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

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*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-1α*), 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-1α* and *ArgK*, provided good support across all of the taxonomic levels examined, whereas support of the more rapidly evolving mt*16S* 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-1α*); Kawakita *et al*. 2003, 2004). Plowright and Stephen (1973) also inferred a close relationship between

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*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-1α F2 copy (*EF-1α*), 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* (Tkalců) 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.wilmattae* 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* (Tkalců), *B. mirus* (Tkalců), *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-1α* 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 874–16SlR (Cameron *et al*. 1992) to obtain ~500 base pairs (bp) from *16S*.

*EF-1α* 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-1α* 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′- TTGCAAAGCTTCRKGATGCATTT-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-1α* | *opsin* | *ArgK* |
|---|---|---|---|---|---|---|---|
| *Alpigenobombus* | *nobilis* Friese | Sichuan, China | 098 | AY737370 | AY739591 | AY739485 | AY739528 |
| | *wurflenii* Radoszkowski | Obergurgl, Austria [Monte Rosa, Italy] | 001 | AY737393 | AY739612, AF492940$^{K}$ (1) | AF493007$^{K}$ | AF492873$^{K}$ |
| *Alpinobombus* | *alpinus* (Linnaeus) | Gurgltal, Austria | 029 | AY737321 | AY739542 | AY739452 | AY741385 |
| | *balteatus* Dahlbom | Kiruna, Sweden | 039 | AY737324 | AY739546 | AY739455 | AY739499 |
| | *hyperboreus* Schönherr | Avesta, Sweden | 070 | AY737345 | AY739565 | AY739470 | AY739513 |
| | *neoboreus* Sladen | Alaska, USA | 188 | AY737369 | AY739590 | AY739484 | AY739527 |
| | *polaris* Curtis | [Kamchatka, Russia] | | | AF492970$^{K}$ | AF493037$^{K}$ | AF492903$^{K}$ |
| *Bombias* | *auricomus* (Robertson) | Illinois, USA [Delaware, USA] | 062 | AY737323 | AY739545, AF492959$^{K}$ (0) | AY739454 | AF492892 |
| *Bombus* | *affinis* Cresson | Illinois, USA | 167 | AY737320 | AY739541 | AY739451 | AY739497 |
| | *cryptarum* (Fabricius) | Erzincan, Turkey | 127 | AY737332 | AY739554 | AY739461 | AY739504 |
| | *hypocrita* Pérez | Kyushu, Japan [Tokushima, Japan] | 123 | AY737347 | AY739568, AF492956$^{K}$ (1) | AF493023$^{K}$ | AF492889$^{K}$ |
| | *ignitus* Smith | Beijing, China [Oita, Japan] | 096 | AY737348 | AY739569, AF492965$^{K}$ (1) | AF493032$^{K}$ | AF492898$^{K}$ |
| | *lucorum* (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) |
| | *moderatus* Cresson | Alberta, Canada | 163 | AY737366 | AY739587 | AY739481 | AY739524 |
| | *occidentalis* Greene | New Mexico, USA | 025 | AY737371 | AY739592 | AY739486 | AY739529 |
| | *patagiatus* Nylander | Sichuan, China [Primorsky, Russia] | 111 | AY737372 | AY739593, AF492953$^{K}$ (1) | AF493020$^{K}$ | AF492886$^{K}$ |
| | *sporadicus* Nylander | Abisco, Sweden | 193 | AY737381 | AY739601 | AY739491 | AY739534 |
| | *terrestris* (Linnaeus) | San Quirico, Italy [L'Aquila, Italy] | 003 | AY737386 | AY739605, AF492955$^{K}$ (0) | AF493022$^{K}$ | AF492888$^{K}$ |
| | *terricola* Kirby | Ontario, Canada [Quebec, Canada] | 205 | AY737387 | AY739606, AF492952$^{K}$ (0) | AF493019$^{K}$ | AF492885$^{K}$ |
| *Confusibombus* | *confusus* Schenck | Dorres, France | 083 | AY737331 | AY739553 | AY739460 | AY739503 |
| *Cullumanobombus* | *rufocinctus* Cresson | Alberta, Canada [Quebec, Canada] | 186 | AY737377 | AY739597, AF492967$^{K}$ (0) | AF493034$^{K}$ | AF492900$^{K}$ |
| *Fervidobombus* | *pensylvanicus* (DeGeer) | [Missouri, USA] [California, USA] | | AY268410$^{C}$ | AF492929$^{K}$ | AY268388$^{C}$ | AF492862$^{K}$ |
| *Festivobombus* | *festivus* Smith | Sichuan, China | 104 | AY737336 | AY739558 | AY739465 | AY739508 |
| *Kallobombus* | *soroeensis* (Fabricius) | Eyne, France [Cesana, Italy] | 136 | AY737380 | AY739600, AF492941$^{K}$ (0) | AF493008$^{K}$ | AF492874$^{K}$ |
| *Megabombus* | *argillaceus* (Scopoli) | Kayseri, Turkey | 058 | AY737322 | AY739544 | AY739453 | AY739498 |
| *Melanobombus* | *friseanus* Skorikov | Sichuan, China | 105 | AY737340 | AY739560 | AY739467 | AY739510 |
| | *keriensis* Morawitz | Sichuan, China | 114 | AY737353 | AY739574 | AY739474 | AY739517 |
| | *ladakhensis* Richards | Sichuan, China | 158 | AY737354 | AY739575 | AY739475 | AY739518 |
| | *lapidarius* (Linnaeus) | San Quirico, Italy [Windsor, UK] | 006 | AY737355 | AY739576, AF492938$^{K}$ (1) | AF493005$^{K}$ | AF492871$^{K}$ |
| | *rufofasciatus* Smith | Sichuan, China | 133 | AY737378 | AY739598 | AY739489 | AY739532 |
| | *erzurumensis* (Özbek) | Artvin Prov., Turkey | 126 | AY737334 | AY739556 | AY739463 | AY739506 |
| *Mendacibombus* | *mendax* Gerstaecker | Gurgltal, Austria [Monte Rosa, Italy] | 019 | AY737363 | AY739584, AF492957$^{K}$ (0) | AF493024$^{K}$ | AF492890$^{K}$ |
| *Psithyrus* | *maxillosus* Klug | Kayseri, Turkey | 074 | AY737361 | AY739582 | AY739480 | AY739523 |
| | *vestalis* (Geoffroy) | Kent, England | 169 | AY737390 | AY739609 | AY739495 | AY739538 |
| *Pyrobombus* | *ardens* Smith | Dae-Dong, S. Korea [Nara, Japan] | 131 | AF364822$^{B}$ | AY739543, AF492964$^{K}$ (1) | AF493031$^{K}$ | AF492897$^{K}$ |
| | *beaticola* (Tkalců) | [Nagano, Japan] | | | AF492963$^{K}$ | AF493030$^{K}$ | AF492896$^{K}$ |
| | *bifarius* Cresson | New Mexico, USA [Seattle, USA] | 208 | AY737325 | AY739547, AF492943$^{K}$ (1) | AF493010$^{K}$ | AF492876$^{K}$ |
| | *bimaculatus* Cresson | Arkansas, USA | 218 | AY737326 | AY739548 | AY739456 | AY739500 |
| | *biroi* Vogt | Ketmen Mts., Kazakhstan | 210 | AY737327 | AY739549 | AY739457 | |
| | *brodmannicus* Vogt | Artvin Prov., Turkey | 077 | AY737328 | AY739550 | AY739458 | AY739501 |

(*continued next page*)

**Table 1.** (continued)

| Subgenus | Species | Collection locality | No. | *16S* | *EF-1α* | *opsin* | *ArgK* |
|---|---|---|---|---|---|---|---|
| | *caliginosus* (Frison) | California, USA [Seattle, USA] | 150 | AY737329 | AY739551, AF492968[K] (0) | AF493035[K] | AF492901[K] |
| | *centralis* Cresson | Washington, USA [Colorado, USA] | 146 | AY737330 | AY739552, AF492981[K] (2) | AY739459 | AY739502 |
| | *cingulatus* Wahlberg | [Kamchatka, Russia] | | | AF492948[K] | AF493015[K] | AF492881[K] |
| | *ephippiatus* Say | Chiapas, Mexico | 198 | AY737333 | AY739554 | AY739462 | AY739505 |
| | *flavescens* (Smith) | Mei-fang, Taiwan [Habon, Taiwan] | 181 | AY737337 | AF492950[K] | AF493017[K] | AF492883[K] |
| | *flavifrons* Cresson | California, USA [Seattle, USA] | 095 | AY737338 | AF492949[K] | AF493016[K] | AF492882[K] |
| | *frigidus* Smith | Alaska, USA | 185 | AY737339 | AY739559 | AY739466 | AY739509 |
| | *haematurus* Kriechbaumer | Trabzon, Turkey | 211 | AY739613 | | | |
| | *huntii* Greene | Washington, USA [Colorado, USA] | 151 | AY737344 | AY739563, AF492978[K] (0) | AF493045[K] | AF492911[K] |
| | *hypnorum* (Linnaeus) | Klösterle, Austria [Piemonte, Italy] | 078 | AY737346 | AY739566, AF492946[K] (1) | AF493013[K] | AF492879[K] |
| | | Sichuan, China | 207 | AY739614 (9) | AY739567 (5,4) | | |
| | *impatiens* Cresson | Illinois, USA [Quebec, Canada] | 060 | AY737349 | AY739570, AF492942[K] (0) | AF493009[K] | AF492875[K] |
| | *infirmus* (Tkalců) | Sichuan, China | 157 | AY737350 | AY739571 | AY739471 | AY739514 |
| | *infrequens* (Tkalců) | Sichuan, China | 140 | AY737351 | AY739572 | AY739472 | AY739515 |
| | *jonellus* (Kirby) | Lappland Co., Sweden | 079 | AY737352 | AY739573 | AY739473 | AY739516 |
| | *lapponicus* (Fabricius) | Kiruna, Sweden | 103 | AY737356 | AY739577 | AY739476 | AY739519 |
| | *lemniscatus* Skorikov | Sichuan, China | 161 | AY737357 | AY739578 | AY739477 | AY739520 |
| | *lepidus* Skorikov | Sichuan, China | 155 | AY737358 | AY739579 | AY739478 | AY739521 |
| | *luteipes* Richards | Pokhara, Nepal | 195 | AY739615 | | | |
| | *melanopygus* Nylander | California, USA [Seattle, USA] | 215 | AY737362 | AY739583, AF492944[K] (0) | AF493011[K] | AF492877[K] |
| | *mixtus* Cresson | Washington, USA [Seattle, USA] | 024 | AY737365 | AY739586, AF492947[K] (3) | AF493014[K] | AF492880[K] |
| | *modestus* Eversmann | Sichuan, China | 160 | AY737367 | AY739588 | AY739482 | AY739525 |
| | *monticola* Smith | Eyne, France | 176 | AY737368 | AY739589 | AY739483 | AY739526 |
| | *perplexus* Cresson | Ontario, Canada [Quebec, Canada] | 166 | AY737373 | AY739594, AF492945[K] (0) | AF493012[K] | AF492878[K] |
| | *picipes* Richards | Sichuan, China | 180 | AY737374 | AY739595 | AY739487 | AY739530 |
| | *pratorum* (Linnaeus) | Klösterle, Austria [Sunningdale, UK] | 075 | AY737375 | AF492966[K] | AF493033[K] | AF492899[K] |
| | *pyrenaeus* Pérez | Gurgltal, Austria | 035 | AY737376 | AY739596 | AY739488 | AY739531 |
| | *sitkensis* Nylander | California, USA | 144 | AY737379 | AY739599 | AY739490 | AY739533 |
| | *sonani* (Frison) | [Alishan, Taiwan] | | | AF492951[K] | AF493018[K] | AF492884[K] |
| | *sylvicola* Kirby | New Mexico, USA | 108 | AY737384 | AY739604 | AY739493 | AY739536 |
| | *ternarius* Say | Nova Scotia, Canada [New York, USA] | 116 | AY737385 | AF492979[K] | AF493046[K] | AF492912[K] |
| | *vagans* Smith | Wisconsin, USA | 044 | AY737388 | AY739607 | AY739494 | AY739537 |
| | *vandykei* (Frison) | Washington, USA [California, USA] | 149 | AY737389 | AY739608, AF492982[K] (0) | AF493049[K] | AF492915[K] |
| | *vosnesenskii* Radoszkowski | Washington, USA [California, USA] | 112 | AY737391 | AY739610, AF492980[K] (0) | AF493047[K] | AF492913[K] |
| | *wilmattae* Say | Chiapas, Mexico | 199 | AY737392 | AY739611 | AY739496 | AY739539 |
| *Rhodobombus* | *mesomelas* (Gerstaecker) | Switzerland [L'Aquila, Italy] | 037 | AY737364 | AY739585, AF492936[K] (4) | AF493003[K] | AF492869[K] |
| *Robustobombus* | *hortulanus* Friese | Magdalena, Colombia | 200 | AY737342 | AY739562 | AY739468 | AY739511 |
| *Rufipedibombus* | *eximius* Smith | Alishan, Taiwan | 049 | AY737335 | AY739557 | AY739464 | AY739507 |
| *Separatobombus* | *griseocollis* (DeGeer) | Illinois, USA [New York, USA] | 082 | AY737341 | AY739561, AF492972[K] (0) | AF493039[K] | AF492905[K] |
| *Sibiricobombus* | *sulfureus* Friese | Kayseri, Turkey | 064 | AY737383 | AY739603 | AY739492 | AY739535 |
| *Subterraneobombus* | *subterraneus* (Linnaeus) | Uppland Co., Sweden [L'Aquila, Italy] | 046 | AY737382 | AY739602, AF492960[K] (0) | AF493027[K] | AF492893[K] |
| *Thoracobombus* | *humilis* 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 *Rh*1 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-1α* and *opsin* sequences from GenBank (shown in Table 1), submitted mostly by Kawakita *et al*. (2003). We sequenced most of our taxa for *EF-1α* 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-1α*, 57–60°C/72°C for *opsin* and 48–50°C/72°C for *ArgK*. We used Eppendorf Master*Taq*® 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-1α* 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-1α*, 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-1α* intron (GTR+Γ), *EF-1α* exon (GTR+I+Γ), *EF-1α* 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 5 000 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 ≥ 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, ≤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 ≥ 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-1α* 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-1α*. Of 2861 total characters in the combined analysis, 527 characters were parsimony informative, 114 belonging to *16S*, 154 to *EF-1α*, 92 to *opsin* and 138 to *ArgK* (Table 2). There were 0–4 nucleotide differences between our *EF-1α* 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 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *16S* | 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 |
| *EF-1α* | 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 |
| *opsin* | 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 |
| *ArgK* | 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 |
| *opsin* intron | 75.3 | | | 39 | 178 | 21.9 | | | | 58.16 | 0.48 |
| *opsin* exon | 55.5 | | | 53 | 502 | 10.6 | | | | 20.46 | 0.13 |
| *opsin* pos1 | 61.0 | | | 8 | 167 | 4.8 | | | | 5.50 | 0.20 |
| *opsin* pos2 | 59.8 | | | 5 | 167 | 3.0 | | | | 5.00 | 0.45 |
| *opsin* pos3 | 45.8 | | | 40 | 168 | 23.8 | | | | 9.01 | 0.08 |
| *EF-1α* intron | 67.9 | | | 69 | 238 | 29.0 | | | | 36.33 | 0.15 |
| *EF-1α* exon | 54.9 | | | 85 | 523 | 16.3 | | | | 69.98 | 0.20 |
| *EF-1α* pos1 | 40.6 | | | 3 | 175 | 1.7 | | | | 2.77 | 0.29 |
| *EF-1α* pos2 | 58.0 | | | 2 | 174 | 1.1 | | | | –3.15 | –0.49 |
| *EF-1α* pos3 | 66.2 | | | 80 | 174 | 46.0 | | | | 71.24 | 0.22 |
| *EF-1α* gap | | 0.633 | 0.890 | 19 | 19 | 100.0 | | | | 30.26 | 0.90 |
| *ArgK* intron | 74.5 | | | 98 | 399 | 24.6 | | | | 87.58 | 0.30 |
| *ArgK* exon | 50.9 | | | 40 | 530 | 7.5 | | | | 36.39 | 0.28 |
| *ArgK* pos1 | 43.1 | | | 3 | 177 | 1.7 | | | | 1.00 | 0.13 |
| *ArgK* pos2 | 62.5 | | | 0 | 176 | 0.0 | | | | 0.00 | 0.00 |
| *ArgK* pos3 | 47.2 | | | 37 | 177 | 20.9 | | | | 34.37 | 0.29 |
| *ArgK* 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 ≥ X; $B_x$, no. nodes in Bayesian tree resolved with posterior probabilities ≥ 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. 1*B–D*; single gene parsimony phylogenies not shown). For *16S*, the Bayesian tree is unresolved at the basal nodes (Fig. 1*A*). 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-1α v. opsin*, $P = 0.88$; *EF-1α 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-1α* ($P = 0.04$) 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. 1*A*, *B*, *D*) indicate that (*B. bimaculatus*+*B. lapponicus*)+*B. monticola* is supported only by *16S* data and is contradicted by *EF-1α* 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-1α*: 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. 1*A*) 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-1α*: 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. 1*A*, *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-1α* 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-1α* 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-1α* 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. 1*A*). 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

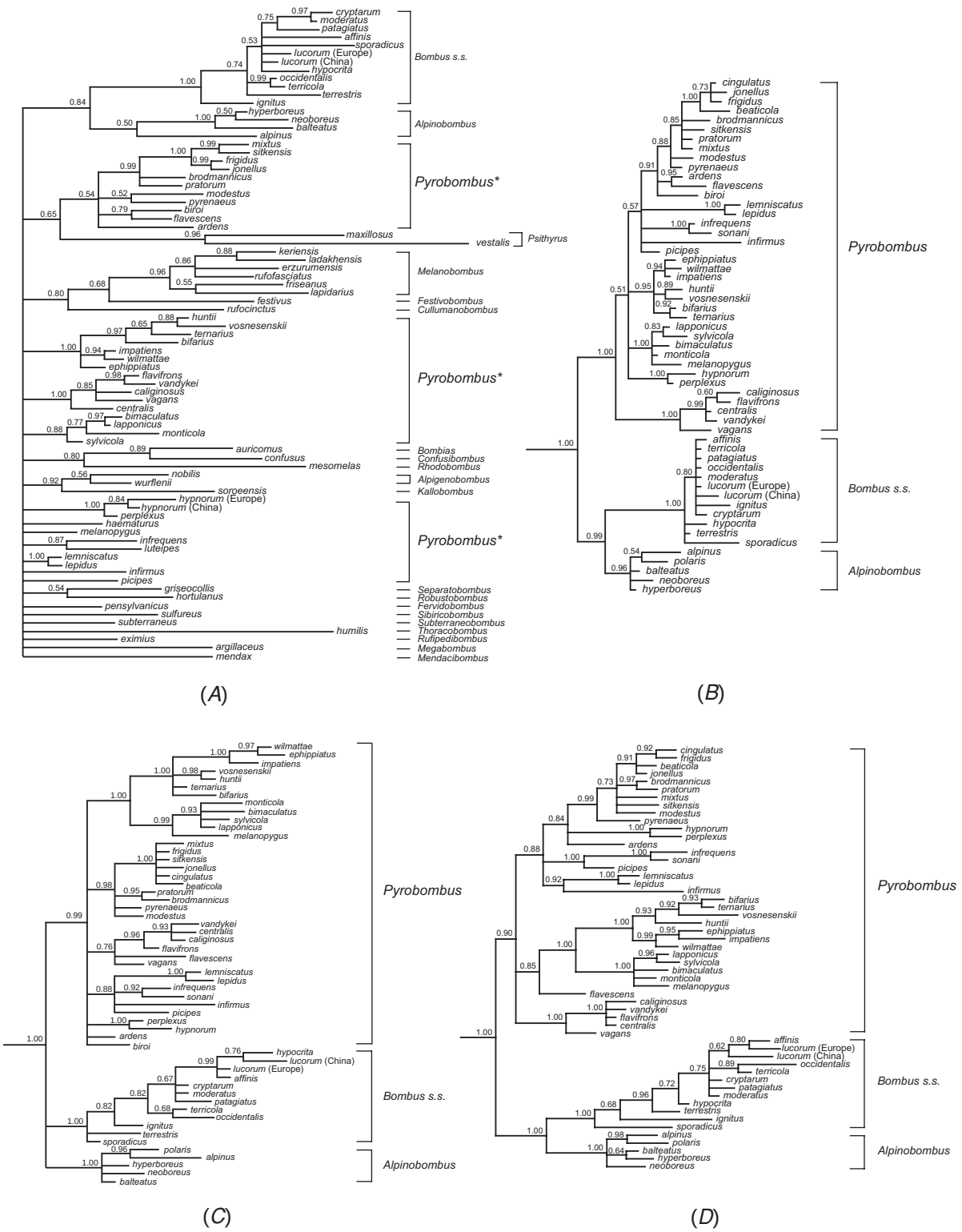


**Fig. 1.** Bayesian individual gene phylogenies. *A*, *16S*; *B*, *EF-1α*; *C*, *opsin*; *D*, *ArgK*. Each phylogeny is estimated from 35 003 trees (2 runs: 2 000 000 generations, four chains, sampling every 100 trees, burn-in = 5000 trees; 1 run: same but 1 000 000 generations) using flat priors, models 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-1α*: 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.

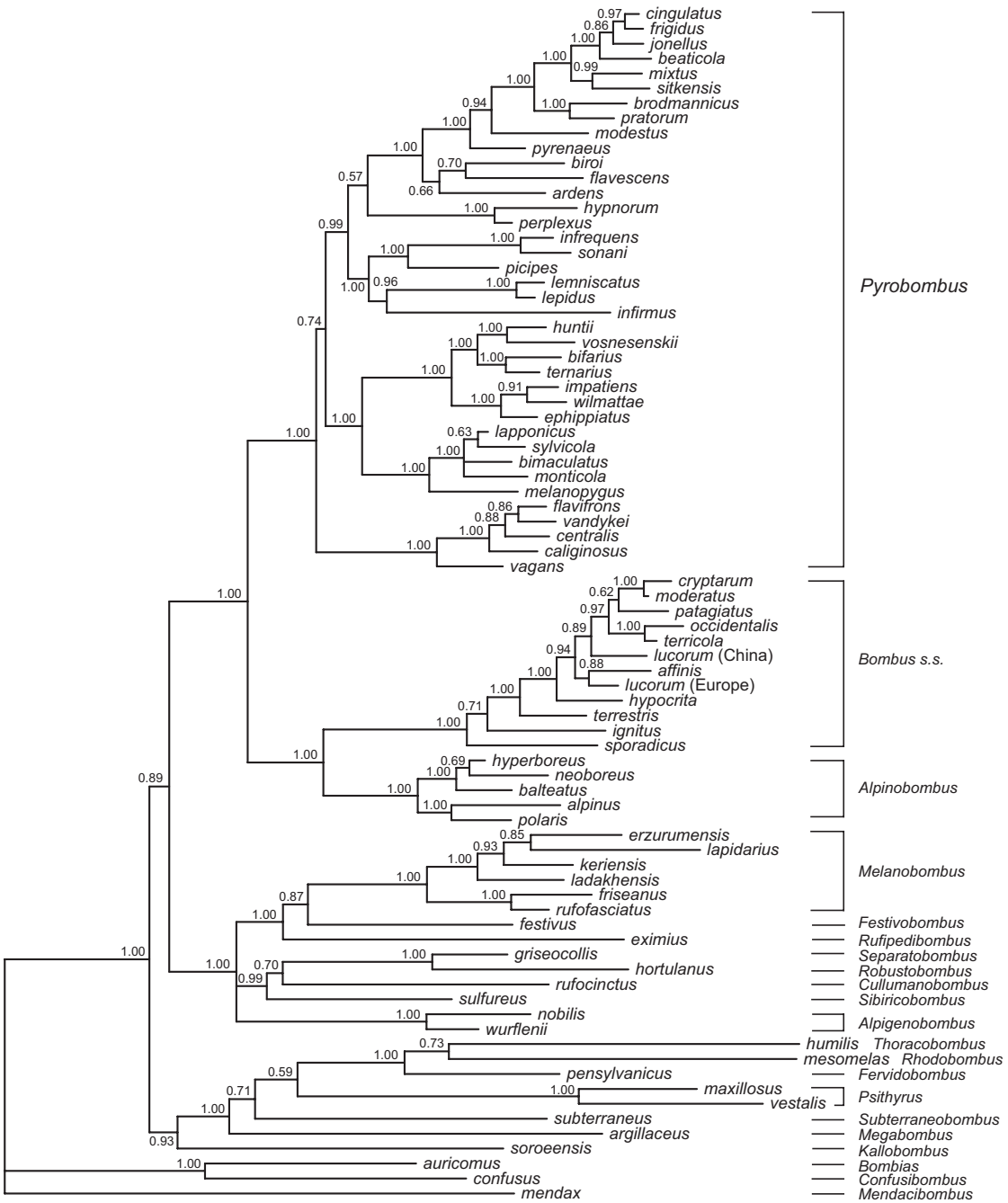


**Fig. 2.** Bayesian phylogeny based on the combined dataset (*16S* + *EF-1α* + *opsin* + *ArgK*). Phylogeny based on 35001 trees (4000000 generations; 8 chains; sampling every 100 trees; burn-in = 5000 trees) using flat priors, mixed models and partitioning by gene, exon/intron and gap characters when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

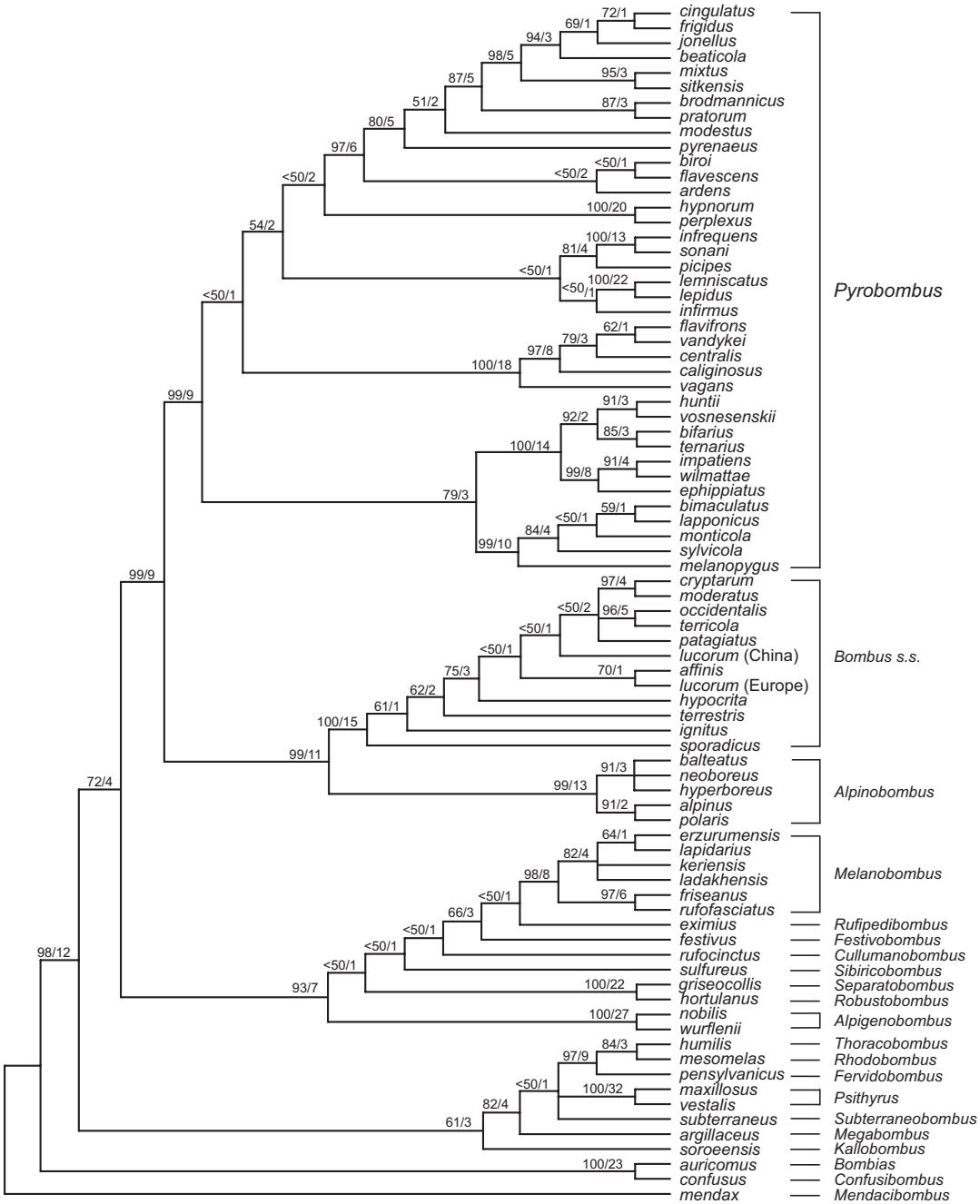


**Fig. 3.** Parsimony-based phylogeny of the combined dataset (*16S* + *EF-1α* + opsin + *ArgK*). Strict consensus tree of 16 most parsimonius trees obtained using a heuristic search (1000 random additions, tree bisection reconnection (TBR) branch swapping). Numbers above branches indicate bootstrap 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-1α* 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-1α* is only slightly AT biased (58.5%). For all three nuclear genes, the AT bias is higher in the introns ($\overline{X} = 72.6\%$) than the exons ($\overline{X} = 53.8\%$).

*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-1α*.

*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-1α* (≤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-1α* 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-1α* gap-coded characters. This suggests that gap characters support

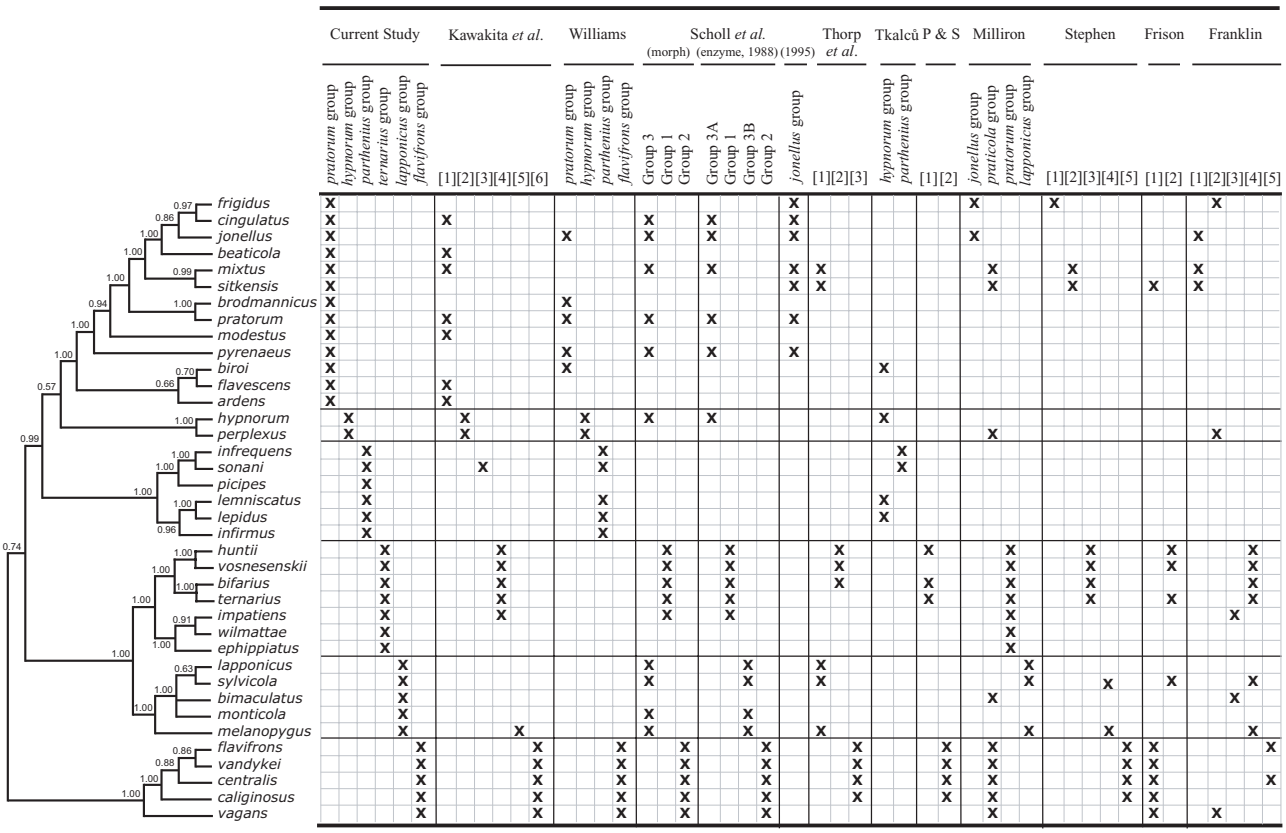


**Fig. 4.** Synopsis of species-groups discernable from the phylogeny in this study (Bayesian phylogeny at left) in relation to species-groups demarcated explicitly by Franklin (1912), Frison (1923, 1927*a*, 1927*b*), Milliron (1971), Plowright and Stephen (P & S) (1973), Scholl *et al*. (1988, including some unpublished data presented in a poster (split into results based on morphology or enzymes); 1995), Stephen (1957), Thorp *et al*. (1983), Tkalců (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-1α*, 0.968 for *ArgK*). Only two of eleven *ArgK* gap characters contained homoplasy. For *EF-1α*, 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 Koulianos 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, 1927*a*, *b*; Stephen 1957; Milliron 1971; Plowright and Stephen 1973; Tkalců 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 (1927*a*) 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 Guatamala – *B. wilmattae,* Labougle 1990). Their close relationship may enhance interspecific 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 mt*16S* 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-1α* 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-1α* 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-1α* and may be promising for resolving deeper phylogenetic relationships in insects.

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