

Molecular phylogeny of the bumble bee subgenus *Pyrobombus* (Hymenoptera: Apidae: *Bombus*) with insights into gene utility for lower-level analysis

Heather M. Hines^{A,C}, Sydney A. Cameron^A and Paul H. Williams^B

^ADepartment of Entomology, 320 Morrill Hall, 505 South Goodwin Ave.,
University of Illinois, Urbana, IL 61801, USA.

^BDepartment of Entomology, The Natural History Museum, London SW7 5BD, United Kingdom.

^CCorresponding author. Email: hhines@life.uiuc.edu

Abstract. Comprising nearly 20% of all bumble bees, the subgenus *Pyrobombus* is distributed across diverse habitats in the Northern Hemisphere and exhibits considerable morphological and behavioural variation relative to other subgenera. Its size and variation have led to questions concerning its monophyly and intrasubgeneric relationships, but too few known morphological synapomorphies and insufficient taxon sampling have precluded robust answers to these questions. To obtain a robust phylogeny of the group, we obtained DNA sequences for 36 of the 43 species from four genes (mitochondrial *16S* rRNA and three nuclear genes: elongation factor – 1α (*EF-1\alpha*), long wavelength rhodopsin (*LW Rh* or *opsin*) and arginine kinase (*ArgK*)). Both Bayesian and parsimony phylogenies are well resolved and indicate a monophyletic *Pyrobombus* when assessed against representatives of 20 additional subgenera. The more conserved nuclear genes, especially *EF-1\alpha* and *ArgK*, provided good support across all of the taxonomic levels examined, whereas support of the more rapidly evolving *mt16S* was restricted mostly to close relationships at the tips of the tree. The exon regions of *ArgK* were the most conserved and may be promising for higher-level phylogenetics. We discuss species relationships within *Pyrobombus* and its sister-group, *Bombus s.s.* + *Alpinobombus*, in relation to previous taxonomic studies.

Introduction

Bumble bees (*Bombus* Latreille) play a vital role in the pollination of many native and cultivated plant species and have been the subject of considerable investigation on foraging and social behaviours. The taxonomic interest they have inspired has resulted in >2800 formally recognised specific and subspecific names and classification of the species into 38 subgenera, of which 50% comprise only one or two species (Williams, 1998). Yet, despite the taxonomic attention, the species-level relationships of the bumble bees within the larger subgenera, including *Pyrobombus* Dalla Torre, *Thoracobombus* Dalla Torre and *Psithyrus* Lepeletier, are poorly known. Save for the phylogenetic examination of *Fervidobombus* Skorikov (Cameron and Williams 2003), recent systematic research on bumble bees has focused more on higher-level relationships among subgenera (Kawakita *et al.* 2003, 2004).

Of the 38 *Bombus* subgenera, *Pyrobombus* is the largest, containing 43 of the 239 bumble bee species recognised by Williams (1998). Relative to other subgenera, *Pyrobombus* species are diverse in morphology (e.g. tongue length and wing venation Lutz 1916; Medler 1962; Richards 1968) and behaviour (e.g. nest site preference and emergence times Sakagami 1976). They are also broadly distributed across the

Northern Hemisphere in a variety of habitats, from desert to arctic tundra. *Pyrobombus* is thus an ideal group for testing the influences of adaptation and phylogenetic history on ecological and morphological traits and for assessing widespread biogeographic dispersal patterns.

Several morphological characters are diagnostic for *Pyrobombus* (Richards 1968; Williams 1991), yet numerous studies have concluded that the subgenus may not be monophyletic. Medler (1962) suggested that *Pyrobombus* might be an unnatural group because the species possess a wide range of variation in mouthpart structures and wing length indices. A genus-wide phenetic study of wing venation by Plowright and Stephen (1973) resulted in a polyphyletic *Pyrobombus*, with some species more closely related to species of the subgenera *Melanobombus* Dalla Torre, *Bombus s.s.* Latreille, or *Kallobombus* Dalla Torre. Small-scale molecular studies of bumble bees (11–19 taxa) reported species of *Melanobombus* to fall within *Pyrobombus* (Pedersen 1996; Koulianos 1999; Koulianos and Schmid-Hempel 2000). More extensive molecular analyses suggest *Pyrobombus* is monophyletic and most closely related to *Bombus s.s.* and *Alpinobombus* (Pedersen 2002 (*EF-1\alpha*); Kawakita *et al.* 2003, 2004). Plowright and Stephen (1973) also inferred a close relationship between

Pyrobombus and *Bombus s.s.*, but morphological characters from other studies suggest more distant relationships among *Bombus s.s.*, *Alpinobombus* and *Pyrobombus* (Ito 1985; Williams 1985, 1994; Chen and Wang 1997). The relationships within *Pyrobombus* and *Bombus s.s.* have commercial relevance because they include the species that have been marketed for pollination (*B. terrestris* (Linnaeus) (*Bombus s.s.*), *B. occidentalis* Greene (*Bombus s.s.*) and *B. impatiens* Cresson (*Pyrobombus*)). *Bombus terrestris* is also a model species used throughout Europe for studies of *Bombus* social behaviour.

The disagreement over the taxonomic status and phylogenetic relationships of *Pyrobombus* is likely the result of an insufficient number of characters and incomplete taxon sampling. The most comprehensive molecular study of *Bombus* to date (Kawakita *et al.* 2004) included less than half of the known *Pyrobombus* species in their higher-level study. The principal goal of our investigation was to obtain a robust phylogeny of the species and intrasubgeneric groups within *Pyrobombus*. To this end, we analyse relationships among 36 of the 43 recognised *Pyrobombus* species and an additional four taxa of uncertain species status, using nucleotide sequences from four genes: mitochondrial 16S rRNA (16S) and three nuclear genes: elongation factor-1 α F2 copy (*EF-1 α*), long-wavelength rhodopsin (LW *Rh* or *opsin*) and arginine kinase (*ArgK*). We also examine the sister-group relationships to *Pyrobombus*, including all but two of the species of *Bombus s.s.* and *Alpinobombus* (Williams 1998) and several species of *Melanobombus* and other subgenera proposed as close relatives to *Pyrobombus* in prior studies. Additional new character information from DNA sequences and a near complete *Pyrobombus* species representation allows a more confident assessment of monophyly and provides additional synapomorphies to resolve the internal structure of the subgenus.

The second goal of our study was to assess the utility of nuclear genes for lower-level phylogenetic analysis. Maximising phylogenetic resolution requires selection of appropriate genes for the taxonomic level and divergence history of the group of interest. DNA sequences from mitochondrial and rRNA genes have been used extensively for analysis of both higher and lower-level relationships within insects, primarily because of their ease of amplification and the availability of universal primers (Simon *et al.* 1994). More recently, there has been greater emphasis on the use of nuclear protein-encoding genes for resolving deeper phylogenetic relationships within insects (Friedlander *et al.* 1992, 1994, 1996; Cho *et al.* 1995; Brower and DeSalle 1998; Mardulyn and Cameron 1999; Moulton and Wiegmann 2004). Baker *et al.* (2001) and Lin and Danforth (2004) found nuclear genes to be more useful than mitochondrial genes for inferring insect relationships because they have less base composition bias, slower rates of nucleotide substitution, lower levels of homoplasy and contribute more to tree

resolution. Although the use of nuclear genes has been advocated for assessing higher-level relationships, their utility at lower taxonomic levels has been less explored. In this study, we examine the utility of 16S and the three described nuclear genes for resolving relationships within species-groups and near relatives.

Materials and methods

Taxa examined

To assess intrasubgeneric and sister-group relationships of *Pyrobombus*, we analysed sequences from single exemplar specimens of 81 *Bombus* species (Table 1). This included 36 of the 43 species of *Pyrobombus* recognised by Williams (1998) as well as four uncertain species: *B. infrequens* (Tkalcü) and *B. sonani* (Frison) (until recently known from very few specimens and included within a broader concept of *B. parthenius* Richards), *B. sylvicola* Kirby (a Nearctic taxon possibly conspecific with the Old World *B. lapponicus* (Fabricius)) and *B. wilmatiae* Say (questionably conspecific with *B. ephippiatus* Say). To assess nucleotide sequence variation within the widespread *B. hypnorum* (Linnaeus), we sequenced two individuals, one from China and another from Austria. The seven *Pyrobombus* species missing from the analysis include five species from central Asia (*B. abnormis* (Tkalcü), *B. mirus* (Tkalcü), *B. parthenius*, *B. rotundiceps* Friese and *B. subtypicus* (Skorikov)), *B. sandersoni* Franklin (eastern North America) and *B. oceanicus* Friese (northern Japan). We included an additional 41 species from 20 other *Bombus* subgenera, targeting groups thought to be near *Pyrobombus* based on previous studies. This includes six of 14 species of *Melanobombus*, all five species of *Alpinobombus* and eight of the 10 species of *Bombus s.s.* recognised by Williams (1998). In *Bombus s.s.* we also sequenced several taxa of uncertain status including *B. cryptarum*, *B. moderatus* (members of the *lucorum* species-complex), *B. occidentalis* (= *B. terricola* Kirby?) and *B. lucorum s.s.* (Linnaeus) specimens from China, France and Turkey. Specimen vouchers for DNA sequences were deposited at the Illinois Natural History Museum in Champaign, Illinois, USA.

Gene selection

We sequenced fragments of four genes: 16S rRNA, *opsin*, *EF-1 α* and *ArgK*. The mitochondrial 16S has been used to assess relationships to the ordinal level in insects (e.g. Yoshizawa and Johnson 2003). Its high evolutionary rate, however, makes it potentially more reliable for lower-level phylogenetic analyses (Whitfield and Cameron 1998). We used primers 16SWb (Dowton and Austin 1994) and 874-16SIR (Cameron *et al.* 1992) to obtain ~500 base pairs (bp) from 16S.

EF-1 α is involved in the binding of charged tRNAs at the ribosome during translation. It has been used to infer relationships at multiple levels in insects (Cho *et al.* 1995; Rokas *et al.* 2002; Danforth *et al.* 2004; Lin and Danforth 2004). Two copies of *EF-1 α* occur in bees (Danforth and Ji 1998). We obtained ~720 bp of the F2 copy, which includes an intron ~200 bp in length. This fragment was amplified with primers F2-ForH (5'-GGRCAYAGAGATTTCATCAAGAAC-3') and F2-RevH2 (5'-TTGCAAGCTTCRKGATGCATTT-3'), designed from sequences obtained using primers F2-rev1 (Danforth *et al.* 1999) and HaF2For1 (Sipes and Wolf 2001). These primers partially overlap with those used by Kawakita *et al.* (2003).

Long-wavelength rhodopsin is a member of a class of light-absorbing receptor proteins involved in colour vision in animals. It has been useful for resolving Cretaceous age divergences within Hymenoptera, including the families Cynipidae (Rokas *et al.* 2002) and Halictidae (Danforth *et al.* 2004) and for inferring relationships among the corbiculate bees (Apinae) (Mardulyn and Cameron 1999; Cameron and Mardulyn 2001, 2003; Michel-Salzat and Whitfield 2004, although see

Table 1. Species examined and their collection localities, voucher numbers and GenBank accession numbers

Sequences obtained from previous studies are indicated by letters after accession numbers and their localities are indicated in brackets. Numbers in parantheses refer to the number of base differences between sequences from this study and Kawakita *et al.* (2003) or between specimens from different localities (*B. hypnorum*, *B. lucorum*)

Subgenus	Species	Collection locality	No.	16S	EF-1 α	opsin	ArgK
<i>Alpigenobombus</i>	<i>nobilis</i> Friese	Sichuan, China	098	AY737370	AY739591	AY739485	AY739528
	<i>wurflenii</i> Radoszkowski	Obergurgl, Austria [Monte Rosa, Italy]	001	AY737393	AY739612, AF492940 ^K (1)	AF493007 ^K	AF492873 ^K
<i>Alpinobombus</i>	<i>alpinus</i> (Linnaeus)	Gurgltal, Austria	029	AY737321	AY739542	AY739452	AY741385
	<i>balteatus</i> Dahlbom	Kiruna, Sweden	039	AY737324	AY739546	AY739455	AY739499
	<i>hyperboreus</i> Schönherr	Avesta, Sweden	070	AY737345	AY739565	AY739470	AY739513
	<i>neoboreus</i> Sladen	Alaska, USA	188	AY737369	AY739590	AY739484	AY739527
	<i>polaris</i> Curtis	[Kamchatka, Russia]			AF492970 ^K	AF493037 ^K	AF492903 ^K
<i>Bombias</i>	<i>auricomus</i> (Robertson)	Illinois, USA [Delaware, USA]	062	AY737323	AY739545, AF492959 ^K (0)	AY739454	AF492892
<i>Bombus</i>	<i>affinis</i> Cresson	Illinois, USA	167	AY737320	AY739541	AY739451	AY739497
	<i>cryptarum</i> (Fabricius)	Erzincan, Turkey	127	AY737332	AY739554	AY739461	AY739504
	<i>hypocrita</i> Pérez	Kyushu, Japan [Tokushima, Japan]	123	AY737347	AY739568, AF492956 ^K (1)	AF493023 ^K	AF492889 ^K
	<i>ignitus</i> Smith	Beijing, China [Oita, Japan]	096	AY737348	AY739569, AF492965 ^K (1)	AF493032 ^K	AF492898 ^K
	<i>lucorum</i> (Linnaeus)	Ayder, Turkey; Egat, France [Udine, Italy]	217	AY737360	AY739581, AF492954 ^K (0)	AF493021 ^K	AF492887 ^K
		Sichuan, China	184	AY737359 (9)	AY739580 (3)	AY739479 (3)	AY739522 (6)
	<i>moderatus</i> Cresson	Alberta, Canada	163	AY737366	AY739587	AY739481	AY739524
	<i>occidentalis</i> Greene	New Mexico, USA	025	AY737371	AY739592	AY739486	AY739529
	<i>patagiatus</i> Nylander	Sichuan, China [Primorsky, Russia]	111	AY737372	AY739593, AF492953 ^K (1)	AF493020 ^K	AF492886 ^K
	<i>sporadicus</i> Nylander	Abisko, Sweden	193	AY737381	AY739601	AY739491	AY739534
	<i>terrestris</i> (Linnaeus)	San Quirico, Italy [L'Aquila, Italy]	003	AY737386	AY739605, AF492955 ^K (0)	AF493022 ^K	AF492888 ^K
	<i>terricola</i> Kirby	Ontario, Canada [Quebec, Canada]	205	AY737387	AY739606, AF492952 ^K (0)	AF493019 ^K	AF492885 ^K
<i>Confusibombus</i>	<i>confusus</i> Schenck	Dorres, France	083	AY737331	AY739553	AY739460	AY739503
<i>Cullumanobombus</i>	<i>rufocinctus</i> Cresson	Alberta, Canada [Quebec, Canada]	186	AY737377	AY739597, AF492967 ^K (0)	AF493034 ^K	AF492900 ^K
<i>Fervidobombus</i>	<i>pensylvanicus</i> (DeGeer)	[Missouri, USA] [California, USA]		AY268410 ^C	AF492929 ^K	AY268388 ^C	AF492862 ^K
<i>Festivobombus</i>	<i>festivus</i> Smith	Sichuan, China	104	AY737336	AY739558	AY739465	AY739508
<i>Kallobombus</i>	<i>soroensis</i> (Fabricius)	Eyne, France [Cesana, Italy]	136	AY737380	AY739600, AF492941 ^K (0)	AF493008 ^K	AF492874 ^K
<i>Megabombus</i>	<i>argillaceus</i> (Scopoli)	Kayseri, Turkey	058	AY737322	AY739544	AY739453	AY739498
<i>Melanobombus</i>	<i>friseanus</i> Skorikov	Sichuan, China	105	AY737340	AY739560	AY739467	AY739510
	<i>keriensis</i> Morawitz	Sichuan, China	114	AY737353	AY739574	AY739474	AY739517
	<i>ladakhensis</i> Richards	Sichuan, China	158	AY737354	AY739575	AY739475	AY739518
	<i>lapidarius</i> (Linnaeus)	San Quirico, Italy [Windsor, UK]	006	AY737355	AY739576, AF492938 ^K (1)	AF493005 ^K	AF492871 ^K
	<i>rufofasciatus</i> Smith	Sichuan, China	133	AY737378	AY739598	AY739489	AY739532
<i>Mendacibombus</i>	<i>erzurumensis</i> (Özbek)	Artvin Prov., Turkey	126	AY737334	AY739556	AY739463	AY739506
	<i>mendax</i> Gerstaecker	Gurgltal, Austria [Monte Rosa, Italy]	019	AY737363	AY739584, AF492957 ^K (0)	AF493024 ^K	AF492890 ^K
<i>Psithyrus</i>	<i>maxillosus</i> Klug	Kayseri, Turkey	074	AY737361	AY739582	AY739480	AY739523
<i>Pyrobombus</i>	<i>vestalis</i> (Geoffroy)	Kent, England	169	AY737390	AY739609	AY739495	AY739538
	<i>ardens</i> Smith	Dae-Dong, S. Korea [Nara, Japan]	131	AF364822 ^B	AY739543, AF492964 ^K (1)	AF493031 ^K	AF492897 ^K
	<i>beaticola</i> (Tkalcü)	[Nagano, Japan]			AF492963 ^K	AF493030 ^K	AF492896 ^K
	<i>bifarius</i> Cresson	New Mexico, USA [Seattle, USA]	208	AY737325	AY739547, AF492943 ^K (1)	AF493010 ^K	AF492876 ^K
	<i>bimaculatus</i> Cresson	Arkansas, USA	218	AY737326	AY739548	AY739456	AY739500
	<i>biroi</i> Vogt	Ketmen Mts., Kazakhstan	210	AY737327	AY739549	AY739457	
	<i>brodmannicus</i> Vogt	Artvin Prov., Turkey	077	AY737328	AY739550	AY739458	AY739501

(continued next page)

Table 1. (continued)

Subgenus	Species	Collection locality	No.	<i>I6S</i>	<i>EF-1α</i>	<i>opsin</i>	<i>ArgK</i>
	<i>caliginosus</i> (Frison)	California, USA [Seattle, USA]	150	AY737329	AY739551, AF492968 ^K (0)	AF493035 ^K	AF492901 ^K
	<i>centralis</i> Cresson	Washington, USA [Colorado, USA]	146	AY737330	AY739552, AF492981 ^K (2)	AY739459	AY739502
	<i>cingulatus</i> Wahlberg	[Kamchatka, Russia]			AF492948 ^K	AF493015 ^K	AF492881 ^K
	<i>ephippiatus</i> Say	Chiapas, Mexico	198	AY737333	AY739554	AY739462	AY739505
	<i>flavescens</i> (Smith)	Mei-fang, Taiwan [Habon, Taiwan]	181	AY737337	AF492950 ^K	AF493017 ^K	AF492883 ^K
	<i>flavifrons</i> Cresson	California, USA [Seattle, USA]	095	AY737338	AF492949 ^K	AF493016 ^K	AF492882 ^K
	<i>frigidus</i> Smith	Alaska, USA	185	AY737339	AY739559	AY739466	AY739509
	<i>haematurus</i> Kriechbaumer	Trabzon, Turkey	211	AY739613			
	<i>huntii</i> Greene	Washington, USA [Colorado, USA]	151	AY737344	AY739563, AF492978 ^K (0)	AF493045 ^K	AF492911 ^K
	<i>hypnorum</i> (Linnaeus)	Klösterle, Austria [Piemonte, Italy]	078	AY737346	AY739566, AF492946 ^K (1)	AF493013 ^K	AF492879 ^K
		Sichuan, China	207	AY739614 (9)	AY739567 (5,4)		
	<i>impatiens</i> Cresson	Illinois, USA [Quebec, Canada]	060	AY737349	AY739570, AF492942 ^K (0)	AF493009 ^K	AF492875 ^K
	<i>infirmus</i> (Tkalčů)	Sichuan, China	157	AY737350	AY739571	AY739471	AY739514
	<i>infrequens</i> (Tkalčů)	Sichuan, China	140	AY737351	AY739572	AY739472	AY739515
	<i>jonellus</i> (Kirby)	Lapland Co., Sweden	079	AY737352	AY739573	AY739473	AY739516
	<i>lapponicus</i> (Fabricius)	Kiruna, Sweden	103	AY737356	AY739577	AY739476	AY739519
	<i>lemniscatus</i> Skorikov	Sichuan, China	161	AY737357	AY739578	AY739477	AY739520
	<i>lepidus</i> Skorikov	Sichuan, China	155	AY737358	AY739579	AY739478	AY739521
	<i>luteipes</i> Richards	Pokhara, Nepal	195	AY739615			
	<i>melanopygus</i> Nylander	California, USA [Seattle, USA]	215	AY737362	AY739583, AF492944 ^K (0)	AF493011 ^K	AF492877 ^K
	<i>mixtus</i> Cresson	Washington, USA [Seattle, USA]	024	AY737365	AY739586, AF492947 ^K (3)	AF493014 ^K	AF492880 ^K
	<i>modestus</i> Eversmann	Sichuan, China	160	AY737367	AY739588	AY739482	AY739525
	<i>monticola</i> Smith	Eyne, France	176	AY737368	AY739589	AY739483	AY739526
	<i>perplexus</i> Cresson	Ontario, Canada [Quebec, Canada]	166	AY737373	AY739594, AF492945 ^K (0)	AF493012 ^K	AF492878 ^K
	<i>picipes</i> Richards	Sichuan, China	180	AY737374	AY739595	AY739487	AY739530
	<i>pratorum</i> (Linnaeus)	Klösterle, Austria [Sunningdale, UK]	075	AY737375	AF492966 ^K	AF493033 ^K	AF492899 ^K
	<i>pyrenaes</i> Pérez	Gurgltal, Austria	035	AY737376	AY739596	AY739488	AY739531
	<i>sitkensis</i> Nylander	California, USA	144	AY737379	AY739599	AY739490	AY739533
	<i>sonani</i> (Frison)	[Alishan, Taiwan]			AF492951 ^K	AF493018 ^K	AF492884 ^K
	<i>sylicola</i> Kirby	New Mexico, USA	108	AY737384	AY739604	AY739493	AY739536
	<i>ternarius</i> Say	Nova Scotia, Canada [New York, USA]	116	AY737385	AF492979 ^K	AF493046 ^K	AF492912 ^K
	<i>vagans</i> Smith	Wisconsin, USA	044	AY737388	AY739607	AY739494	AY739537
	<i>vandykei</i> (Frison)	Washington, USA [California, USA]	149	AY737389	AY739608, AF492982 ^K (0)	AF493049 ^K	AF492915 ^K
	<i>vosnesenskii</i> Radoszkowski	Washington, USA [California, USA]	112	AY737391	AY739610, AF492980 ^K (0)	AF493047 ^K	AF492913 ^K
	<i>wilmattae</i> Say	Chiapas, Mexico	199	AY737392	AY739611	AY739496	AY739539
<i>Rhodobombus</i>	<i>mesomelas</i> (Gerstaecker)	Switzerland [L'Aquila, Italy]	037	AY737364	AY739585, AF492936 ^K (4)	AF493003 ^K	AF492869 ^K
<i>Robustobombus</i>	<i>hortulanus</i> Friese	Magdalena, Colombia	200	AY737342	AY739562	AY739468	AY739511
<i>Rufipedibombus</i>	<i>eximius</i> Smith	Alishan, Taiwan	049	AY737335	AY739557	AY739464	AY739507
<i>Separatobombus</i>	<i>griseocollis</i> (DeGeer)	Illinois, USA [New York, USA]	082	AY737341	AY739561, AF492972 ^K (0)	AF493039 ^K	AF492905 ^K
<i>Sibiricobombus</i>	<i>sulfureus</i> Friese	Kayseri, Turkey	064	AY737383	AY739603	AY739492	AY739535
<i>Subterraneobombus</i>	<i>subterraneus</i> (Linnaeus)	Uppland Co., Sweden [L'Aquila, Italy]	046	AY737382	AY739602, AF492960 ^K (0)	AF493027 ^K	AF492893 ^K
<i>Thoracobombus</i>	<i>humilis</i> Illiger	Llo, France	056	AY737343	AY739563	AY739469	AY739512

^K = Kawakita *et al.* (2003); ^C = Cameron and Williams (2003); ^B = J. S. Bae *et al.*, 2001, GenBank (unpublished).

Ascher *et al.* 2001), including relationships among some *Bombus* (Cameron and Williams 2003). We used *opsin* primers developed by Mardulyn and Cameron (1999) to obtain ~680 bp, including two introns totaling 178 bp. Although *opsin* occurs in two copies in bees (Spaethe and Briscoe 2004), only the LW *Rh1* copy was amplified in this study.

ArgK is a phosphogen kinase similar to the vertebrate creatine kinase. Collier (1990) found *ArgK* evolved more slowly and had lower heterozygosity than seven other proteins in *Drosophila melanogaster*. This gene was developed for *Bombus* by Kawakita *et al.* (2003), thus some sequences were available from GenBank for this study. To generate *ArgK* sequences, we used the first of their two pairs of unlabelled primer sequences, from which we obtained ~860 bp containing an intron ~325 bp in length.

In addition to *ArgK* sequences, we obtained several *EF-1 α* and *opsin* sequences from GenBank (shown in Table 1), submitted mostly by Kawakita *et al.* (2003). We sequenced most of our taxa for *EF-1 α* and confirmed that these taxa corresponded to the same species as those sequenced by Kawakita *et al.* (2003) by comparing base pair differences (Table 1) and constructing a phylogeny using Bayesian analysis. We renamed two of the species from GenBank based on their geographic distribution: *B. parthenius* from Taiwan was renamed *B. sonani* and *B. nevadensis* Cresson from the eastern United States was renamed *B. auricomus* (Robertson).

DNA extraction, amplification and sequencing

We extracted tissue from specimens preserved in 95–100% ethanol maintained at 4°C. Thoracic muscle was removed through a small opening cut into the pleuron, thereby keeping the specimens intact as vouchers. We used legs and mesosomal muscle to extract DNA from a few pinned specimens (*B. luteipes* Richards, *B. biroi* Vogt, *B. haematurus* Kriechbaumer) with a maximum age of 21 years (*B. luteipes*). Tissue was digested for four or more hours in proteinase K at 45–55°C. DNA was extracted using either a standard phenol-chloroform protocol or a QIAGEN DNeasy® Tissue Kit (QIAGEN, Valencia, CA, USA).

Standard conditions for PCR amplification were: initial denaturation for 3 min. at 94°C; 36 cycles of 60 s denaturation at 94°C, 60 s annealing at 48–60°C and 60 s elongation at 68 or 72°C; and a final extension for 5 min. at 72°C. Annealing/elongation temperatures for each gene were 48°C/68°C for *16S*, 53–56°C/72°C for *EF-1 α* , 57–60°C/72°C for *opsin* and 48–50°C/72°C for *ArgK*. We used Eppendorf MasterTaq® or HotMaster™ Taq (Eppendorf, Westbury, NY, USA) polymerase for most reactions. Polymerase chain reaction products were purified primarily with the QIAGEN QIAquick® PCR Purification Kit. *ArgK* primers often yielded extra, shorter fragments and the *opsin* and *ArgK* primers were prone to primer-dimers. In these cases, PCR products were purified by gel extraction using the QIAGEN QIAquick® Gel Extraction Kit. We performed cycle-sequencing reactions on both forward and reverse strands using Applied Biosystems BigDye® Terminator version 3.0 or version 3.1 (Applied Biosystems, Foster City, CA, USA) and PCR primers. Sequencing products were purified using either an ethanol precipitation and sequenced with an ABI 377 sequencer (Applied Biosystems; W. M. Keck Center for Comparative Genomics at the University of Illinois, Urbana, IL, USA) or were sent to the Keck Center for purification and direct sequencing on an ABI 3730XL sequencer (Applied Biosystems).

Alignment

We edited and aligned sequences in BioEdit version 5.0.9 (Hall 1999) and manually adjusted them. For *16S*, all questionably aligned regions, including ~50 bp from four hypervariable AT-rich regions, were excluded from the analysis. All alignments were edited again after compilation to confirm rare base substitutions and assess *EF-1 α* bases that differed from those reported by Kawakita *et al.* (2003). Sequences are available in GenBank (accession numbers in Table 1).

Phylogenetic analyses

We analysed each gene separately and in combination, using Bayesian and maximum parsimony methods. Two taxa represented solely by *16S* (*B. luteipes*, *B. haematurus*) were excluded from combined analyses because they were missing a substantial amount of data. For *ArgK* and *EF-1 α* , each gap region was coded as a single character weighted equally to a base substitution using the simple coding method of Simmons and Ochoterena (2000). All trees were rooted with the subgenus *Mendacibombus* Skorikov (*B. mendax*), which was indicated as the sister-group to the remaining *Bombus* subgenera by morphological (Williams 1985, 1994; Ito 1985) and molecular data (Kawakita *et al.* 2003).

Bayesian inference

Bayesian analyses were implemented in MrBayes version 3.0b4 (Ronquist and Huelsenbeck 2003) using models based on Akaike information criteria (AIC) in Modeltest (Posada and Crandall 1998). Each gene was partitioned into exons, introns and gap characters when relevant. The models applied to each partition were: *16S* (general time reversible (GTR) + proportion of invariable sites (I) + gamma distribution (Γ)), *EF-1 α* intron (GTR+ Γ), *EF-1 α* exon (GTR+I+ Γ), *EF-1 α* gap characters (standard morphology), *opsin* intron (GTR), *opsin* exon (Hasegawa–Kishino–Yano (HKY) + Γ), *ArgK* intron (GTR+I), *ArgK* exon (GTR+I) and *ArgK* gap characters (standard morphology). Three independent Bayesian analyses were run for each gene. Two runs were performed with 2 000 000 generations and four chains, using flat priors and mixed models, saving trees every 100 generations; a third analysis was run for 1 000 000 generations, with all other variables as in the first two runs. We plotted log-likelihood values to examine the point at which they reached stationarity and discarded all trees before this point (burn-in). Trees from the first 499 900 generations (5000 trees), a conservative estimate of burn-in for all analyses, were removed from each run. Trees remaining after burn-in from all three runs converged on similar values and were combined for a total of 35 003 trees for each gene. All genes and partitions were combined and run as a single analysis in MrBayes using 4 000 000 generations, eight chains, trees saved every 100 generations, flat priors, mixed models and a burn-in of 5000 trees. The combined analysis was run on an IBM p-series 690 supercomputer at the National Center for Supercomputing Applications (University of Illinois Urbana-Champaign, Champaign, IL). An additional analysis with 2 000 000 generations, four chains and a burn-in of 5000 trees resulted in the same tree topology. Clade support was estimated for each analysis using posterior probabilities calculated from Bayesian analyses.

Parsimony analyses

Strict consensus trees were constructed from analyses of individual genes and from all genes combined using parsimony criteria in PAUP* (Swofford 2001). For the single-gene trees, heuristic searches were performed using 1000 random additions (RA) and TBR branch swapping, keeping a maximum of 500 trees per RA $\geq$ a tree length of 1. For all analyses except *ArgK*, 1000 random additions were performed without exceeding tree storage capabilities. *ArgK* was analysed saving a maximum of 75 trees per RA sequence for 1000 RA. The analysis of all genes combined was performed with 1000 RA and TBR branch swapping, employing no maximum tree limits. Clade support values for parsimony analyses were estimated using nonparametric bootstrapping calculated in PAUP* (1000 replicates, simple addition, $\leq$ 500 trees saved per replicate) and Bremer support values (Bremer 1988) calculated in TreeRot version 2 (Sorenson 1999). To determine the congruence and combinability of individual gene datasets we performed incongruence length difference (ILD) tests (Farris *et al.* 1995) on gene pairs in PAUP* (100 replicates; heuristic search with 10 RA, TBR branch swapping, a maximum of 500 trees $\geq$ a tree length of 1 saved per RA) after removing uninformative characters.

Gene utility

To determine the contribution of each gene and intragene partition to the phylogeny based on the combined genes, we calculated partitioned Bremer support (PBS) (Baker and DeSalle 1997) for three partitioning levels (gene, intron/exon and codon position) using TreeRot version 2 (Sorenson 1999). The PBS values for each partition were standardised by dividing by the minimum number of steps for that partition (PBS/min), as done by Baker *et al.* (2001). This measures the contribution of each partition to the resulting tree topology relative to the amount of phylogenetic information (minimum steps) provided. PBS values also provide information on concordance among the partitions, with negative values for a partition indicating support for an alternative relationship to that supported by the combined dataset. Overall tree resolution provided by each gene was estimated by counting the total number of resolved nodes. Overall clade support was measured by counting the number of nodes with posterior probabilities 1) ≥ 0.75 and 2) ≥ 0.95 (Bayesian) and the number of nodes with bootstrap values 1) ≥ 50 and 2) ≥ 70 (parsimony). These support levels were chosen based on the suggested equivalency of a 70 bootstrap value to a 95% confidence interval by Hillis and Bull (1993). Rates of nucleotide substitutions for each partition were compared using uncorrected pairwise distance ranges and the number of parsimony informative characters relative to the total number of characters (obtained in PAUP*). Coded gap characters were not included in the number and percent parsimony informative characters for each gene. Homoplasy in each gene was measured by the consistency index (CI) and retention index (RI) from the individual gene analyses. It was also calculated in a separate run for the each of the gap character partitions. Because high AT bias effectively reduces the number of character states available and thus can contribute to higher levels of homoplasy under similar substitution rates, the percentage of A+T nucleotides in each partition was calculated using base frequencies obtained in PAUP* or MacClade (Maddison and Maddison 2000). This was done with the hypervariable regions included for *16S*.

Results

Data characteristics

The *EF-1 α* alignments contain few questionably aligned and, therefore, questionably gap-coded regions. *16S* (excluding hypervariable indel regions) and *opsin* were easily aligned because they have no parsimony-informative gaps. *ArgK*, in spite of several long indels, was straightforward to align, with the exception of sequences for *B. ignitus*, which had a unique 30 bp region with alignment ambiguities and *B. mendax* Gerstaecker, *B. confusus* Schenck and *B. auricomus*, which contain long indels (such as a unique 325 bp region in *B. auricomus*) of uncertain alignment in the intron sequences. The unalignable regions were removed and treated as missing data (Kawakita *et al.* 2003). There were 11 parsimony informative indels for *ArgK* and 19 for *EF-1 α* . Of 2861 total characters in the combined analysis, 527 characters were parsimony informative, 114 belonging to *16S*, 154 to *EF-1 α* , 92 to *opsin* and 138 to *ArgK* (Table 2). There were 0–4 nucleotide differences between our *EF-1 α* sequences for a given species and those reported by Kawakita *et al.* (2003) for the same species (Table 1). In most cases, the same species from the two studies occur as sister taxa or as unresolved relative to other species (phylogeny not shown). Two species that are not resolved as sister taxa when comparing our sequences to those of Kawakita *et al.* (2003) are *B. patagiatus* Nylander and *B. centralis* Cresson, but this is based on only a few nucleotide differences (Table 1).

Table 2. Summary of gene utility tests

Partition	% A+T	CI	RI	PI chars	Total chars	% PI chars	MP trees	Resolved nodes (P/P ₅₀ /P ₇₀ ;B/B ₇₅ /B ₉₅)	Pairwise distance range	PBS	PBS/min
<i>16S</i>	78.6	0.216	0.560	114	461	24.7	4005	64/20/13; 45/33/17	0.004–0.123	94.41	0.11
<i>EF-1α</i>	58.5	0.416	0.761	154	761	20.2	241	52/33/22; 45/36/25	0.000–0.095	136.99	0.22
<i>opsin</i>	60.7	0.587	0.839	92	680	13.5	18523	35/33/21; 44/40/29	0.000–0.063	78.60	0.28
<i>ArgK</i>	59.7	0.538	0.798	138	929	14.9	70878	41/32/18; 54/45/30	0.000–0.067	131.39	0.30
<i>opsin</i> intron	75.3			39	178	21.9				58.16	0.48
<i>opsin</i> exon	55.5			53	502	10.6				20.46	0.13
<i>opsin</i> pos1	61.0			8	167	4.8				5.50	0.20
<i>opsin</i> pos2	59.8			5	167	3.0				5.00	0.45
<i>opsin</i> pos3	45.8			40	168	23.8				9.01	0.08
<i>EF-1α</i> intron	67.9			69	238	29.0				36.33	0.15
<i>EF-1α</i> exon	54.9			85	523	16.3				69.98	0.20
<i>EF-1α</i> pos1	40.6			3	175	1.7				2.77	0.29
<i>EF-1α</i> pos2	58.0			2	174	1.1				–3.15	–0.49
<i>EF-1α</i> pos3	66.2			80	174	46.0				71.24	0.22
<i>EF-1α</i> gap		0.633	0.890	19	19	100.0				30.26	0.90
<i>ArgK</i> intron	74.5			98	399	24.6				87.58	0.30
<i>ArgK</i> exon	50.9			40	530	7.5				36.39	0.28
<i>ArgK</i> pos1	43.1			3	177	1.7				1.00	0.13
<i>ArgK</i> pos2	62.5			0	176	0.0				0.00	0.00
<i>ArgK</i> pos3	47.2			37	177	20.9				34.37	0.29
<i>ArgK</i> gap		0.917	0.968	11	11	100.0				9.16	0.59
Combined	62.4	0.356	0.689	527	2861	18.4	16	73/58/46; 75/63/50	0.001–0.065	441.00	1.00

CI, consistency index; RI, retention index; PI chars, no. parsimony informative characters; MP trees, no. most parsimonious trees; P, no. nodes resolved in parsimony tree; B, no. nodes resolved in Bayesian tree; P_x, no. nodes in parsimony tree with bootstrap values $\geq X$; B_x, no. nodes in Bayesian tree resolved with posterior probabilities $\geq X$; PBS, partitioned Bremer support; PBS/min, partitioned Bremer support/minimum no. steps.

Tree resolution

The phylogenies based on the individual nuclear genes are relatively well resolved and well supported (Fig. 1B–D; single gene parsimony phylogenies not shown). For *16S*, the Bayesian tree is unresolved at the basal nodes (Fig. 1A). The *16S* parsimony strict consensus tree is mostly resolved (tree not shown), but only 20 of the 64 nodes are supported with bootstrap values ≥ 50 (Table 2) and all support falls near the tips of the tree. Results from the ILD tests indicate no significant incongruence between the datasets (*EF-1 α* v. *opsin*, $P = 0.88$; *EF-1 α* v. *ArgK*, $P = 0.97$; *opsin* v. *ArgK*, $P = 0.89$; *16S* v. *ArgK*, $P = 0.17$; *16S* v. *opsin* = 0.64) when the $P < 0.01$ significance level is used (Cunningham, 1997), but when $P < 0.05$ is used *16S* and *EF-1 α* ($P = 0.04$) are incongruent. Because *16S* provides valuable information at the tips of the tree and its incongruence was not consistently or strongly supported, all four datasets were combined in analyses.

The combined data from the four genes provide nearly completely resolved Bayesian (Fig. 2) and maximum parsimony (Fig. 3) phylogenies with many high clade support values among species (Table 2). The strict consensus parsimony tree of the combined data is based on 16 most parsimonious trees; many fewer than the number of trees obtained from individual gene analyses (Table 2).

Phylogenies inferred from the combined-gene parsimony and Bayesian analyses are largely congruent, with inconsistencies occurring in regions of poor support (bootstrap values (BV) ≤ 59). For example, Bayesian and parsimony results differ topologically with respect to their identification of the species-group comprising the root of the *Pyrobombus* clade: Bayesian analysis identifies *B. flavifrons* Cresson–*B. vagans* Smith (posterior probability (PP) = 0.74) and parsimony identifies *B. huntii* Greene–*B. melanopygus* Nylander (BV < 50). However, the low support values for both Bayesian and parsimony analyses suggest this node is effectively unsupported. Another incongruence occurs within the clade *B. lapponicus*–*B. monticola* Smith: with parsimony, *B. lapponicus* and *B. bimaculatus* Cresson are sister taxa (BV = 59); with Bayesian analysis, *B. lapponicus* and *B. sylvicola* are sister taxa (PP = 0.63). PBS values and individual gene tree topologies (Fig. 1A, B, D) indicate that (*B. bimaculatus*+*B. lapponicus*)+*B. monticola* is supported only by *16S* data and is contradicted by *EF-1 α* and *ArgK*, which support a sister-group relationship between *B. lapponicus* and *B. sylvicola*. *Bombus lapponicus* and *B. sylvicola* are considered conspecific by Williams (1998).

Subgeneric relationships and monophyly

Pyrobombus monophyly is supported by the individual nuclear-gene trees (*EF-1 α* : posterior probability (PP) = 1.00, bootstrap (BV) = 90; *opsin*: PP = 0.99, BV = 54; *ArgK*: PP = 0.90, BV ≤ 50) and is strongly supported in the combined analyses (PP = 1.00 and BV = 99). The *16S* parsimony tree

(not shown) results in a *Pyrobombus* clade with several other subgenera contained within it. The *16S* Bayesian tree (Fig. 1A) suggests paraphyly of *Pyrobombus* only through a poorly supported sister relationship with the *Psithyrus* clade.

The clade *Bombus s.s.*+*Alpinobombus* is a well supported monophyletic clade in the combined analyses (PP = 1.00, BV = 99). This clade is the sister-group to *Pyrobombus* in both the combined analyses and in each of the nuclear gene trees (*EF-1 α* : PP = 1.00, BV = 95; *opsin*: PP = 1.00, BV = 70; *ArgK*: PP = 1.00, BV = 68). The monophyly of *Bombus s.s.* and *Alpinobombus* individually is also well supported (PP = 1.00/1.00, BV = 100/99, respectively). *Melanobombus* is monophyletic and more distantly related to *Pyrobombus*.

Specific and intraspecific relationships

Our Bayesian results show strong support (PP = 1.00) for six *Pyrobombus* species-groups: the *flavifrons*, *lapponicus*, *ternarius*, *parthenius*, *hypnorum* and *pratorum*-groups (Fig. 4). Well supported higher groups include a clade comprising the *hypnorum*, *pratorum* and *parthenius*-groups (PP = 0.99, BV = 54) and a clade comprising the *lapponicus* and *ternarius*-groups (PP = 1.00, BV = 79). The *parthenius*-group is only strongly supported by the Bayesian analysis.

Although *B. ephippiatus* and *B. wilmattae* were considered conspecific by Williams (1998), they were resolved as (*B. impatiens*+*B. wilmattae*)+*B. ephippiatus* in this study. PBS values and tree topologies (Fig. 1A, C, D) reveal that *B. impatiens*+*B. wilmattae* is highly supported by *16S*, but is contradicted by *opsin* (which supports *B. ephippiatus*+*B. wilmattae*) and *ArgK* (which supports *B. impatiens*+*B. ephippiatus*).

There are five nucleotide differences (0.7% pairwise distance) in *EF-1 α* between *B. hypnorum* (subgenus *Pyrobombus*) from Europe and China and nine differences (1.8%) for *16S* (Table 1). This is more intraspecific variation than found between all comparisons of *EF-1 α* sequences of our specimens with those of Kawakita *et al.* (2003, 2004) and is equal to the number of *16S* bp differences observed between *B. lucorum* specimens from China and Europe. *B. hypnorum* from Austria and Italy are sister populations in the *EF-1 α* Bayesian tree (phylogeny not shown), but *B. hypnorum* from China could not be resolved relative to the North American *B. perplexus* Cresson. For *16S*, Chinese and European specimens of *B. hypnorum* are sister clades separate from *B. perplexus* (Fig. 1A). The considerable genetic variability between Chinese and European specimens of *B. hypnorum* suggests that more than one species could be involved, although the possibility that this is a single widespread species with DNA variation must be considered.

Most of the phylogenetic structure within *Bombus s.s.* receives good support in the combined Bayesian phylogeny although many of these clades are not strongly supported with parsimony and are somewhat inconsistent between genes (Figs 1, 2, 3). Our results expand the *lucorum*-complex

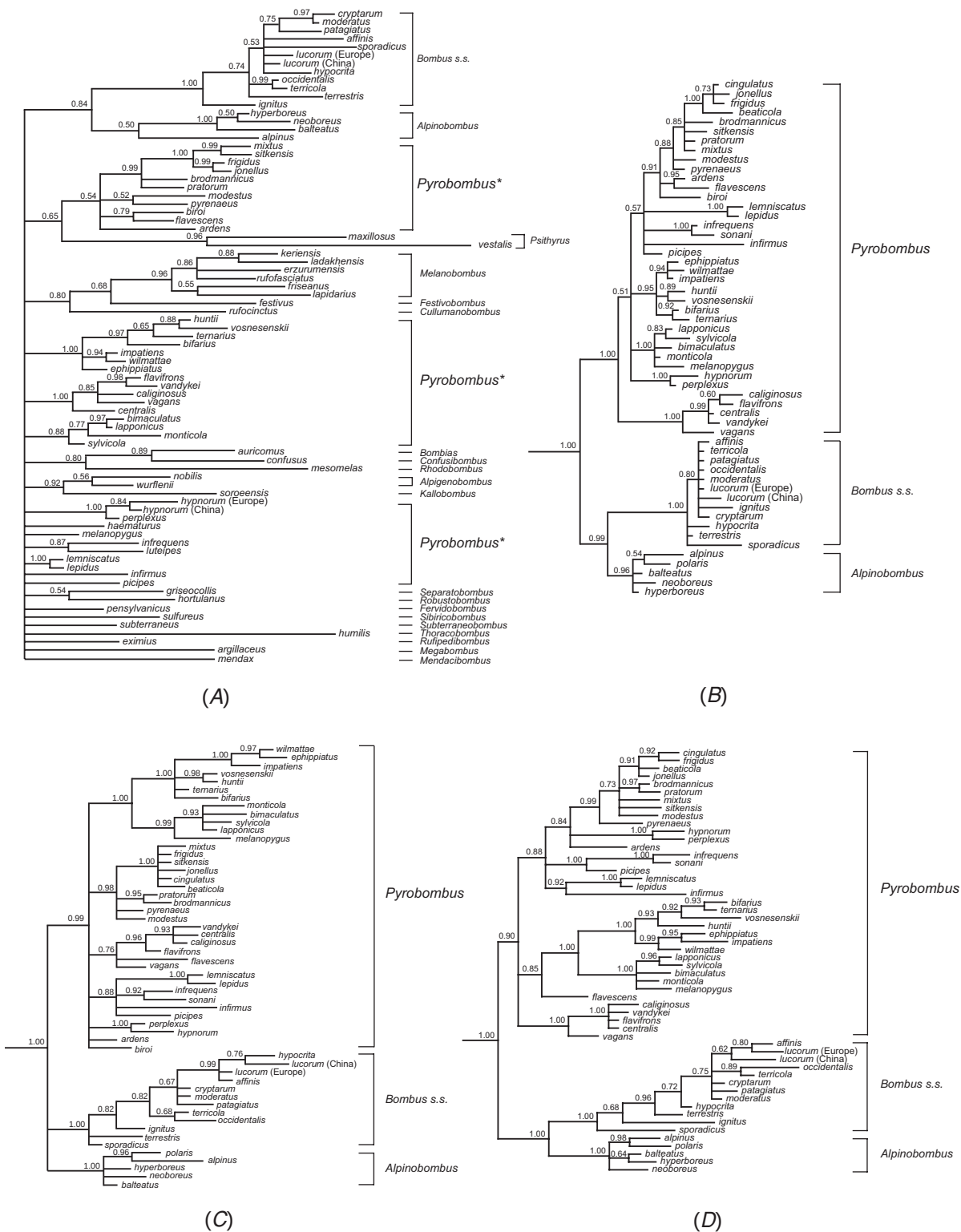


Fig. 1. Bayesian individual gene phylogenies. A, 16S; B, EF-1α; C, opsin; D, ArgK. Each phylogeny is estimated from 35 000 trees (2 runs: 2 000 000 generations, four chains, sampling every 100 trees, burn-in = 5 000 trees; 1 run: same but 1 000 000 generations) using flat priors, models specified in Modeltest using Akaike information criteria (AIC) and partitioning by exons, introns and gap characters when appropriate. Clade support values are Bayesian posterior probabilities. Asterisks indicate clades not resolved as monophyletic. Taxa external to the *Pyrobombus*, *Bombus s.s.* and *Alpinobombus* clades have been pruned for EF-1α, opsin and ArgK trees, but could not be removed for 16S because *Pyrobombus* is not resolved as monophyletic.

to include all but four of the *Bombus* s.s. taxa (Figs 2, 3). Most notably, *Bombus lucorum* from China differs from *B. lucorum* s.s. from Europe by 21 bp (*16S*: 9, *EF-1 α* : 3, *opsin*: 3, *ArgK*: 6) and they are polyphyletic in combined analyses (Figs 2, 3). The European *B. lucorum* is sister to *B. affinis* Cresson, an eastern Nearctic species previously not considered a member of the *lucorum*-complex. Support

values for relationships within *Alpinobombus* are high in both Bayesian and parsimony analyses with the exception of the lack of good support between *B. balteatus*, *B. neoboreus* and *B. hyperboreus*.

Gene utility

Gene utility statistics are given in Table 2.

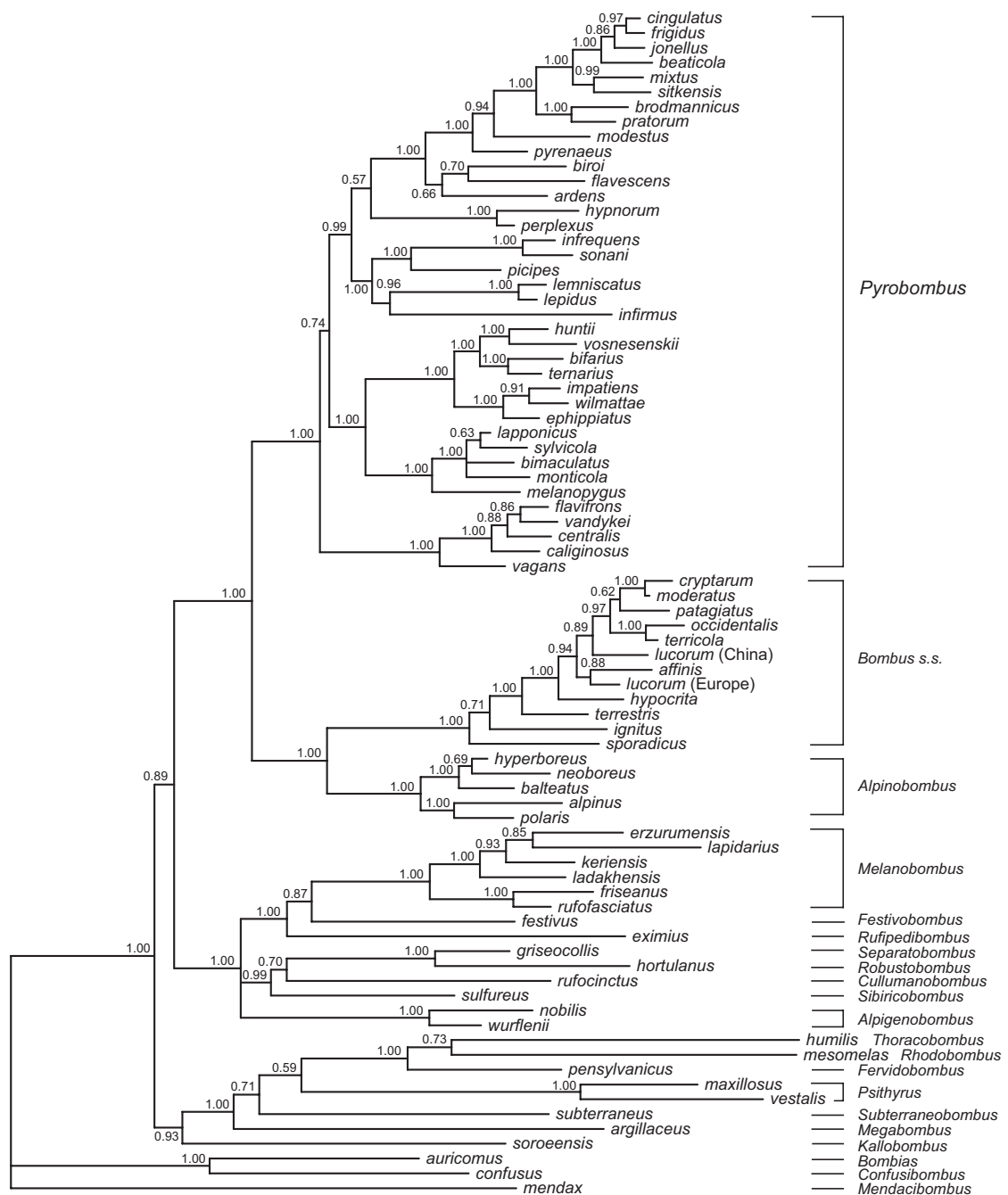


Fig. 2. Bayesian phylogeny based on the combined dataset (*16S* + *EF-1 α* + *opsin* + *ArgK*). Phylogeny based on 35001 trees (4000000 generations; 8 chains; sampling every 100 trees; burn-in = 5000 trees) using flat priors, mixed models and partitioning by gene, exon/intron and gap characters when applicable. Clade support values are Bayesian posterior probabilities.

16S. *16S* has the highest percentage of parsimony informative characters (24.7%) and the highest pairwise distances (≤ 0.123). It also has the highest level of homoplasy ($RI = 0.560$) and AT bias (78.6%), exceeding those of the nuclear genes and partitions. Although trees from *16S* are equally resolved (Bayesian analysis) or more so (parsimony analysis) than those estimated from individual nuclear gene data, they have fewer well supported clades (e.g. 13 clades

have $BV \geq 70$, compared to 22, 21 and 18 for nuclear genes). *16S* has a low PBS value (lower only in *opsin*) and the lowest PBS/min.

EF-1 α . *EF-1 α* exhibits the highest rate of substitutional change among the nuclear genes, as indicated by the percentage of parsimony informative characters (20.2%) and pairwise distances (≤ 0.095). Third positions and introns contain 97% of the parsimony informative characters,

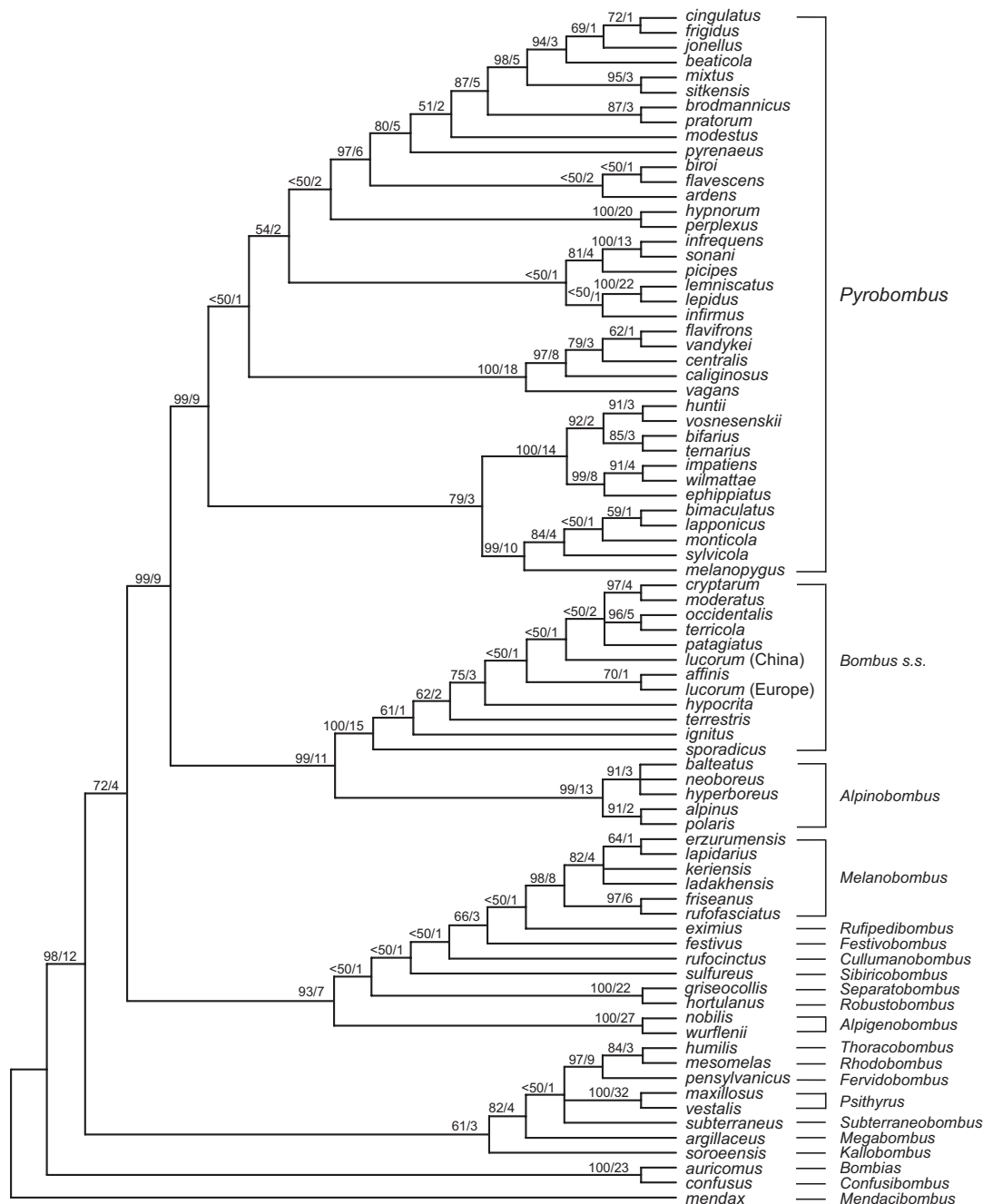


Fig. 3. Parsimony-based phylogeny of the combined dataset (*16S* + *EF-1 α* + *opsin* + *ArgK*). Strict consensus tree of 16 most parsimonious trees obtained using a heuristic search (1000 random additions, tree bisection reconnection (TBR) branch swapping). Numbers above branches indicate bootstrap values in front of the slash followed by Bremer support values.

although they comprise only 54% of the total characters. It is the most homoplasious of the nuclear genes ($RI = 0.761$) and, accordingly, the PBS/min value is the lowest among the three nuclear genes. Nonetheless, *EF-1 α* results in the highest gene tree resolution of the nuclear genes using parsimony and contributes most to resolving the combined phylogeny (indicated by PBS). This is probably because it provides more total parsimony informative characters than any other gene. *EF-1 α* is only slightly AT biased (58.5%). For all three nuclear genes, the AT bias is higher in the introns ($\bar{X} = 72.6\%$) than the exons ($\bar{X} = 53.8\%$).

Opsin. *Opsin* provides the least tree resolution of the nuclear genes and the lowest PBS value of all genes. Yet, the PBS/min for *opsin* is the second highest among the genes, probably because it has the lowest level of homoplasy ($RI = 0.839$). The highest percentage of parsimony informative characters and most of the contribution to the *opsin* phylogeny comes from the introns, which have the greatest PBS and PBS/min values within the gene. The percentage of parsimony informative characters for codon positions 1 and

2 is higher, both overall and relative to third codon positions, than for those of *ArgK* and *EF-1 α* .

ArgK. Of the parsimony informative characters for *ArgK*, 71% are confined to the intron, which comprises only 43% of the total characters. *ArgK* exons are more conserved than those of the other nuclear genes, having only 7.5% parsimony informative characters and no informative characters in second codon positions. The overall substitution rate of *ArgK*, as measured by pairwise distance ranges (≤ 0.067) and percent parsimony informative characters (14.9%) falls between those of *opsin* (≤ 0.063 , 13.5%) and *EF-1 α* (≤ 0.095 , 20.2%), as does the level of homoplasy ($RI = 0.798$). The Bayesian *ArgK* tree has the highest level of resolution of any of the gene trees. *ArgK* has PBS values lower only than *EF-1 α* and contributes the highest PBS/min of all genes. Although the intron has more than twice the PBS value of the exon, the intron and exon (particularly third positions) provide nearly equal PBS/min.

Gap characters. The highest PBS/min values of all partitions are contained within the *ArgK* and *EF-1 α* gap-coded characters. This suggests that gap characters support

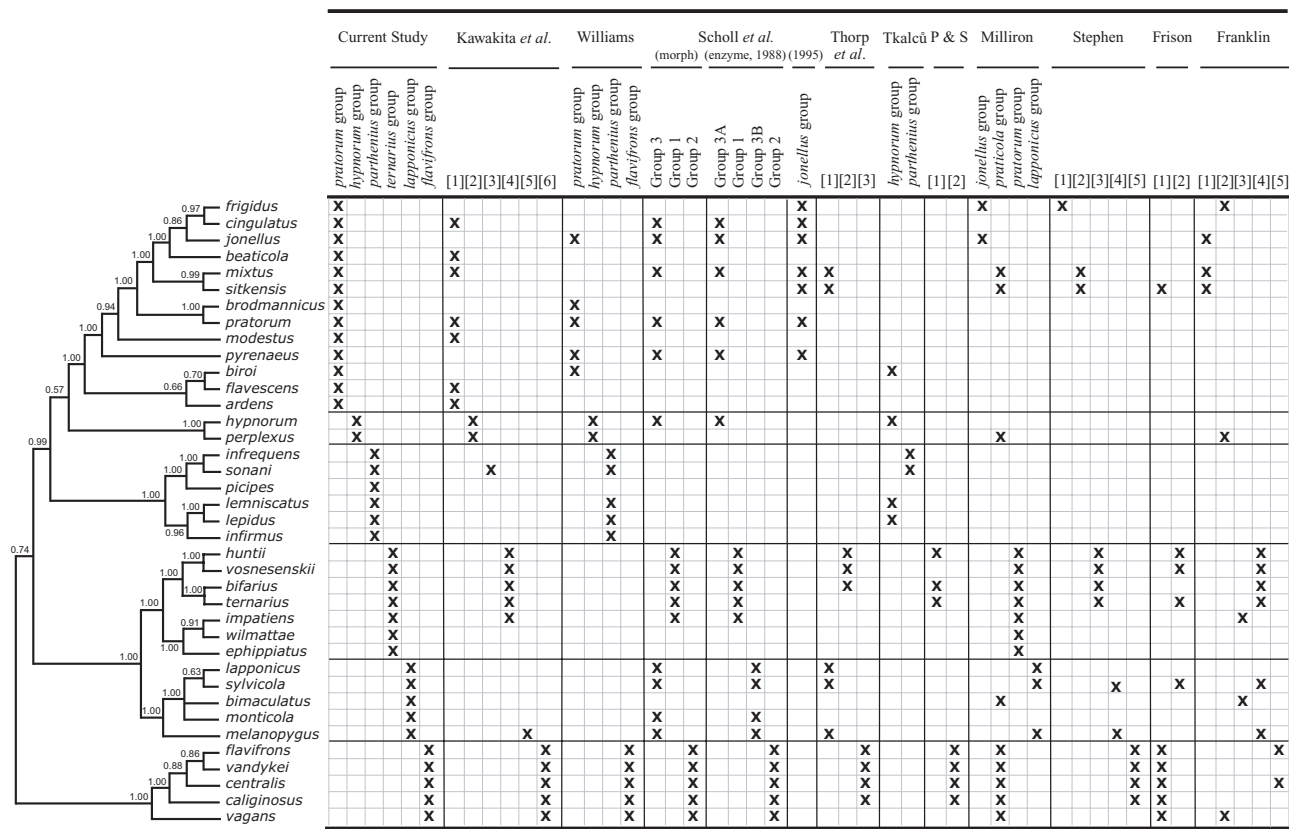


Fig. 4. Synopses of species-groups discernable from the phylogeny in this study (Bayesian phylogeny at left) in relation to species-groups demarcated explicitly by Franklin (1912), Frison (1923, 1927a, 1927b), Milliron (1971), Plowright and Stephen (P & S) (1973), Scholl *et al.* (1988, including some unpublished data presented in a poster (split into results based on morphology or enzymes); 1995), Stephen (1957), Thorp *et al.* (1983), Tkalcu (1974, 1989) and Williams (1991). Although Kawakita *et al.* (2004) did not discuss division of *Pyrobombus* into groups, we have presented results from their phylogeny in relation to our groups to facilitate comparison of general results, including taxon sampling. Groups in brackets were unnamed by the authors.

relationships concordant with those of base-substitution characters, which make a much higher contribution to the combined phylogeny (indicated by PBS values). Gap characters also exhibited the lowest levels of homoplasy ($RI = 0.890$ for *EF-1 α* , 0.968 for *ArgK*). Only two of eleven *ArgK* gap characters contained homoplasy. For *EF-1 α* , 12 of 19 characters contained some homoplasy, but most of these also contained useful phylogenetic signal.

Discussion

Bombus relationships

Our investigation of *Pyrobombus*, which includes 84% of the recognised species, provides strong evidence for its monophyly. *Melanobombus* appears to be more distantly related to *Pyrobombus*, in contrast to previous studies by Plowright and Stephen (1973), Pedersen (1996) and Koulouanos and Schmid-Hempel (2000). The non-monophyly of *Pyrobombus* in these earlier reports is likely the result of insufficient character and taxon representation given that other studies with larger taxon sampling by Pedersen (2002) and Kawakita *et al.* (2004) also recover monophyly.

Historically, *Pyrobombus* has been loosely and inconsistently divided into natural 'groups' or 'complexes' using morphological (e.g. Franklin 1912; Frison 1923, 1927a, b; Stephen 1957; Milliron 1971; Plowright and Stephen 1973; Tkalcu 1974, 1989; Thorp *et al.* 1983; Williams 1991), behavioural (Plowright and Stephen 1973) and enzyme mobility data (Scholl *et al.* 1988, 1995). Only Plowright and Stephen (1973) and Scholl *et al.* (1988, 1995) performed phylogenetic analyses to obtain species-groups. The six species groups supported by our data are compared with those of previous studies in Fig. 4. Several authors have recognised the *ternarius*, *lapponicus* and *flavifrons*-groups, although not consistently by the same names and species contents. The placement of *B. bimaculatus* within the *lapponicus*-group with strong support is a result unique to this study. Scholl *et al.* (1988) and Thorp *et al.* (1983) did not distinguish members of the *lapponicus*-group from the *pratorum*-group using male genitalic characters, although Scholl *et al.* (1988) concluded they were distinct based on enzyme data. Frison (1927a) and Franklin (1912) linked members of the *lapponicus*-group to the *ternarius*-group, indicated also by our results. The *parthenius*-group was similarly recognised by Williams (1991) based on morphology, but he excluded *B. picipes*. The *hypnorum* and *pratorum*-groups have been least consistently classified in the literature (Fig. 4), but the species contents of both groups receive good support in our study. The sister-group relationship between them, however, is not well supported, so we have classified them separately. Scholl *et al.* (1988) also united these two groups based on enzyme mobilities. With respect to the *hypnorum*-group, Williams (1991) noted the similarities between *B. perplexus* and *B. hypnorum*, but also found them

difficult to place relative to other groups based on morphology. Kawakita *et al.* (2004) did not address intrasubgeneric taxonomic issues but we have assigned their taxa to species-groups based on their phylogeny to facilitate comparison between studies (Fig. 4). The groups are largely consistent with our results, despite having approximately half the taxon representation.

We can reliably conclude, on the basis of our relatively thorough taxon sampling of several alleged sister-groups to *Pyrobombus*, that *Bombus s.s.* + *Alpinobombus* is the sister clade. All three of these subgenera include many cold-adapted species and have a Holarctic distribution. Franklin (1954) noted four traits, which he considered convergent, that unite these groups: female-like corbiculae on the male hind tibiae; reduced 'claspers' of the male genitalia; short to average length of male antennal flagellae and an early seasonal lifecycle. Phylogenies constructed from morphological characters have not placed these three groups together, although Plowright and Stephen (1973) grouped *Bombus s.s.* within *Pyrobombus* and Williams (1985, 1994) resolved them as moderately close relatives. Chen and Wang (1997) reported a sister-group relationship between *Bombus s.s.* and *Alpinobombus* but considered them distantly related to *Pyrobombus*.

Within *Bombus s.s.*, the *lucorum* species-complex (including *B. lucorum s.s.*, *B. cryptarum*, *B. magnus*, *B. moderatus*) has a wide distribution throughout most of Eurasia and the north-western Nearctic (Williams 1991: 184). These taxa have been the subject of considerable investigation regarding their species status (e.g. Pekkarinen 1979; de Jonghe and Rasmont 1983; Scholl and Obrecht 1983; Pamilo *et al.* 1984; Rasmont 1984). Our study expands the *lucorum*-complex to include the conventional *B. cryptarum*+*B. moderatus* and *B. lucorum s.s.*, along with *B. affinis*, *B. terricola*+*B. occidentalis* and *B. patagiatus*. The close relationship between *B. cryptarum* and *B. moderatus*, which has also been recovered using enzyme data (Scholl *et al.* 1990), is especially interesting given the considerable geographic distance that separates *B. cryptarum* (Europe and western Asia) and *B. moderatus* (north-western Nearctic). With the uncertainty in identifying Asian members of the traditional *lucorum*-complex (Williams 1991), it is possible that close relatives of these two taxa occur in eastern parts of Asia.

In the past 15 years, bumble bees have been reared on a large commercial scale for greenhouse pollination. *B. impatiens* (subgenus *Pyrobombus*) is reared in the United States and *B. terrestris* (subgenus *Bombus s.s.*) is reared in Europe. These make good species for commercial use because they are widely distributed, produce relatively large colonies, are efficient pollinators for numerous greenhouse crops and are easily reared in captivity. Our results reveal *B. impatiens* to be most closely related to *B. ephippiatus* and *B. wilmattae*. *Bombus impatiens*, which is widely distributed throughout the eastern United States and south-east Canada, is being

imported for pollination purposes into the ranges of its sister species (northern Mexico to north-west South America – *B. ephippiatus*; Chiapas and Guatemala – *B. wilmattae*, Labougle 1990). Their close relationship may enhance inter-specific competition, facilitate the spread of potential parasites carried by *B. impatiens* and could have negative implications if these species can interbreed (cf. Thorp 2003). *B. ephippiatus*, which is widespread and biologically similar to *B. impatiens*, would be an excellent alternative species for commercial rearing within its native range in Mexico.

Gene utility

To generalise about the potential utility of these genes for resolving phylogenetic relationships at a given taxonomic level requires knowledge of the divergence times of *Bombus*. Reliable *Bombus* fossils dating to the Miocene (24–5 million years ago) have been discovered in Russia, China and Washington, USA (Zeuner and Manning 1976; Zhang 1990; Rasnitsyn and Michener 1991). Eocene fossils recovered from Baltic amber (~44 million years old) did not contain *Bombus* (Engel 2001) and Oligocene (34–24 million years ago) fossils questionably belong to *Bombus* (Zeuner and Manning 1976). This places the likely diversification of *Bombus* somewhere between 40 and 20 million years ago. The relatively rapid evolutionary rate of mitochondrial DNA suggests it should be useful for resolving relationships among the recently diverging bumble bees. In fact, the higher substitution rate of *mt16S* resulted in more character homoplasy, which made it difficult to resolve relationships deeper than close intrasubgeneric species-groups. Moreover, *16S* supported several relationships that were inconsistent with the nuclear genes and subgeneric classification. The *16S* fragment was smaller than those of the nuclear genes (~500 bp compared with ~680–860 bp), but adding a few hundred more base pairs would be unlikely to resolve problems with homoplasy in the deeper relationships. The nuclear gene, *opsin*, was rather conserved, providing the lowest PBS values to the combined phylogeny and the least parsimony informative characters across all genes. *ArgK* and *EF-1 α* were the most useful genes, providing a good balance between character information and homoplasy at this lower level.

We found gap-coded characters of *ArgK* and *EF-1 α* to exhibit relatively low levels of homoplasy and to be useful for resolving *Bombus* relationships, as demonstrated in Kawakita *et al.* (2003). However, the introns of *ArgK*, which comprise a large portion of the fragment, were highly variable in length and difficult to align in certain regions for the earlier diverging subgenera *Mendacibombus*, *Confusibombus* Ball and *Bombias* Robertson, so the gaps may be less informative at suprageneric levels. *ArgK* exon regions evolved more slowly than either *opsin* or *EF-1 α* and may be promising for resolving deeper phylogenetic relationships in insects.

Acknowledgments

Special thanks to Pierre Rasmont and Bjorn Cederberg for providing multiple specimens, identifications and guidance. We are also grateful to A. Murat Aytekin, Yvan Barbier, Pierre Rasmont, Michael Terzo, James Whitfield, Chen Xuexin and Tang Ya for providing specimens and help in the field and to the following for providing specimens: J. van Asperen de Boer, Oistein Berg, Won Young Choi, Terry Griswold, Martin Hauser, Randall Hepburn, Kevin Holston, Robin Owen, Claus Rasmussen, Adolf Scholl, Robbin Thorp, Remy Vandame and Natapot Warrit. Thanks to Alice Michel-Salzat, Andy Deans and Sudhakar Pamidighantam for technical assistance and to Kevin Johnson, Gene Robinson and James Whitfield for comments and suggestions. This project was supported by an NRI USDA grant (2002–35302–11553) to S. A. C., the H. H. Ross Memorial award and the Francis M. and Harlie M. Clark grant to H. M. H.

References

- Ascher, J. S., Danforth, B. N., and Ji, S. (2001). Phylogenetic utility of the major opsin in bees (Hymenoptera: Apoidea): a reassessment. *Molecular Phylogenetics and Evolution* **19**, 76–93. doi:10.1006/mpev.2001.0911
- Baker, R. H., and DeSalle, R. (1997). Multiple sources of character information and the phylogeny of Hawaiian drosophilids. *Systematic Biology* **46**, 654–673.
- Baker, R. H., Wilkinson, G. S., and DeSalle, R. (2001). Phylogenetic utility of different types of molecular data used to infer evolutionary relationships among stalk-eyed flies (Diopsidae). *Systematic Biology* **50**, 87–105. doi:10.1080/106351501750107512
- Bremer, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**, 795–803.
- Brower, A. V. Z., and DeSalle, R. (1998). Patterns of mitochondrial versus nuclear DNA sequence divergence among nymphalid butterflies: the utility of wingless as a source of characters for phylogenetic inference. *Insect Molecular Biology* **7**, 73–82. doi:10.1046/j.1365-2583.1998.71052.x
- Cameron, S. A., and Mardulyn, P. (2001). Multiple molecular data sets suggest independent origins of highly eusocial behavior in bees (Hymenoptera: Apinae). *Systematic Biology* **50**, 194–214. doi:10.1080/10635150151125851
- Cameron, S. A., and Mardulyn, P. (2003). The major opsin gene is useful for inferring higher level phylogenetic relationships of the corbiculate bees. *Molecular Phylogenetics and Evolution* **28**, 610–613. doi:10.1016/S1055-7903(03)00055-1
- Cameron, S. A., and Williams, P. H. (2003). Phylogeny of bumble bees in the New World subgenus *Fervidobombus* (Hymenoptera: Apidae): congruence of molecular and morphological data. *Molecular Phylogenetics and Evolution* **28**, 552–563. doi:10.1016/S1055-7903(03)00056-3
- Cameron, S. A., Derr, J. N., Austin, A. D., Wooley, J. B., and Wharton, R. A. (1992). The application of nucleotide sequence data to phylogeny of the Hymenoptera: a review. *Journal of Hymenopteran Research* **1**, 63–79.
- Chen, X., and Wang, S. (1997). A study on phylogenetic relationships among the subgenera of bumble bees (Hymenoptera: Apidae). *Acta Entomologica Sinica* **4**, 325–336.

- Cho, S., Mitchell, A., Regier, J. C., Mitter, C., Poole, R. W., Friedlander, T. P., and Zhao, S. (1995). A highly conserved nuclear gene for low-level phylogenetics: elongation factor-1 α recovers morphology-based tree for heliothine moths. *Molecular Biology and Evolution* **12**, 650–656.
- Collier, G. E. (1990). Evolution of arginine kinase within the genus *Drosophila*. *The Journal of Heredity* **81**, 177–181.
- Cunningham, C. W. (1997). Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**, 733–740.
- Danforth, B. N., and Ji, S. (1998). Elongation factor-1 α occurs as two copies in bees: implications for phylogenetic analysis of EF-1 α sequences in insects. *Molecular Biology and Evolution* **15**, 225–235.
- Danforth, B. N., Brady, S. G., Sipes, S. D., and Pearson, A. (2004). Single-copy nuclear genes recover Cretaceous-age divergences in bees. *Systematic Biology* **53**, 309–326. doi:10.1080/10635150490423737
- Danforth, B. N., Sauquet, H., and Packer, L. (1999). Phylogeny of the bee genus *Halictus* (Hymenoptera: Halictidae) based on parsimony and likelihood analyses of nuclear EF-1 α sequence data. *Molecular Phylogenetics and Evolution* **13**, 605–618. doi:10.1006/mpev.1999.0670
- Dowton, M., and Austin, A. D. (1994). Molecular phylogeny of the insect order Hymenoptera: apocritan relationships. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9911–9915.
- Engel, M. S. (2001). A monograph of the Baltic amber bees and evolution of the Apoidea (Hymenoptera). *Bulletin of the American Museum of Natural History* **259**, 1–192. doi:10.1206/0003-0090(2001)259<0001:AMOTBA>2.0.CO;2
- Farris, J. D., Källersjö, M., Kluge, A. G., and Bult, C. (1995). Constructing a significance test for incongruence. *Systematic Biology* **44**, 570–572.
- Franklin, H. J. (1912). The Bombidae of the New World. *Transactions of the American Entomological Society* **38**, 177–486.
- Franklin, H. J. (1954). The evolution and distribution of American bumblebee kinds. *Transactions of the American Entomological Society* **80**, 43–51.
- Friedlander, T. P., Regier, J. C., and Mitter, C. (1992). Nuclear gene sequences for higher level phylogenetic analysis: 14 promising candidates. *Systematic Biology* **41**, 483–490.
- Friedlander, T. P., Regier, J. C., and Mitter, C. (1994). Phylogenetic information content of five nuclear gene sequences in animals: initial assessment of character sets from concordance and divergence studies. *Systematic Biology* **43**, 511–525.
- Friedlander, T. P., Regier, J. C., Mitter, C., and Wagner, D. L. (1996). A nuclear gene for higher level phylogenetics: phosphoenolpyruvate carboxykinase tracks Mesozoic-age divergences within Lepidoptera (Insecta). *Molecular Biology and Evolution* **13**, 594–604.
- Frison, T. H. (1923). Systematic and biological notes on bumblebees (Bremidae; Hymenoptera). *Transactions of the American Entomological Society* **48**, 307–326.
- Frison, T. H. (1927a). A contribution to our knowledge of the relationships of the Bremidae of America north of Mexico (Hymenoptera). *Transactions of the American Entomological Society* **53**, 51–78.
- Frison, T. H. (1927b). Records and descriptions of western bumblebees (Bremidae). *Proceedings of the California Academy of Sciences* **16**, 365–380.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.
- Hillis, D. M., and Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**, 182–192.
- Ito, M. (1985). Supraspecific classification of bumblebees based on the characters of male genitalia. *Contributions from the Institute of Low Temperature Science. Series B* **20**, 1–143.
- de Jonghe, R., and Rasmont, P. (1983). Kreuzungsexperiment mit Hummeln des Genus *Bombus* Latreille sensu stricto (Hymenoptera, Apidae). *Phegea, Antwerpen* **11**, 7–10.
- Kawakita, A., Sota, T., Ascher, J. S., Ito, M., Tanaka, H., and Kato, M. (2003). Evolution and phylogenetic utility of alignment gaps within intron sequences of three nuclear genes in bumble bees (*Bombus*). *Molecular Biology and Evolution* **20**, 87–92. doi:10.1093/molbev/msg007
- Kawakita, A., Sota, T., Ito, M., Ascher, J. S., Tanaka, H., Kato, M., and Roubik, D. W. (2004). Phylogeny, historical biogeography, and character evolution in bumble bees (*Bombus*: Apidae) based on simultaneous analysis of three nuclear gene sequences. *Molecular Phylogenetics and Evolution* **31**, 799–804. doi:10.1016/j.ympev.2003.12.003
- Koulianos, S. (1999). Phylogenetic relationships of the bumblebee subgenus *Pyrobombus* (Hymenoptera: Apidae) inferred from mitochondrial cytochrome b and cytochrome oxidase I sequences. *Annals of the Entomological Society of America* **92**, 355–358.
- Koulianos, S., and Schmid-Hempel, P. (2000). Phylogenetic relationships among bumble bees (*Bombus*, Latreille) inferred from mitochondrial cytochrome b and cytochrome oxidase I sequences. *Molecular Phylogenetics and Evolution* **14**, 335–341. doi:10.1006/mpev.1999.0621
- Labougle, J. M. (1990). *Bombus* of México and Central America (Hymenoptera, Apidae). *Kansas University Science Bulletin* **54**, 35–73.
- Lin, C., and Danforth, B. N. (2004). How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined datasets. *Molecular Phylogenetics and Evolution* **30**, 686–702. doi:10.1016/S1055-7903(03)00241-0
- Lutz, F. E. (1916). The geographic distribution of Bombidae (Hymenoptera), with notes on certain species of boreal America. *Bulletin of the American Museum of Natural History* **35**, 501–521.
- Maddison, D. R., and Maddison, W. P. (2000). 'MacClade 4: Analysis of Phylogeny and Character Evolution. Version 4.0.' (Sinauer Associates: Sunderland, MA, USA.)
- Mardulyn, P., and Cameron, S. A. (1999). The major opsin in bees (Insecta: Hymenoptera): a promising nuclear gene for higher level phylogenetics. *Molecular Phylogenetics and Evolution* **12**, 168–176. doi:10.1006/mpev.1998.0606
- Medler, J. T. (1962). Morphometric studies on bumble bees. *Annals of the Entomological Society of America* **55**, 212–218.
- Michel-Salzat, A., and Whitfield, J. B. (2004). Preliminary evolutionary relationships within the parasitoid wasp genus *Cotesia* (Hymenoptera: Braconidae: Microgastrinae): combined analysis of four genes. *Systematic Entomology* **29**, 371–382. doi:10.1111/j.0307-6970.2004.00246.x
- Milliron, H. E. (1971). A monograph of the Western Hemisphere bumblebees (Hymenoptera: Apidae: Bombinae). I. The genera *Bombus* and *Megabombus* subgenus *Bombias*. *Memoirs of the Entomological Society of Canada* **82**, 1–80.
- Moulton, J. K., and Wiegmann, B. M. (2004). Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged eumoneuran Diptera (Insecta). *Molecular Phylogenetics and Evolution* **31**, 363–378. doi:10.1016/S1055-7903(03)00284-7
- Pamilo, P., Varvio-Aho, S., and Pekkari, A. (1984). Genetic variation in bumblebees (*Bombus*, *Psithyrus*) and putative sibling species of *Bombus lucorum*. *Hereditas* **101**, 245–251.
- Pedersen, B. V. (1996). A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletier) and bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Molecular Phylogenetics and Evolution* **5**, 289–297. doi:10.1006/mpev.1996.0024

- Pedersen, B. V. (2002). European bumblebees (Hymenoptera: Bombini) – phylogenetic relationships inferred from DNA sequences. *Insect Systematics & Evolution* **33**, 361–386.
- Pekkarinen, A. (1979). Morphometric, colour and enzyme variation in bumblebees (Hymenoptera, Apidae, Bombus) in Fennoscandia and Denmark. *Acta Zoologica Fennica* **158**, 1–60.
- Plowright, R. C., and Stephen, W. P. (1973). A numerical taxonomic analysis of the evolutionary relationships of *Bombus* and *Psithyrus* (Apidae: Hymenoptera). *Canadian Entomologist* **105**, 733–743.
- Posada, P., and Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics (Oxford, England)* **14**, 817–818. doi:10.1093/bioinformatics/14.9.817
- Rasmont, P. (1984). Les bourdons du genre *Bombus* Latr. sensu stricto en Europe occidentale et centrale (Hymenoptera, Apidae). *Spixiana* **7**, 135–160.
- Rasnitsyn, A. P., and Michener, C. D. (1991). Miocene fossil bumble bees from the Soviet Far East with comments on the chronology and distribution of fossil bees (Hymenoptera: Apidae). *Annals of the Entomological Society of America* **84**, 583–589.
- Richards, O. W. (1968). The subgeneric division of the genus *Bombus* Latr. (Hymenoptera: Apidae). *Bulletin of the British Museum (Natural History). Entomology* **22**, 209–276.
- Rokas, A., Nylander, J. A. A., Ronquist, F., and Stone, G. N. (2002). A maximum-likelihood analysis of eight phylogenetic markers in gallwasps (Hymenoptera: Cynipidae): implications for insect phylogenetic studies. *Molecular Phylogenetics and Evolution* **22**, 206–219. doi:10.1006/mpev.2001.1032
- Ronquist, F., and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics (Oxford, England)* **19**, 1572–1574. doi:10.1093/bioinformatics/btg180
- Sakagami, S. F. (1976). Specific differences in the bionomic characters of bumblebees. A comparative review. *Journal of the Faculty of Science, Hokkaido University (Zoology)* **20**, 390–447.
- Scholl, A., and Obrecht, E. (1983). Enzymeelektrophoretische untersuchungen zur artabgrenzung im *Bombus lucorum*-komplex (Apidae, Bombini). *Apidologie* **14**, 65–78.
- Scholl, A., Obrecht, E., and Owen, R. E. (1990). The genetic relationships between *Bombus moderatus* Cresson and the *Bombus lucorum* auct. species complex (Hymenoptera: Apidae). *Canadian Journal of Zoology* **68**, 2264–2268.
- Scholl, A., Obrecht, E., and Thorp, R. W. (1988). Biochemical systematics in bumble bees: the subgenus *Pyrobombus* (Hymenoptera: Apidae). In 'Proceedings of the XVIII International Congress of Entomology'. pp. 63. (Vancouver, BC, Canada.)
- Scholl, A., Thorp, R. W., Bishop, J. A., and Obrecht, E. (1995). The taxonomic status of *Bombus alboanalis* Franklin and its relationship with other taxa of the subgenus *Pyrobombus* from North America and Europe (Hymenoptera: Apidae). *The Pan-Pacific Entomologist* **71**, 113–120.
- Simmons, M. P., and Ochoterena, H. (2000). Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* **49**, 369–381. doi:10.1080/10635159950173889
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., and Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**, 651–701.
- Sipes, S. D., and Wolf, P. G. (2001). Phylogenetic relationships within *Diadasia*, a group of specialist bees. *Molecular Phylogenetics and Evolution* **19**, 144–156. doi:10.1006/mpev.2001.0914
- Sorenson, M. D. (1999). 'TreeRot, Version 2.' (Boston University: Boston, MA, USA.)
- Spaethe, J., and Briscoe, A. D. (2004). Early duplication and functional diversification of the opsin gene family in insects. *Molecular Biology and Evolution* **21**, 1583–1594. doi:10.1093/molbev/msh162
- Stephen, W. P. (1957). Bumble bees of western America (Hymenoptera: Apoidea). Technical Bulletin, Agricultural Experiment Station, Oregon State University No. 40.
- Swofford, D. L. (2001). 'PAUP*, Phylogenetic Analysis Using Parsimony (*and Other Methods).' (Sinauer Associates: Sunderland, MA, USA.)
- Thorp, R. W. (2003). Bumble bees (Hymenoptera: Apidae): commercial use and environmental concerns. In 'For Non-native Crops, Whence Pollinators of the Future?'. (Eds K. Strickler and J. H. Cane.) pp. 21–40. (Thomas Say Publications in Entomology; Proceedings, Entomological Society of America: Lanham, MD, USA.)
- Thorp, R. W., Horning, D. S., Jr, and Dunning, L. L. (1983). Bumble bees and cuckoo bumble bees of California (Hymenoptera: Apidae). *Bulletin of the California Insect Survey* **23**, 1–79.
- Tkalců, B. (1974). Eine Hummel-Ausbeute aus dem Nepal-Himalaya (Insecta, Hymenoptera, Apoidea, Bombinae). *Senckenbergiana Biologica* **55**, 311–349.
- Tkalců, B. (1989). Neue Taxa asiatischer Hummeln (Hymenoptera, Apoidea). *Acta Entomologica Bohemoslovaca* **86**, 39–60.
- Whitfield, J. B., and Cameron, S. A. (1998). Hierarchical analysis of variation in the mitochondrial 16S rRNA gene among Hymenoptera. *Molecular Biology and Evolution* **15**, 1728–1743.
- Williams, P. H. (1985). A preliminary cladistic investigation of relationships among the bumble bees (Hymenoptera, Apidae). *Systematic Entomology* **10**, 239–255.
- Williams, P. H. (1991). The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bulletin of the British Museum (Natural History). Entomology* **60**, 1–204.
- Williams, P. H. (1994). Phylogenetic relationships among bumble bees (*Bombus* Latr.): a reappraisal of morphological evidence. *Systematic Entomology* **19**, 327–344.
- Williams, P. H. (1998). An annotated checklist of bumble bees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini). *Bulletin of the British Museum (Natural History). Entomology* **67**, 79–152.
- Yoshizawa, K., and Johnson, K. P. (2003). Phylogenetic position of Pthiraptera (Insecta: Paraneoptera) and elevated rate of evolution in mitochondrial 12S and 16S rDNA. *Molecular Phylogenetics and Evolution* **29**, 102–114. doi:10.1016/S1055-7903(03)00073-3
- Zeuner, F. E., and Manning, F. J. (1976). A monograph on fossil bees (Hymenoptera: Apoidea). *Bulletin of the British Museum (Natural History). Geology Series* **27**, 151–268.
- Zhang, J. (1990). New fossil species of Apoidea. *Acta Zootaxonomica Sinica* **15**, 83–91. [In Chinese, English summary.]

Manuscript received 30 June 2005, revised and accepted 15 February 2006.