basal clade (*morio* + *excellens* + *dahlbomii*) to be from the Andean and south temperate regions of South America, highly disjunct from the Palearctic sister subgenus *Thoracobombus* and other most closely related subgenera to *Fervidobombus*. The next successive basal clade (*digressus* + *weisi*) is from Mexico and Central America, the next (*californicus* + *fervidus*) from North America. Thus the ancestral distributions, while uncertain, would appear to suggest a southern New World diversification of the group, far from its nearest relatives in the Old World. The remaining clade I is also inferred to have a non-North American ancestor, although in this case a Mexican/Central American ancestor is more likely than a South American ancestor.

4. Discussion

4.1. Congruence of DNA and morphology-based topologies

Three different genes, two mitochondrial and one nuclear-encoding, and morphology data applied to the estimation of *Fervidobombus* relationships showed fairly high levels of congruence based on the ILD analysis. Opsin alone is too conserved to fully resolve the *Fervidobombs* species. Opsin sequences for several species pairs were identical. However, there is little conflict in the data, which exhibits a very high consistency index. Sequencing the entire opsin gene, which would add another two introns, would probably provide the desired overall level of substitutional variation. The 12S gene is poor at this closely related species level. Consistency is low, as reflected by the lack of

Table 5
Partitioned Bremer support (Baker and DeSalle, 1997; Baker et al., 1998) for the Morph + DNA tree shown in Fig. 3

Node	Morphology	Opsin	12S	16S	Total Bremer
MUSC, PASC	2.17	4.34	5.94	16.54	29
morio, exce, dahl	2.77	5.44	−1.96	2.74	9
digr, weisi	1.08	0.10	−0.23	0.05	1
calif, ferv	−1.56	−0.06	2.54	4.08	6
tran, pull, atra	1.27	0.94	−3.46	1.24	0
mexi, medi, stei	−1.73	−0.06	1.54	1.24	1
dili, bell	0.50	−0.06	0.00	−0.44	0
penn, sono	−1.23	0.94	2.54	1.74	4
atrat—bras	−0.73	4.94	−0.96	3.74	11
atrat—ferv	−1.83	0.34	−0.06	2.54	1
atrat—weisi	0.00	3.00	0.00	2.00	5
atrat—morio	2.17	4.34	5.94	16.54	29

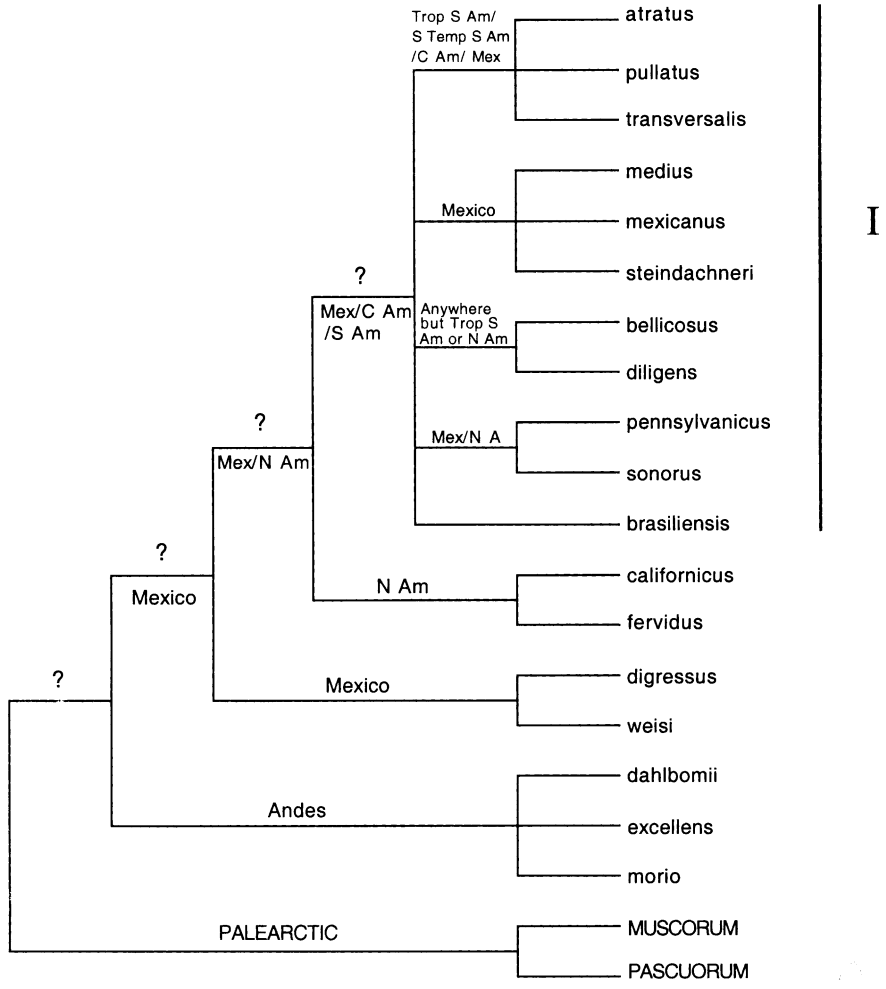

Fig. 4. Dispersal/vicariance analysis with geographic regions optimized onto the All-DNA bootstrap consensus tree. Question marks above branches indicate uncertainty regarding ancestral origin; geographic regions listed below question marks are hypothetical ancestral distributions based on logical inference. Clade I as described in Fig. 1.

resolution at all the deeper nodes in the tree and at many of the tip nodes. Interestingly, and the reason we included the data from this gene, many of the recovered species groups were identical to those recovered for opsin and 16S (Table 4). In contrast to 12S, the 16S gene is quite good at resolving the deeper nodes and many of the tips as well. It also recovers similar relationships to the other two genes. Thus, in spite of relatively low branch support for some levels in the tree for each gene, all genes nonetheless recovered the same

basic species groupings. Simultaneous analysis of all genetic data sets increased support for a number of the congruent clades, although support remained below 50% for many species groupings in the large tip clade (clade I of Figs. 1–4).

The morphology-based consensus tree showed several of the same groupings as those recovered with the DNA trees, but alone there was too little resolution from the small data set of 15 genitalic characters. Combining all of the data partitions resulted in a tree that was entirely congruent with the All DNA tree and, importantly, branch support increased significantly such that all resolved clades showed bootstrap support above 50% (most were between 65 and 100%) as a result of combining all partitions. It is of interest that the genes used in our subgeneric analysis of closely related species have been found to be congruent in analyses of higher level relationships among insects (Cameron and Mardulyn, 2001; Mardulyn and Cameron, 1999; Simon et al., 1994). It may be a general pattern with other genes that if there is strong, congruent signal at higher levels, this should also result in recovery of accurate phylogenetic patterns at lower levels. Although resolution may be less than desirable, the characters should at least not give misleading results.

4.2. Biogeographic history

Examination of the geographic distribution of *Fervidobombus* clades suggests a complex picture of spread and diversification from the Old World into the southern New World. The pattern does not entirely fit a simple model of expansion into northern North America with subsequent spread progressively farther southward (e.g., Williams, 1985). If this were the case, the basal clades should be North American, with the most derived clades found in southern South America. To the contrary, our results show the most basal clade (*dahlbomii* + *excellens* + *morio*) is absent from north of the Panama isthmus, and it contains the most southerly distributed species. However, it is possible that this group has gone extinct from the more northern areas. If we consider only the remaining clades, then the two more basal groups are indeed from north of the Panama isthmus, while the remaining groups (comprising clade I in all figures) are found north, north and south, or entirely south of the isthmus.

Fervidobombus are unique in that some species have spread into lowland tropical rain forest. There is a clear phylogenetic component to their ecological adaptation to rain forest habitat, as the two primary tropical rain forest species (*B. transversalis* and *Bombus pullatus*) appear in the same clade, along with a third species, *B. atratus*, which is widely distributed in South America, including lowland subtropical habitats (Moure and Sakagami, 1962).

Availability of a well-supported phylogeny of *Fervidobombus* will ultimately allow us to examine the pattern of evolution of some of the unusual social behavioral traits found within this species group, which, in turn, should provide fresh insights into causes and constraints in the evolution of social behavior. By knowing the phylogeographic history of these bumble bee species we can find answers to some long-standing questions in social evolution: To what degree is highly social behavior correlated with tropical habitats (Dornhaus and Cameron, 2003), as hypothesized for traits such as recruitment communication systems in the highly eusocial bees (Frisch, 1967; Heinrich, 1979; Seeley, 1995)? Is there a pattern suggesting that social behavior in bumble bees became more complex as species moved into subtropical and tropical regions of the New World? For instance, does colony size and aggressiveness increase from north to south or from high to lower elevation, and to what degree? How common is perenniality in some of these tropical species, and how much interspecific variation occurs with any of these traits? These questions are tractable within a phylogenetic framework, and await further examination of comparative behavior.

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Appendix A

Morphological characters and character states [PV = penis valve, VS = volsella, GS = gonostylus, and GC = gonocoxite, Williams, 1985, 1991]

	PV	VS	GS	GC
Character number		1	111	11
	123456	7890	123	45
Ordered	*	**	*	
AVINOVIELLUS	110000	????	???	00

Appendix A (*continued*)

	PV	VS	GS	GC
MUCIDUS	110000	2010	011	00
MUSCORUM	000000	2011	013	00
PASCUORUM	200000	2011	013	00
fervidus	200000	2010	111	00
californicus	200000	2010	111	00
pennsylvanicus	210000	2010	111	00
sonorus	210000	2010	111	00
excellens	100110	2010	111	00
dahlbomii	100111	2010	111	00
morio	100111	2010	111	00
diligens	110000	1000	111	00
opifex	110000	1000	111	00
bellicosus	110000	1000	121	00
pullatus	000000	1010	110	00
transversalis	010000	1010	100	00
atratus	000000	1110	100	01
digressus	011000	1110	100	11
brasiliensis	000000	0010	111	00
steindachneri	000000	0010	111	00
medius	000000	0010	121	00
weisi	000000	0020	120	00
trinominatus	000?00	0020	13?	00
mexicanus	001000	0020	132	10
brevivillus	011000	0020	132	11

Appendix B

Biogeographic area character state matrix. Stemp SA, southern temperate South America; Trop SA, tropical S. Am.; CentAmer, Central Am.; N.Amer, North Am.

Character	1	2	3	4	5	6
	StempSA	Andes	TropSA	CentAmer	Mexico	N.Amer
atratus	1	1	0	0	0	0
bellicosus	1	1	0	0	0	0
brasiliensis	1	1	0	0	0	0
brevivillus	0	0	1	0	0	0
californicus	0	0	0	0	0	1
dahlbomii	1	1	0	0	0	0
digressus	0	0	0	1	1	0
diligens	0	0	0	1	1	0
excellens	0	1	0	0	0	0
fervidus	0	0	0	0	0	1
medius	0	0	0	1	1	0
mexicanus	0	0	0	1	1	0
morio	1	1	0	0	0	0
opifex	1	1	0	0	0	0
pennsylvanicus	0	0	0	0	1	1
pullatus	0	0	1	1	1	0
sonorus	0	0	0	0	1	1
steindachneri	0	0	0	0	1	0
transversalis	0	0	1	0	0	0
trinominatus	0	0	0	0	1	0
weisi	0	0	0	0	1	0

References

Baker, R.H., DeSalle, R., 1997. Multiple sources of character information and the phylogeny of Hawaiian drosophilids. Syst. Biol. 46, 654–673.

Baker, R.H., Yu, X., DeSalle, R., 1998. Assessing the relative contribution of molecular and morphological characters in simultaneous analysis trees. Mol. Phylogenet. Evol. 9, 427–436.

Cameron, S.A., 1989. Temporal patterns of division of labor among workers in the primitively eusocial bumble bee, *Bombus griseocollis* (Hymenoptera: Apidae). Ethology 80, 137–151.

Cameron, S.A., 1993. Multiple origins of advanced eusociality in bees inferred from mitochondrial DNA sequences. Proc. Natl. Acad. Sci. USA 90, 8687–8691.

Cameron, S.A., Derr, J.N., Austin, A.D., Wooley, J.B., Wharton, R.A., 1992. The application of nucleotide sequence data to phylogeny of the Hymenoptera: a review. J. Hymenop. Res. 1, 63–79.

Cameron, S.A., Jost, M.C., 1998. Mediators of dominance and reproductive success among queens in the periodically polygynous neotropical bumble bee *Bombus atratus* Franklin. Insect. Soc. 45, 135–149.

Cameron, S.A., Mardulyn, P., 2001. Multiple molecular data sets suggest independent origins of highly eusocial behavior in bees (Hymenoptera: Apinae). Syst. Biol. 50, 194–214.

Cameron, S.A., Whitfield, J.B., 1996. Use of walking trails by bees. Nature 379, 125.

Cameron, S.A., Whitfield, J.B., Cohen, M., Thorp, N., 1999. Novel use of walking trails by the Amazonian bumble bee, *Bombus transversalis* (Hymenoptera: Apidae). In: Byers, G.W., Hagen, R.H., Brooks, R.W. (Eds.), Entomological Contributions in Memory of Byron A. Alexander. University of Kansas Natural History Museum Special Publication 24, pp. 187–193.

Chang, B.S.W., Ayers, D., Smith, W.C., Pierce, N.E., 1996. Cloning of the gene encoding honeybee long-wavelength rhodopsin: a new class of insect visual pigments. Gene 173, 215–219.

Cunningham, C.W., 1997. Can three incongruence tests predict when data should be combined? Mol. Biol. Evol. 14, 733–740.

Dias, D., 1958. Contribuição para o conhecimento da bionomia de *Bombus incarum* Franklin da Amazônia (Hymenóptera:Bombidae). Rev. Bras. Entomol. 8, 1–20.

Dornhaus, A., Cameron, S., 2003. A scientific note on food alert in *Bombus transversalis*. Apidologie 34, 87–88.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of incongruence. Cladistics 10, 315–319.

Franklin, H.J., 1912. The Bombidae of the New World. Trans. Am. Entomol. Soc. 38, 177–486.

Franklin, H.J., 1913. The Bombidae of the New World. Trans. Am. Entomol. Soc. 39, 73–200.

Frisch, K. von, 1967. The Dance Language and Orientation of Bees. Harvard University Press, Cambridge, MA.

Gilbert, D., 1996. SeqPup version 0.6. University of Indiana, Bloomington.

Handlirsch, A., 1888. Die Hummelsammlung des k. k. naturhistorischen Hofmuseums. Ann. Naturh. Mus. Wien 3, 209–250.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Heinrich, B., 1979. Bumble Bee Economics. Harvard University Press, Cambridge, MA.

Hobbs, G.A., 1966. Ecology of species of *Bombus* Latr. (Hymenoptera, Apidae) in Southern Alberta IV. Subgenus *Fervidobombus* Skorikov. Can. Entomol. 98, 33–39.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Koulianos, S., Schmid-Hempel, P., 2000. Phylogenetic relationships among bumble bees (*Bombus*, Latreille) inferred from mitochondrial cytochrome *b* and cytochrome oxidase I sequences. Mol. Phylogenet. Evol. 14, 335–341.

Labougle, J.M., 1990. *Bombus* of Mexico and Central America (Hymenoptera, Apidae). Univ. Kansas Sci. Bull. 54, 35–73.

Lambkin, C.L., Lee, M.S.Y., Winterton, S.L., Yeates, D.K., 2002. Partitioned Bremer support and multiple trees. Cladistics 18, 436–444.

Maddison, D.R., Maddison, W.P., 2000. MacClade 4. Sinauer, Sunderland, MA.

Mardulyn, P., Cameron, S.A., 1999. The major opsin LW Rh in bees (Insecta: Hymenoptera): a promising nuclear gene for higher level phylogenetics. Mol. Phylogenet. Evol. 12, 168–176.

Michener, C.D., 1974. The Social Behavior of the Bees. Harvard University Press, Cambridge, MA.

Michener, C.D., LaBerge, W.E., 1954. A large *Bombus* nest from Mexico. Psyche 61, 63–67.

Milliron, H.E., 1962. Taxonomic notes on some American bumble bees (Hymenoptera: Apidae: Bombinae). Can. Entomol. 94, 728–735.

Milliron, H.E., 1973. A monograph of the western hemisphere bumble bees (Hymenoptera: Apidae; Bombinae). II. The genus *Megabombus* subgenus *Megabombus*. Mem. Entomol. Soc. Canada 89, 81–237.

Mitchell, T.B., 1962. Bees of the eastern United States. Vol. 2. Tech. Bull., N. C. Agric. Exp. Stat. 152, 557 pp.

Moure, J.S., Sakagami, S.F., 1962. As mamangabas sociais do Brasil (*Bombus* Latreille) (Hymenoptera, Apoidea). Stud. Entomol. 5, 65–194.

Peters, D.S., 1968. Beitrage zur Kenntnis aculeater Hymenopteren von Mexiko. I. Apinae (Apidae, Apoidea). Senckenbergiana Biol. 49, 237–248.

Ronquist, F., 1996. DIVA version 1.1. Computer program and manual. Available from Uppsala University at: <http://www.ebc.uu.se/systzoo/research/diva/diva.html>.

Ronquist, F., 1997. Dispersal–vicariance analysis: a new approach to the quantification of historical biogeography. Syst. Biol. 46, 195–203.

Sakagami, S.F., Zucchi, R., 1965. Winterhalten einer neotropischen Hummel, *Bombus atratus*, innerhalb des Beobachtungskastens. Ein Beitrag zur Biologie der Hummeln. J. Facul. Sci., Hokkaido Univ. (Vi, Zool.) 15, 712–762.

Seeley, T., 1995. The Wisdom of the Hive: The Social Physiology of Honey Bee Colonies. Harvard University Press, Cambridge, MA.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Sorenson, M.D., 1999. TreeRot.v2, Biology Department, Boston University, Boston.

Swofford, D.L., 1993. PAUP: Phylogenetic Analysis Using Parsimony, version 3.1 (Manual).

Swofford, D.L., 1998. PAUP: Phylogenetic Analysis Using Parsimony, Beta version 4.0b1 for Power PC. Sinauer Associates, Sunderland, MA.

Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M., 1996. Phylogeny reconstruction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer Associates, Sunderland, MA, pp. 407–514.

Taylor, O.M., Cameron, S.A., 2003. Nest construction and architecture of the Amazonian bumble bee (Hymenoptera: Apidae). Apidologie (in press).

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface: flexible

strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Thorp, R.W., Horning Jr., D.S., Dunning, L.L., 1983. Bumble bees and cuckoo bumble bees of California (Hymenoptera: Apidae). Bull. Calif. Insect Surv. 23, viii + 79 pp.

Whitfield, J.B., Cameron, S.A., 1998. Hierarchical analysis of variation in the mitochondrial 16S rRNA gene among Hymenoptera. Mol. Biol. Evol. 15, 1728–1998.

Williams, P.H., 1985. A preliminary cladistic investigation of relationships among the bumble bees (Hymenoptera, Apidae). Syst. Entomol. 10, 239–255.

Williams, P.H., 1991. The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). Bull. Brit. Mus. (NH), Entomol. 60, 1–204.

Williams, P.H., 1994. Phylogenetic relationships among bumble bees (*Bombus* Latr.): a re-appraisal of morphological evidence. Syst. Entomol. 19, 327–344.

Williams, P.H., 1998. An annotated checklist of bumble bees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). Bull. Nat. Hist. Mus. Lond. (Ent.) 67, 79–152. Available from <http://www.nhm.ac.uk/entomology/bombus>.

Yang, Z., 1994. Estimating the pattern of nucleotide substitution. J. Mol. Evol. 39, 105–111.

Zucchi, R., 1973. Aspectos Bionômicos de *Exomalopsis aureopilosa* e *Bombus atratus* incluindo considerações sobre a evolução do comportamento social (Hymenoptera, Apoidea). Doctoral Thesis, Faculdade de Filosofia, Ciencias e Letras de Ribeirão Preto, SP, Brasil, 172 pp.