

Phylogenetic Relationships among Some Bumblebees (Hymenoptera: Apidae) Common in Korea Inferred from Mitochondrial 16S rRNA Sequences

Hyung Joo Yoon*, Myeong Lyeol Lee, Sam Eun Kim, Sang Beom Lee, Iksoo Kim, Jin Sik Bae¹,
Byung Rae Jin¹ and Hung Dae Sohn¹

Department of Agricultural Biology, The National Institute of Agricultural Science & Technology, Rural Development Administration, Suwon 441-100, Korea.

¹College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea.

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Phylogenetic relationships were analyzed among bumblebees using a portion of mitochondrial (mt) 16S ribosomal RNA (16S rRNA). Eight species of true bumblebees and one species of cuckoo bumblebee (*Bombini*, Apidae), collected from Korea were included in the analysis. Also, one species of true bumblebee imported from several foreign countries for pollination was included. The length of mt 16S rRNA sequence ranged from 496 bp to 508 bp and sequence divergence ranged from 1.4% (7 bp) to 15.49% (77 bp). As expected, a high A+T content was observed (78.5% on average). According to the phylogeny tree derived from parsimony and maximum likelihood analysis, a monophyletic *Bombus* species, excluding a single cuckoo bumblebee, *Psithyrus coreanus*, was obtained, but the bootstrap estimate at the node supporting the monophyletic group was very weak (40% or 46%), suggesting a very close relationship of the cuckoo bumblebee to the true bumblebee. Within *Bombus* species belonging to identical subgenera subgeneric specific clustering was formed with high bootstrap values, implying validity of the subgeneric names of each species: *Pyrobombus* for *B. ardens ardens* and *B. modeatus*; *Megabombus* for *B. consobrinus wittenburgi* and *B. koreanus*; and *Bombus s. str.* for *B. ignitus*, *B. hypocrita sapporoensis*, and *B. terrestris*.

Key words: True bumblebee, Cuckoo bumblebee, Phylogeny, Apidae, Mitochondrial DNA, 16S rRNA

*To whom correspondence should be addressed.

Department of Agricultural Biology, The National Institute of Agricultural Science & Technology, Rural Development Administration, Suwon 441-100, Korea. Tel: +82-31-290-8567; E-mail: YoonHJ@rda.go.kr

Introduction

Bumblebees are pollinating insects with the large-sized and colorful body. Their pollinating behavior is greatly appreciated for the green house vegetables like tomatoes. Particularly, *Bombus terrestris* is one of the pollinators utilized world-widely, and has been imported in Korea from Europe since 1993. In Korea, a substantial effort on artificial mass rearing of the endemic species, *B. ignites*, has been made to substitute *B. terrestris* for green house pollination (Yoon *et al.*, 1999; Yoon *et al.*, 2002, 2003).

Bumblebees are distributed world widely including alpine, cool temperate and even arctic environments of the northern continents (Williams, 1989). Until now 239 species are listed but still synoptic revision is undergoing (Williams, 1998). In Korea, 21 species consisted of seven subgenera in the genus *Bombus* (Lee and Dumouchel; 1999) and five species of cuckoo bumblebees (*Psithyrus*) are listed in the checklist of Korean insects (Ent. Soc. Kor. & Kor. Soc. Appl. Ent., 1994).

The association between the subgenera within the genus *Bombus* has somewhat progressed both by morphological (Krüger, 1917, 1920; Milliron, 1961, 1971; Williams, 1985, 1994) and behavioral characters (Sladen, 1899; Sakagami, 1976). Recently, systematists have adapted molecular techniques (Stephen and Cheldelin, 1973; Pekkarinen *et al.*, 1979; Obrecht and Scholl, 1981; Pamilo *et al.*, 1987; Pedersen, 1996) to resolve the phylogenetic relationships among species in the *Bombus*.

Kouliannos and Schmid-Hempel (2000) derived an estimate of the relationships within the genus *Bombus* by comparing the mitochondrial (mt) cytochrome *b* (Cyt *b*) and cytochrome oxidase subunit I (COI) genes from 19 species spanning 13 subgenera. All subgenera with two representatives in their analysis were monophyletic except

for *Fervidobombus*, *Melanobombus*, and *Pyrobombus*.

The remarkable morphological resemblance between cuckoo bumblebees (*Psithyrus*) and true bumblebees (*Bombus*) has been the topic of a number of phylogenetic studies based on morphology and protein electrophoretic data (Milliron, 1971; Plowright and Stephen, 1973; Pekkarinen *et al.*, 1979; Obrecht and Scholl, 1981; Pamilo *et al.*, 1987; Williams, 1985, 1991). These studies indicate, with minor deviations, the monophyly of the cuckoo bumblebees. However, the relationships among the cuckoo bumblebees and the different groups of species of true bumblebees vary from author to author. The attention has, in particular, been concentrated on whether a given *Psithyrus* species shares a common ancestor with its host among the *Bombus* species (Pedersen, 1996).

Pedersen (1996) found that the six cuckoo bumblebees form a monophyletic group within the true bumblebees through phylogenetic analysis of mt CO1. The *Bombus lucorum* group (subgenus *Bombus*) falls out as the sister group to a clade comprising the cuckoo bumblebees and the remaining true bumblebees. In addition to mt Cyt *b* or COI gene, the 16S rRNA gene was proven to be informative for phylogenetic analysis at two different levels: among closely related species or populations, and among tribes, subfamilies, and families of Hymenoptera (Whitfield and Cameron, 1998).

In this study, we provide a molecular study intending to derive an estimate of the relationships among 10 species belonging to the Tribe Bombini, consisted of six subgenera and nine species in the genus *Bombus* and one species of cuckoo bumblebees, *Psithyrus coreanus*, which are common in South Korea, using mt 16S rRNA gene.

Materials and Methods

Sampling and DNA extraction

Each two or three individuals of queens from eight true bumblebee and one cuckoo bumblebee species, *Psithyrus coreanus*, were collected from several locations in Korea during 1999 – 2000 (Table 1). The eight true bumblebees consist of five subgenera in the genus *Bombus*. Imported bumblebee, *Bombus terrestris*, from Netherlands, Belgium, and Israel for the pollination of greenhouse tomatoes was also included. A carpenter bee species, *Xylocopa applendiculata circumvolans*, was used as an outgroup. Samples were kept in -70°C until DNA was extracted.

PCR amplification and sequencing of nucleotides

Total genomic DNA was extracted from each sample by Wizard[®] Genomic DNA Purification kit (Promega, USA) according to the manufacturer's recommendation.

A pair of primers was designed for the amplification of mt 16S rRNA gene of the bumblebees based on the honeybee sequences (*Apis mellifera*) (Simon *et al.*, 1994): LR-N-13943: 5-CACCTGTTTATCAAAAACAT-3 and LR-J-13394: 5-TCGATTTGAACTCAAATCATGT-3. PCR reactions were in 50 μl of PCR buffer (50 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris; pH 8.3) with 1 μl of template DNA solution, 20 pmol of each primer, 1 μl of each dNTP (10 mM), and 1.5 units of *Taq* DNA polymerase (PE applied Biosystem). The PCR was performed using MJ Research thermal controller (Model 60; Watertown, MA). The reaction profile consisted of an initial denaturation for 5 min at 94°C ; 40 cycles of denaturation for 30 sec at 94°C , annealing for 40 sec at 48°C , and extension for 45 sec at 72°C , and one final extension for 7 min 45 sec at 72°C .

The amplified product was loaded on 1% agarose gel and electrophoresed with tris-acetate/EDTA (TAE) buffer. The PCR product visualized under UV light as single band with the expected size on the gel was purified by PCR Purification kit (QUIAGEN, Germany) to eliminate free primers and extra nucleotides. DNA sequencing was performed with ABI 377 Genetic Analyzer (PE applied Biosystems, CA).

Analysis of DNA sequences and phylogenetic relationships

The obtained sequences were aligned according to Clustal X algorithm (Thompson *et al.*, 1997) using IBI MacVector (ver. 6.5). The genetic distances among the bumblebee species were estimated by pairwise comparison.

The phylogenetic relationships were performed using maximum-parsimony (MP) method (Fitch, 1971) and maximum-likelihood (ML) method (Felsenstein, 1981) incorporated in PAUP* (Phylogenetic Analysis Using Parsimony and Other Method* ver. 4.0b10 Swofford, 1998) and PHYLIP (Phylogeny Inference Package, ver. 3.57c; Felsenstein, 1995). MP analysis was performed by heuristic search using tree-bisection-reconnection (TBR) for branch-swapping algorithm, steepest descent option not in effect, stepwise addition option for starting tree, number of trees held at each step during stepwise addition for one, and initial "MaxTrees" setting for 100. Branches were collapsed if maximum branch length is zero. ML heuristic searches were conducted using TBR for branch-swapping algorithm, steepest descent option not in effect, stepwise addition option for starting tree, number of trees held at each step during stepwise addition for one, and initial "MaxTrees" setting for 100. Branches were collapsed if maximum branch length is less than or equal to one. Trees were evaluated using the bootstrap test (Felsenstein, 1985) by 1,000 iterations.

Table 1. A list of species names, animal numbers, trapping localities, sequence length, G+C-content, and GenBank accession numbers

Species	Animal number	Collecting locality (company)	Collecting date	Length (bp)	G+C-content (%)	GenBank accession number
Family Apidae						
Subfamily Apinae						
Tribe Bombini						
<i>Bombus ardens ardens</i>	B146	Muju-gun, Chollabuk Province	April 2000	496	22.2	AF364822
	B160	Busan City	April 2000	496	22.2	
<i>Bombus consobrinus wittenburgi</i>	B9	Chongson-gun, Kangwon Province	May 2000	503	21.9	AF364823
	B10	Chongson-gun, Kangwon Province	May 2000	503	21.9	
<i>Bombus hypocrita sapporoensis</i>	B1	Chongson-gun, Kangwon Province	May 2000	508	21.1	AF364824
	B2	Chongson-gun, Kangwon Province	May 2000	508	21.1	
<i>Bombus ignitus</i>	B66	Muju-gun, Chollabuk Province	April 2000	504	20.4	AF364825
	B85	Chongson-gun, Kangwon Province	May 2000	504	20.4	
<i>Bombus koreanus</i>	B23	Pyongchang-gun, Kangwon Province	May 1999	502	20.9	AF364826
	B25	Pyongchang-gun, Kangwon Province	May 1999	502	20.9	
<i>Bombus modeatus</i>	B30	Chongson-gun, Kangwon Province	June 2000	508	25.0	AF364827
	B300	Chongson-gun, Kangwon Province	June 1999	508	25.0	
<i>Bombus schrencki albidopleuralis</i>	B32	Chongson-gun, Kangwon Province	May 2000	501	19.4	AF364828
	B298	Chongson-gun, Kangwon Province	April 2000	501	19.4	
<i>Bombus ussurensis</i>	B12	Chongson-gun, Kangwon Province	May 2000	500	23.0	AF364829
	B14	Chongson-gun, Kangwon Province	May 2000	500	23.0	
<i>Psithyrus coreanus</i>	B20	Chongson-gun, Kangwon Province	May 2000	505	21.8	AF364830
	B21	Chongson-gun, Kangwon Province	May 2000	505	21.8	
<i>Bombus terrestris</i> *	B39	Netherland (Koppport)	March 2000	505	21.2	AF364831
	B46	Israel (Pollination Service Yad mordechai)	March 2000	505	21.4	AF364832
	B51	Belgium (Biobest)	March 2000	505	21.2	AF364833
Tribe Xylocopini						
<i>Xylocopa applendiculata circumvolans</i> **	B33	Chongson-gun, Kangwon Province	May 2000	503	20.1	AF364834

*This bumblebee species is not indigenous, but imported as tomato pollinators from Europe (Netherlands, Belgium, and Israel).

**The species was used as an outgroup for phylogenetic analysis.

			30			60
<i>B. a. ardens</i> (B146)	GGCTTGATGA	T-AATAATTT	TAAGTCGAT-	CTGCCCAATG	ATTAA----	-TATTAAATG
<i>B. c. wittenburgi</i> (B9)A....G.....T....	..A.....
<i>B. h. sapporoensis</i> (B1)AT...	GT.....A..TTTAAA.	..A..T....
<i>B. ignitus</i> (B66)AT...	A.....A..TTTATAA.	..A..T....
<i>B. koreanus</i> (B23)A....G.....T....	..A.....
<i>B. modeatus</i> (B30)T.....AT.AATAT	T...T....
<i>B. s. albidopleualis</i> (B32)AT...	A.....T...T.A....	..AT..T....
<i>B. ussurensis</i> (B12)	...C.AT...	A.T.....	..G.....TTA....	..A..T....
<i>P. coreanus</i> (B20)	.T...A....	A.TT.....A.....	..A..T....
<i>B. terrestris</i> (B39, B51)	.T...AT...	AT.....A..TTTAAAT.	..A.....
<i>B. terrestris</i> (B46)	.T...AT...	AT.....A..TTTAAAT.	..A.....
<i>X. a. circumvolans</i> (B33)	..T..AT...	AT.....	..GA..A..T....	..A---.....	..A.....A
			90			120
<i>B. a. ardens</i> (B146)	GCTGCAGTAT	AATTAAGTGT	ACAAAGGTAG	CATAATCGAT	TGTTTTTTAA	TTGGAATCTG
<i>B. c. wittenburgi</i> (B9)T..G....A..AAG.A...
<i>B. h. sapporoensis</i> (B1)G.....A..AG.A...
<i>B. ignitus</i> (B66)	C.....A..AA..A...
<i>B. koreanus</i> (B23)	GT..G....A..AA..A...
<i>B. modeatus</i> (B30)C.G....A...
<i>B. s. albidopleualis</i> (B32)A..AA..A..A
<i>B. ussurensis</i> (B12)G....	-T..G....A..A..A..A
<i>P. coreanus</i> (B20)AG.A..A..A
<i>B. terrestris</i> (B39, B51)G....	...G....A..AA..A...
<i>B. terrestris</i> (B46)G....	...G....A..AA..A...
<i>X. a. circumvolans</i> (B33)A	T.A.G....A..	A..A..A..T
			150			180
<i>B. a. ardens</i> (B146)	GAATGAAAGA	AITTAATGAGA	TATATACTGT	CTCATATATA	TTTAATGAAT	TTAAATTTT
<i>B. c. wittenburgi</i> (B9)A.....
<i>B. h. sapporoensis</i> (B1)	G.....A...T...
<i>B. ignitus</i> (B66)	G.....T...
<i>B. koreanus</i> (B23)A.....
<i>B. modeatus</i> (B30)	.T.....A...
<i>B. s. albidopleualis</i> (B32)A...A...
<i>B. ussurensis</i> (B12)	A.A.....
<i>P. coreanus</i> (B20)	.T.....T.....A...
<i>B. terrestris</i> (B39, B51)	G.....	..C.....A...T...
<i>B. terrestris</i> (B46)	G.....	..CC.....A...T...
<i>X. a. circumvolans</i> (B33)G...A.AA.T...A
			210			240
<i>B. a. ardens</i> (B146)	AGTAAAAATG	CTTAAATAAA	TTTATGGGAC	GATAAGACCC	TATAGAAITTT	TATATTAATA
<i>B. c. wittenburgi</i> (B9)	..T.....TT
<i>B. h. sapporoensis</i> (B1)	..T.....	..A...T.G
<i>B. ignitus</i> (B66)G...TT.
<i>B. koreanus</i> (B23)	..T.....TT
<i>B. modeatus</i> (B30)	..G.....	..G...GT.
<i>B. s. albidopleualis</i> (B32)T.T	..AA..T...
<i>B. ussurensis</i> (B12)T.T
<i>P. coreanus</i> (B20)A...T.TA.
<i>B. terrestris</i> (B39, B51)	..T.....	..A...T..
<i>B. terrestris</i> (B46)	..T.....	..A...T..
<i>X. a. circumvolans</i> (B33)A...TTT	A.....T.AT

Fig. 1. Sequences alignment of nine true bumblebee and one cuckoo bumblebee species obtained from 517-bp 16S rRNA gene sequences. Numbers in the parenthesis are animal number. Dots indicate identical sequences to *B. a. ardens* (B 146).

			270			300
<i>B. a. ardens</i> (B146)	T-ATAGATA-	-AAT--AATT	TTTAT--ATA	TTAATAITTA	GTTGGGAGGA	TTGGTAAATT
<i>B. c. wittenburgi</i> (B9)	AT.ATT...A	A.T.TATT..	A...T....A.....
<i>B. h. sapporoensis</i> (B1)	...T...TA	T...TT....	A...A....TAA.....
<i>B. ignitus</i> (B66)	..TATA..TA	T...TT.T..	A..TA....TA.A.....
<i>B. koreanus</i> (B23)	AT.ATT...A	A.T.TATT..	A..T.T....A.....
<i>B. modeatus</i> (B30)GATA....TT.....G...
<i>B. s. albidopleualis</i> (B32)	..-ATA..TA	A--.TATT.A	A...A....	.A.....	A.....	..AA.....
<i>B. ussurensis</i> (B12)	..A.ATAG.TA	TTT.TAT.GG	A..T.T....	A.....	..A.....
<i>P. coreanus</i> (B20)	G.T.TA..TA	A.T.AA...A	A...ATT..	A.....	..A.....
<i>B. terrestris</i> (B39, B51)	..T.TA..TA	T...TTT..	A..GA....TA.....
<i>B. terrestris</i> (B46)	..T.TA..TA	T...TTT..	A..GA....TA.....
<i>X. a. circumvolans</i> (B33)	..A.ATA.ATA	T..A..TT..	A..TATTTA.	.A.....AT.....
			330			360
<i>B. a. ardens</i> (B146)	TATTAAACTT	TATTTTAAAA	TTTTAACITT	AATTTGAGAA	TAA----AT	AATGATCTTG
<i>B. c. wittenburgi</i> (B9)	..G.....-	...A.....	G...AA....	..TTT...T.	T-.....
<i>B. h. sapporoensis</i> (B1)A...-	.A.....	...AA....	...AT..AT.	TT.....T
<i>B. ignitus</i> (B66)	...T.....	...AT...-	.A.A.....	...AA....	...T...-	TT.....T
<i>B. koreanus</i> (B23)	..A.....-	...A.....	G...A....	--TTT...T.	T-.....
<i>B. modeatus</i> (B30)	..GAAT....G...-	..AA.....	G.....	...GATAA..
<i>B. s. albidopleualis</i> (B32)	..A.....T...-	AA.A.....T....	..TAT...TAA
<i>B. ussurensis</i> (B12)	..A.....-	.A.A.....	G...A....	..-TAT...T.	T-.....
<i>P. coreanus</i> (B20)	..TAA.....	...A...-	A..A...A	G...AA..G.	..TT.....-	TTA..G...
<i>B. terrestris</i> (B39, B51)-	.A.....	...AA....	..TA...-	TT.....T
<i>B. terrestris</i> (B46)-	.A.....	...AA....	..TA...-	TT.....T
<i>X. a. circumvolans</i> (B33)	A.A.T.....	..A.--....	...A.T...A	...AA..T.	A..TATATTA	..T...ATAAA
			390			420
<i>B. a. ardens</i> (B146)	T-ATTATAAA	TTATAAGATT	AAATTACCTT	AGGGATAACA	GCGTAATATC	TTTTGAAGA
<i>B. c. wittenburgi</i> (B9)G..	..A.A...AAT...G
<i>B. h. sapporoensis</i> (B1)	...A..A...	..TAT...A.T....
<i>B. ignitus</i> (B66)	...A..A...	..A...A.T....
<i>B. koreanus</i> (B23)A.A...AAT....
<i>B. modeatus</i> (B30)A.G...G.G....
<i>B. s. albidopleualis</i> (B32)	..T.....	...A..A.A.T....
<i>B. ussurensis</i> (B12)G..	-A.....A.T...G
<i>P. coreanus</i> (B20)A...A.AG....
<i>B. terrestris</i> (B39, B51)	...G..A...	..G.A...G.T....
<i>B. terrestris</i> (B46)	...G..A...	..G.A...G.T....
<i>X. a. circumvolans</i> (B33)	..T...TG.	...AT.....	C.....
			450			480
<i>B. a. ardens</i> (B146)	TCCTATTGAT	AGAGATGATT	GCGACCTCGA	TGTTGAATTA	AGATAAATTT	TAAATGAAGA
<i>B. c. wittenburgi</i> (B9)	..A...A..	..A.....G.....
<i>B. h. sapporoensis</i> (B1)	..A.....A	..A..G....
<i>B. ignitus</i> (B66)	..A.....A	..A.....G...
<i>B. koreanus</i> (B23)	..A...A..	..A.....G.....
<i>B. modeatus</i> (B30)	..GA.....	..A..G....T.....	...T...
<i>B. s. albidopleualis</i> (B32)	..A...A..	..A...T..GG.....
<i>B. ussurensis</i> (B12)	..A.....	..A.....GG...G..
<i>P. coreanus</i> (B20)	..AA...T.A	..A.....T.....	...C..G
<i>B. terrestris</i> (B39, B51)A	..A.....
<i>B. terrestris</i> (B46)A	..A.....
<i>X. a. circumvolans</i> (B33)	..AA.....	..AG.....A...	...T...

Fig. 1. Continued.

			510	517
<i>B. a. ardens</i> (B146)	AATTTAATAA	TT-AAGTCTG	TTCGACTTTT	AAAATCTC
<i>B. c. wittenburgi</i> (B9)	.G.....A..
<i>B. h. sapporoensis</i> (B1)
<i>B. ignitus</i> (B66)
<i>B. koreanus</i> (B23)	.G.....A..
<i>B. modeatus</i> (B30)	.G.....G.
<i>B. s. albidopleualis</i> (B32)	...C...AT.
<i>B. ussurensis</i> (B12)	.G.....A..
<i>P. coreanus</i> (B20)	.G.....	...G.....
<i>B. terrestris</i> (B39, B51)
<i>B. terrestris</i> (B46)
<i>X. a. circumvolans</i> (B33)	TG.....A.T	..T.....

Fig. 1. Contined.

Results and Discussion

Nucleotide variation among bumblebees

A region of mitochondrial (mt) 16S rRNA gene was PCR-amplified and sequenced from nine species of bumblebees (eight species of true bumble bees, consisted of five subgenera in the genus *Bombus* and one species of cuckoo bumblebee, *Psithyrus coreanus*) and one outgroup species, *X. a. circumvolans*. The amplified product varied in their length ranging from 496 bp to 508 bp (Table 1). Length variation in insect mt 16S rRNA has been reported previously, and main reason for the variation was several small indels (Simon *et al.*, 1994). Our sequence analysis of the bumblebee species showed scattered 24 indels in several regions within the genome, instead of clustering of indels (Fig. 1).

The average frequencies of each nucleotide are A = 39.2, C = 13.7, G = 7.8 and T = 39.3% (78.5% of A + T content) among the sequences, indicating clearly higher usage of A + T nucleotides in the 16S rRNA. A high A + T content in the invertebrate mt genome has been repeatedly reported. A recent complete insect mt genome sequences showed that A + T content of insect 16S rRNA ranges from 74.5% to 85.3% (Bae *et al.*, 2004), and the highest value was observed in the honeybee, *Apis mellifera* (Crozier and Crozier, 1993). Because our 16S rRNA sequences are partial of the gene, an accurate bumblebee A + T content in not available yet.

All the species, except for *B. terrestris*, revealed no sequence variation in the two individuals. However, the three individuals of *B. terrestris*, which were imported from other countries for green house pollination revealed two haplotypes, containing one nucleotide difference between them.

The sequences diversity among nine *Bombus* and one *Psithyrus* species ranged from 1.4% (7 bp) to 15.49% (77 bp). The minimum sequence divergence was observed in a

comparison between *B. consobrinus wittenburgi* and *B. koreanus*, belonging to the same subgenus *Magabombus* (7 bp). On the other hand, the maximum sequence divergence was observed in a comparison between *P. coreanus* and *B. terrestris* (animal number B46). The outgroup species, *X. a. circumvolans* showed the sequence divergence from 14.98% (to *B. koreanus*) to 17.61% (*B. terrestris*, animal number B46) to the ingroup. The 16S RNA sequence divergence within other *Bombus* genus, *Fervidobombus*, was reported to be 0.36% ~ 8.5% (Sydney and Williams, 2003). Somewhat larger sequence divergence in our samples appears to be stemmed due to composition of several subgenera in the genus *Bombus*.

Phylogenetic relationships

With the introduction of gaps, the 16S rRNA data set used for analysis contain 518 aligned sites. Of these sites, 345 sites are constant, 73 variable sites are parsimony-uninformative, and 100 variable sites are parsimony informative. The phylogenetic relationships among nine species of *Bombus* comprising six subgenera and one cuckoo bumblebee species, *Psithyrus coreanus*, are depicted in Fig. 2. Analyses run with transition: transversion weightings of several ratios in the MP analysis did not affect topology of the tree. Thus, the result of unordered analysis is presented (Fig. 2A). Unweighted parsimony analysis of the 518 bp 16S rRNA sequences resulted in a single most parsimonious tree. In all cases, the species belonging to identical subgenus formed a strong subgroup. For example, three species of subgenus *Bombus s. str.* formed a strong subgroup with a high bootstrap value (97%) and the same is true for other subgenera containing multiple species (*Pyrobombus* and *Megabombus*; Fig. 2A).

There has been a substantial debate on phylogenetic relationships of the cuckoo bumblebees, and the main point has been whether *Psithyrus* is a monophyletic or polyphyletic group and the relationship of *Psithyrus* with

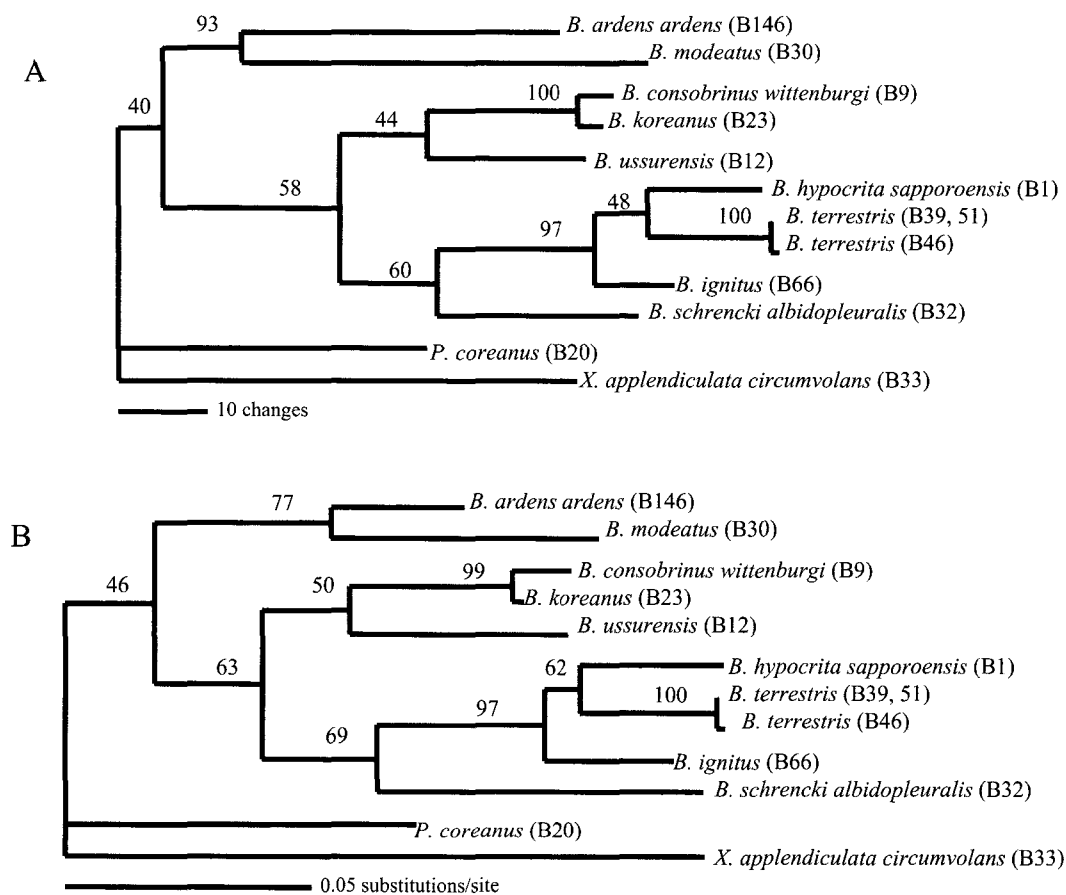


Fig. 2. Phylogenetic analysis by maximum-parsimony (A) and maximum-likelihood methods (B). The analyses were performed with nine true bumblebees and one cuckoo bumblebee species using mitochondrial 16S rRNA sequences. In both analyses a carpenter bee species, *Xylocopa applendiculata circumvolans*, was used as an outgroup. Numbers on each node indicate the number of times the nodes was supported in an analysis of 1,000 bootstrap replicate datasets. The maximum parsimony tree (A) is a single most parsimony tree from the heuristic search and characteristics of the tree are as follows: tree length = 316, a consistency index equal to 0.668, and a homoplasy index equal to 0.332.

true bumblebees (Milliron, 1971; Plowright and Stephen, 1973; Pekkarinen *et al.*, 1979; Obrecht and Scholl, 1981; Williams, 1985, 1991; Pamilo *et al.*, 1987). Morphologically, the parasitic cuckoo bumblebees show high resemblance to the given species of host among the *Bombus* species.

Our phylogenetic analysis among a few species of *Bombus* and one species of *Psithyrus* resulted in the placement of *P. coreanus* outside from all remaining ingroup species of true bumblebees, and at the same time, forming of a very weak monophyletic group consisted of eight species of true bumblebees belonging to *Bombus* (40% to support a monophyletic group of *Bombus*; Fig. 2A). Overall similar topology was obtained by ML analysis, although the bootstrap values supporting nodes vary (Fig. 2B).

Pedersen (1996) reported that the cuckoo bumblebees form a monophyletic group within the true bumblebee groups using mt COI gene. Because our samples only

include one species of *Psithyrus* and limited number of *Bombus* species it is not possible to infer any firm conclusion on the relationships of *Psithyrus* within *Bombus*. Further, it is not possible to decide whether *Psithyrus* is monophyletic or polyphyletic group with one species of *Psithyrus*. Nevertheless, our data allow us to infer that *Psithyrus* is not distant enough to be considered as an independent group considering a very weak bootstrap support for a monophyletic *Bombus* species (Fig. 2A). Furthermore, the pairwise comparison data suggest that the sequence divergence of *Psithyrus* is not always sharply differentiated compared to those within true bumblebee species (Table 2). Thus, there is a possibility for the *Psithyrus* species to be placed within *Bombus* group, if multiple species of *Psithyrus* are included in the analysis.

In summary, we analyzed a portion of mt 16S rRNA gene to understand phylogenetic relationships among *Bombus* species. Mt 16S rRNA gene sequences showed a high

Table 2. Pairwise comparisons of 16S rRNA gene sequences among 11 bumble bees

	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>B. a. ardens</i> (146)	-	9.13	10.93	9.56	8.35	7.27	11.63	10.61	11.56	9.76	9.96	15.98
2. <i>B. c. wittenburgi</i> (B9)	45	-	10.38	11.20	1.40	12.45	11.09	7.80	13.20	11.20	11.40	15.73
3. <i>B. h. sapporoensis</i> (B1)	54	52	-	5.56	10.42	15.11	10.20	11.65	12.85	5.35	5.55	16.70
4. <i>B. ignitus</i> (B66)	47	56	28	-	10.84	13.00	9.44	11.07	12.88	5.56	5.75	16.02
5. <i>B. koreanus</i> (B23)	41	7	52	54	-	11.49	10.32	7.42	12.45	10.84	11.04	14.98
6. <i>B. modeatus</i> (B30)	36	62	76	65	57	-	15.49	13.54	12.32	14.60	14.80	17.37
7. <i>B. s. albidopleualis</i> (B32)	57	55	51	47	51	77	-	10.55	14.17	10.64	10.84	17.04
8. <i>B. ussurensis</i> (B12)	52	39	58	55	37	67	52	-	12.68	11.07	11.27	16.63
9. <i>P. coreanus</i> (B20)	57	66	64	64	62	61	70	63	-	13.28	13.48	16.80
10. <i>B. terrestris</i> (B39, B51)	48	56	27	28	54	73	53	55	66	-	0.20	17.41
11. <i>B. terrestris</i> (B46)	49	57	28	29	55	74	54	56	67	1	-	17.61
12. <i>X. a. circumvolans</i> (B33)	78	78	83	79	74	86	84	82	83	86	87	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

A + T-content and indels. Without exception, the true bumblebee species were clustered together with the species belonging to the same subgenus with high bootstrap values. Due to the limitation of the bumblebee species included any firm conclusion is not possible, but our data suggest that *Psithyrus* may not be an independent phylogenetic groups in the relationship to the genus *Bombus*.

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