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Population Genetic Structure of the Bumblebee, *Bombus ignitus* (Hymenoptera: Apidae), Based on Mitochondrial COI Gene and Nuclear Ribosomal ITS2 Sequences

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Abstract

The bumblebee, *Bombus ignitus* (Hymenoptera: Apidae), is a valuable natural resource that is widely utilized for greenhouse pollination in South Korea. Understanding the magnitude of genetic diversity and geographic relationships is of fundamental importance for long term preservation and utilization. As a first step, we sequenced a partial COI gene of mitochondrial DNA (mtDNA) corresponding to the "DNA barcode" region and the complete internal transcribed spacer 2 (ITS2) of nuclear ribosomal DNA from 88 individuals collected in nine South Korean localities. The complete ITS2 sequences were longest among known insects, ranging in size from 2,034 bp ~ 2,052 bp, harboring two duplicated 112-bp long repeats. The 658-bp long mtDNA sequences provided only six haplotypes with a maximum sequence divergence of 0.61% (4 bp), whereas the ITS sequences provided 84 sequence types with a maximum sequence divergence of 1.02% (21 sites). The combination of the current COI data with those of published data suggest that the *B. ignitus* in South Korea and China are genetically a large group, but those in Japan can be roughly separated into another group. Overall, a very high per generation migration ratio, a very low level of genetic fixation, and no discernable hierarchical population were found to exist among the South Korean populations of *B. ignitus*, which suggests panmixia. This finding is consistent with our understanding of the dispersal capability of the species.

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Introduction

The European bumblebee, *Bombus terrestris*, has been commercially introduced in several parts of the world including South Korea and neighboring countries (Iwasaki, 1995; Mitsuhata, 2000). Despite its importance for crop pollination, the side effects, such as competition among bumblebee species (Inoue *et al*., 2008) and disturbance of pollination of other

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bumblebees adapted to a specific crop (Dohzono *et al*., 2008) have also been reported. In South Korea, an interspecific hybridization, genetic contamination, and competitive copulation between *B. terrestris* and *B. ignitus* have also been reported under laboratory conditions (Yoon *et al*., 2009). Thus, a substantial effort has been directed at establishing an artificial mass rearing system for *B. ignitus* in order to substitute the *B. terrestris* (Yoon *et al*., 2002).

Currently, *B. ignitus* is under commercialized by several companies in South Korea. Once field-released, the bumblebee populations that have been adapted to local environment may possibly be replaced by artificial ones, which could reduce local genetic diversity and fitness (Kawecki and Ebert, 2004). Therefore, knowledge on genetic diversity and geographic relationships of *B. ignitus* is essential for long-term preservation and artificial selection. Nevertheless, studies on bumblebee in this regard have been extremely limited for South Korean populations (Lee *et al*., 2006). Under such circumstances, Tokoro *et al*. (2010) recently reported that *B. ignitus* occurring in Japan is slightly, but obviously different from those of South Korea and China by sequencing 1,048 bp of the CO gene of mitochondrial DNA (mtDNA). However, this conclusion was made on the basis of a single population from each Korea and China, and, thus, further confirmation may be required using additional populations.

In this study, therefore, we collected a total of 88 individuals of *B. ignitus* from nine South Korean localities, and sequenced 658 bp of the mitochondrial COI gene and complete internal transcribed spacer 2 (ITS2) from nuclear ribosomal RNA. The sequence information was subjected to the analysis of population genetic structure with the inference of genetic diversity and genetic relationships for South Korean populations. Further, COI sequences were combined with those of Tokoro *et al*. (2010) to better understand the geographic variation of the Korean populations relative to Japanese populations. The COI region we employed corresponded to the "DNA Barcode" region (Hebert *et al*., 2003), which has been used to provide insight into the patterning of within-specific genomic diversity (Hajibabaei *et al*., 2007). The nuclear ITS2, which is located between the 5.8S and 28S rRNA genes, has been broadly investigated in population genetic and contemporary gene flow studies (Mukabayire *et al*., 1999; Marcilla *et al*., 2001).

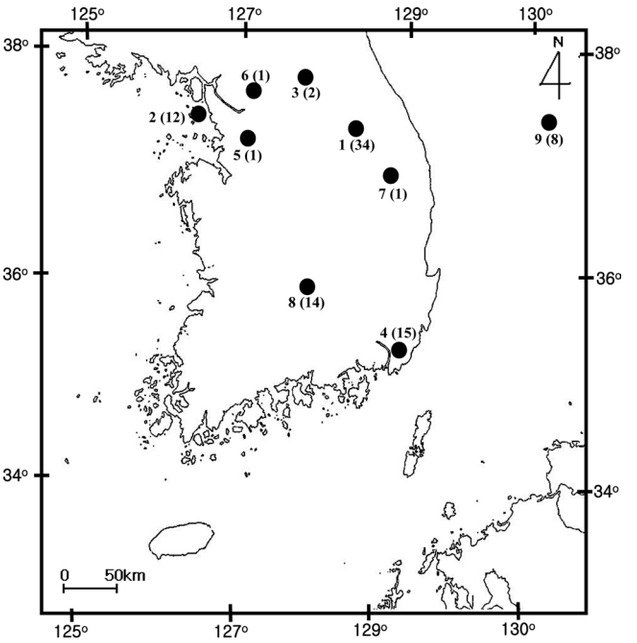


Fig. 1. Sampling location of *Bombus ignitus* in Korea. General locality names and number of sequence indicated are as follows: 1, Jeongseon, Baekjeon-ri, Gangwondo Province; 2, Youngheungdo, Incheon City; 3, Chuncheon City, Gangwondo Province; 4, Busan City; 5, Suwon City, Gyeonggido Province; 6, Namyangju, Gyeonggido Province; 7, Taebaek City, Gangwondo Province; 8, Jungseon, Gangwondo Province; and 9. Muju, Jeollabukdo Province.

Materials and Methods

Insects

A total of 88 *B. ignitus* was collected from nine localities around the beautiful corydalis *Colidalis speciosa* (Fumariaceae) and the cherry blossom *Prunus yedoensis* (Rosaceae) in South Korea. Although most sampling was performed for only one day, those from Jeongseon (locality 1) were mingled with two days samples, resulting in the highest sampling size (Table 1). Regardless of similar sampling effort, more individuals were collected successfully in some localities when compared with others. Sampling locality, number of individuals, date of collection, and GenBank accession numbers for individual COI and ITS2 sequences are provided in Table 1 and the locality map is shown in Fig. 1.

Table 1. Inventory of samples and summary of sequencing results

Collecting locality	Sample number	COI			ITS2			
		Haplotype	A+T contents (%)	GenBank number	Sequence type	Sequence size (bp)	G+C contents (%)	GenBank number
1. Jeongseon	BI0732	BARBI01	77.1	HQ228365	ITSBI02	2044	49.7	HQ228458
Gangwondo	BI0734	BARBI01	77.1	HQ228366	ITSBI03	2044	49.7	HQ228459
Province	BI0736	BARBI01	77.1	HQ228367	ITSBI04	2044	49.7	HQ228460
(34; 06. 29, 2007;	BI0770	BARBI01	77.1	HQ228368	ITSBI05	2045	49.7	HQ228461
04. 24, 2008)	BI0771	BARBI01	77.1	HQ228369	ITSBI06	2045	49.5	HQ228462
	BI1034	BARBI01	77.1	HQ228370	ITSBI07	2045	49.7	HQ228463
	BI1035	BARBI01	77.1	HQ228371	ITSBI08	2045	49.6	HQ228464
	BI1036	BARBI02	77.2	HQ228444	ITSBI09	2044	49.6	HQ228465
	BI1037	BARBI01	77.1	HQ228372	ITSBI10	2047	49.5	HQ228466
	BI1038	BARBI01	77.1	HQ228373	ITSBI11	2034	49.5	HQ228467
	BI1039	BARBI01	77.1	HQ228374	ITSBI12	2046	49.5	HQ228468
	BI1040	BARBI04	77.2	HQ228450	ITSBI13	2049	49.6	HQ228469
	BI1041	BARBI01	77.1	HQ228375	ITSBI14	2042	49.8	HQ228470
	BI1043	BARBI01	77.1	HQ228376	ITSBI01	2051	49.5	HQ228457
	BI1044	BARBI01	77.1	HQ228377	ITSBI15	2043	49.5	HQ228471
	BI1045	BARBI01	77.1	HQ228378	ITSBI16	2047	49.7	HQ228472
	BI1046	BARBI01	77.1	HQ228379	ITSBI17	2045	49.5	HQ228473
	BI1047	BARBI02	77.2	HQ228445	ITSBI18	2044	49.7	HQ228474
	BI1048	BARBI01	77.1	HQ228380	ITSBI19	2048	49.6	HQ228475
	BI1049	BARBI01	77.1	HQ228381	ITSBI20	2041	49.9	HQ228476
	BI1050	BARBI01	77.1	HQ228382	ITSBI21	2047	49.7	HQ228477
	BI1449	BARBI01	77.1	HQ228383	ITSBI51	2045	49.5	HQ228507
	BI1450	BARBI01	77.1	HQ228384	ITSBI52	2042	49.6	HQ228508
	BI1451	BARBI01	77.1	HQ228385	ITSBI53	2045	49.5	HQ228509
	BI1453	BARBI01	77.1	HQ228386	ITSBI54	2044	49.5	HQ228510
	BI 1454	BARBI01	77.1	HQ228387	ITSBI55	2048	49.8	HQ228511
	BI 1455	BARBI01	77.1	HQ228388	ITSBI56	2044	49.7	HQ228512
	BI 1458	BARBI01	77.1	HQ228389	ITSBI57	2044	49.9	HQ228513
	BI1459	BARBI05	77.1	HQ228451	ITSBI58	2042	49.6	HQ228514
	BI1460	BARBI01	77.1	HQ228390	ITSBI59	2043	49.7	HQ228515
	BI1461	BARBI01	77.1	HQ228391	ITSBI60	2044	49.6	HQ228516
	BI1462	BARBI01	77.1	HQ228392	ITSBI61	2046	49.5	HQ228517
	BI1463	BARBI01	77.1	HQ228393	ITSBI62	2052	49.6	HQ228518
	BI1464	BARBI06	77.4	HQ228452	ITSBI63	2045	49.5	HQ228519

Table 1. Continued

Collecting locality	Sample number	COI			ITS2			
		Haplotype	A+T contents (%)	GenBank number	Sequence type	Sequence size (bp)	G+C contents (%)	GenBank number
2. Youngheungdo	BI0851	BARBI01	77.1	HQ228394	ITSBI22	2043	49.7	HQ228478
Incheon City	BI0852	BARBI01	77.1	HQ228395	ITSBI23	2045	49.6	HQ228479
(12; 04. 28, 2007)	BI0853	BARBI01	77.1	HQ228396	ITSBI24	2049	49.6	HQ228480
	BI0854	BARBI01	77.1	HQ228397	ITSBI01	2051	49.5	HQ228453
	BI0855	BARBI01	77.1	HQ228398	ITSBI25	2045	49.5	HQ228481
	BI0856	BARBI01	77.1	HQ228399	ITSBI26	2052	49.6	HQ228482
	BI0857	BARBI01	77.1	HQ228400	ITSBI27	2043	49.7	HQ228483
	BI0858	BARBI02	77.2	HQ228446	ITSBI28	2049	49.6	HQ228484
	BI0859	BARBI01	77.1	HQ228401	ITSBI29	2048	49.7	HQ228485
	BI0860	BARBI01	77.1	HQ228402	ITSBI30	2048	49.7	HQ228486
	BI0861	BARBI01	77.1	HQ228403	ITSBI31	2045	49.5	HQ228487
	BI0862	BARBI01	77.1	HQ228404	ITSBI32	2048	49.5	HQ228488
3. Chuncheon City								
Gangwondo	BI1052	BARBI01	77.1	HQ228405	ITSBI33	2047	49.5	HQ228489
Province	BI1053	BARBI01	77.1	HQ228406	ITSBI34	2046	49.7	HQ228490
(2; 04.28, 2007)								
4. Busan City	BI1056	BARBI01	77.1	HQ228407	ITSBI35	2051	49.5	HQ228491
(15; 05. 07, 2008)	BI1057	BARBI01	77.1	HQ228408	ITSBI36	2045	49.5	HQ228492
	BI1434	BARBI01	77.1	HQ228409	ITSBI37	2045	49.3	HQ228493
	BI1435	BARBI01	77.1	HQ228410	ITSBI38	2045	49.6	HQ228494
	BI1437	BARBI01	77.1	HQ228411	ITSBI39	2044	49.5	HQ228495
	BI1438	BARBI01	77.1	HQ228412	ITSBI40	2045	49.5	HQ228496
	BI1439	BARBI01	77.1	HQ228413	ITSBI41	2044	49.8	HQ228497
	BI1440	BARBI01	77.1	HQ228414	ITSBI42	2041	49.7	HQ228498
	BI1441	BARBI01	77.1	HQ228415	ITSBI43	2042	49.6	HQ228499
	BI1442	BARBI01	77.1	HQ228416	ITSBI44	2047	49.5	HQ228500
	BI1443	BARBI01	77.1	HQ228417	ITSBI45	2042	49.6	HQ228501
	BI1444	BARBI01	77.1	HQ228418	ITSBI46	2043	49.6	HQ228502
	BI1445	BARBI01	77.1	HQ228419	ITSBI47	2043	49.6	HQ228503
	BI1446	BARBI01	77.1	HQ228420	ITSBI48	2045	49.7	HQ228504
	BI1448	BARBI01	77.1	HQ228421	ITSBI49	2047	49.6	HQ228505

Table 1. Continued

Collecting locality	Sample number	COI			ITS2			
		Haplotype	A+T contents (%)	GenBank number	Sequence type	Sequence size (bp)	G+C contents (%)	GenBank number
5. Suwon City								
Gyeonggido	BI1031	BARBI03	77.2	HQ228449	ITSBI01	2051	49.5	HQ228454
Province								
(1; 05. 04, 2007)								
6. Namyangju								
Gyeonggido	BI1032	BARBI01	77.1	HQ228422	ITSBI50	2044	49.7	HQ228506
Province								
(1; 06. 21, 2007)								
7. Taebaek City								
Gangwondo	BI1058	BARBI02	77.2	HQ228447	ITSBI01	2051	49.5	HQ228455
Province								
(1; 06. 10, 2007)								
8. Muju	BI1418	BARBI01	77.1	HQ228423	ITSBI64	2044	49.6	HQ228520
Jeollabukdo	BI1419	BARBI01	77.1	HQ228424	ITSBI65	2046	49.6	HQ228521
Province	BI1420	BARBI01	77.1	HQ228425	ITSBI66	2042	49.8	HQ228522
(14; 04. 25, 2008)	BI1421	BARBI01	77.1	HQ228426	ITSBI67	2046	49.6	HQ228523
	BI1422	BARBI01	77.1	HQ228427	ITSBI68	2044	49.6	HQ228524
	BI1423	BARBI01	77.1	HQ228428	ITSBI01	2051	49.5	HQ228456
	BI1425	BARBI01	77.1	HQ228429	ITSBI69	2044	49.8	HQ228525
	BI1426	BARBI01	77.1	HQ228430	ITSBI70	2045	49.5	HQ228526
	BI1428	BARBI01	77.1	HQ228431	ITSBI71	2043	49.7	HQ228527
	BI1429	BARBI01	77.1	HQ228432	ITSBI72	2047	49.6	HQ228528
	BI1430	BARBI02	77.2	HQ228448	ITSBI73	2045	49.6	HQ228529
	BI1431	BARBI01	77.1	HQ228433	ITSBI74	2046	49.3	HQ228530
	BI1432	BARBI01	77.1	HQ228434	ITSBI75	2043	49.7	HQ228531
	BI1433	BARBI01	77.1	HQ228435	ITSBI76	2040	49.8	HQ228532
9. Ulleungdo	BI1970	BARBI01	77.1	HQ228436	ITSBI77	2051	49.5	HQ228533
Gyeongsangbuk	BI1971	BARBI01	77.1	HQ228437	ITSBI78	2047	49.6	HQ228534
Province	BI1972	BARBI01	77.1	HQ228438	ITSBI79	2044	49.8	HQ228535
(8; 05. 09, 2009)	BI1973	BARBI01	77.1	HQ228439	ITSBI80	2044	49.6	HQ228536

Table 1. Continued

Collecting locality	Sample number	COI			ITS2			
		Haplotype	A+T contents (%)	GenBank number	Sequence type	Sequence size (bp)	G+C contents (%)	GenBank number
	BI1974	BARBI01	77.1	HQ228440	ITSBI81	2045	49.6	HQ228537
	BI1975	BARBI01	77.1	HQ228441	ITSBI82	2044	49.6	HQ228538
	BI1976	BARBI01	77.1	HQ228442	ITSBI83	2044	49.7	HQ228539
	BI1977	BARBI01	77.1	HQ228443	ITSBI84	2042	49.5	HQ228540

Within-parentheses indicate number of individuals collected at each locality and collection date.

DNA extraction, primer, PCR, and sequencing

Total DNA was extracted with a Wizard Genomic DNA Purification Kit, in accordance with the manufacturer's instructions (Promega, USA). A 658-bp region of the mitochondrial COI gene corresponding to the "DNA Barcode" region (Herbert *et al*., 2003) was amplified using a pair of primer sets reported by Kim *et al*. (2009). For the amplification of ITS2, the primers NG02955 and AB052895 located on the 5.8S and 28S rDNAs, respectively, were successfully used (Ji *et al*., 2003). In order to complete the entire ITS2 the internal primer BITSF, 5'-CGTAGTGTTGTCCTCGTGACCGA-3', was designed from the conserved region of the *B. ignitus* ITS2 sequences.

For amplification of the COI gene, PCR was conducted under the following conditions: an initial denaturation step at 94°C for 7 min, a 35-cycle amplification (94°C for 1 min, 54~56°C for 1 min, and 72°C for 1 min), and the final extension step for 7 min at 72°C. For amplification of ITS2, PCR was conducted under the following conditions: an initial denaturation step at 94°C for 7 min, a 35-cycle amplification (94°C for 40 sec, 55~64°C for 20 sec, and 72°C for 20 sec), and the final extension step for 2 min at 72°C. Although COI amplicons were directly sequenced, those of ITS2 were cloned into a pGEM-T Easy vector (Promega, USA). XL1-Blue competent cells (Stratagene, USA) were transformed with the ligated DNA, and the resultant plasmid DNA from one clone per individual was isolated with a Plasmid Miniprep Kit (Dyne Bio Inc., Korea). Sequencing was performed using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer (PE Applied Biosystems, USA).

Sequence analysis

COI gene and ITS2 sequenc es were delimitated and aligned using MAFFT ver. 6 (Katoh *et al*., 2002). The boundary decision to remove 5.8S at the 3′ end and 28S at the 5′ end of the ITS2 sequence was made using the Hidden Markov Model-based ITS2 annotation software (Keller *et al*.,, 2009). When the homologous sequences from two individuals differed by one or more nucleotide bases (for both COI and ITS2) or one insertion/deletion (indel) position (for ITS2), the sequences were considered different haplotypes (for COI) or sequence types (for ITS2). Haplotype or sequence type designations were applied to new sequences as they were discovered (i.e., BARBI01, BARBI02, and BARBI03 for COI, and ITSBI01, ITSBI02, ITSBI03 and so on for ITS2).

For the alignment of the indel-containing ITS2 region sequences, GBlocks version 0.91b software (Castresana, 2000) was used to select conserved regions for the subsequent phylogenetic and population level analyses. Resultantly, a total of 84 ITS2 sequence types with 2,034~2,052 positions, including gaps from 84 ITS2 sequence types with ~2,068 positions were obtained, conserving 96% of the original sequences.

Phylogenetic analysis

To determine the relationships among the COI haplotypes, among ITS2 sequence types, and to detect any describable groups, phylogenetic analysis was conducted via the maximum-parsimony (MP) method (Fitch, 1971) using PAUP* (Phylogenetic Analysis Using Parsimony and

Other Method*) ver. 4.0b10 software (Swofford, 2002) and Bayesian Inference (BI) method using MrBayes ver. 3.1 (Huelsenbeck and Ronquist, 2001), respectively. The analysis for the MP method was conducted by heuristic search. The reliability of the trees was assessed by 1,000 iterations of bootstrapping (Felsenstein, 1985).

For the BI analysis, the substitution model was selected by comparing Akaike information criterion scores (Akaike, 1974) using Modeltest ver. 3.7 (Posada and Crandall, 1998). The best-fit model selected was GTR (Lanave *et al*., 1984) + I + G for the COI gene and K80 + G (Kimura, 1980) for ITS2. The confidence values of the BI tree were presented as the Bayesian posterior probabilities in percentages (BPP). The homologous regions of the within-generic species B. ardens were adapted, respectively, from Kim *et al*. (2009) for the COI gene and Oh *et al*. (2009) for ITS2 as an outgroup to root the trees.

Network construction

Haplotype (COI) or sequence type (ITS2) relationships were determined using the median-joining algorithm (Bandelt *et al*., 1999) incorporated in SplitsTree ver. 4.11.3 (Huson and Bryant, 2006). This method adds to the network median vectors (consensus sequences) by starting with the minimum spanning trees combined within a single network. Such vectors can be interpreted as possibly extinct unsampled sequences or extinct ancestral sequences (Bandelt *et al*., 1999).

Population genetic estimates

Haplotype and nucleotide diversity, both of which are reflective of genetic diversity within each locality, were determined using Arlequin ver. 3.5 (Excoffier and Lischer, 2010), whereas maximum sequence divergence within each locality was estimated by extracting within-locality estimates of unrooted pairwise distances from PAUP* ver. 4.0b (Swofford, 2002). The population pairwise genetic distance (F_{ST}) and a permutation test for its significance (1,000 bootstraps) were evaluated using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). Pairwise *Nm* (the product of the effective population size, *Ne*, and migration rate, *m*) values were employed to estimate the pairwise F_{ST} based on the equilibrium relationship: $F_{ST} = 1 / (2Nm + 1)$ for the COI gene and $F_{ST} = 1 / (4Nm + 1)$ for ITS2. For these estimates only populations with more than two individuals, possessing more than two haplotypes for the COI gene or more than two sequence types for ITS2 were subjected to analyses. Genetic relationships among populations and sets of populations were assessed by the Holsinger and Mason-Gamer (H-MG) method (1996). A detailed rationale of this method is described in the original study of Holsinger and Mason-Gamer (1996).

Results

Sequence analysis

A total of six COI haplotypes (BARBI01 ~ BARBI06) and 84 ITS2 sequence types (ITSBI01 ~ ITSBI84) were obtained by sequencing 88 individuals of *B. ignitus* (Table 1). Although the COI gene contained no indels, presenting all identical 658 bp, ITS2 was variable in length, ranging from 2,034 to 2,052 bp. The length of *B. ignitus* ITS2 was the longest among known insects as far as available data are considered (Table 2). Such a long ITS2 sequence in *B. ignitus* derives, in part, from the presence of a total of 112-bp long, two repeat units, repeated at the beginning region within ~420 bp of *B. ignitus* ITS2 (Fig. 2). An uncorrected pairwise comparison between pairs of haplotypes demonstrated that the sequence divergence ranged from 0.15 to 0.61% (1 ~ 4 bp) among the six haplotypes (Table 3). From the ITS2 sequences a total of 84 sequence types, a sequence divergence ranging from 0.04% to 1.02% was obtained (one ~21 positions; data not shown). In contrast to the higher sequence type diversity of ITS2, the maximum sequence divergence among sequence types was only slightly higher compared to the COI gene (1.02% vs. 0.61%).

Among the six haplotypes, four were found in a single locality as a single individual (BIBAR03, BIBAR04, BIBAR05, and BIBAR06), but the haplotype BIBAR02 was found in four localities (localities 1, 2, 7, and 8) in a total of five individuals and BIBAR01 was found in seven localities in a total of 79 individuals, accounting for 89.8% of the samples (Table 1). Thus, the distribution of *B. ignitus* haplotypes can be summarized as a restricted local distribution in most haplotypes, with a wide distribution only in a limited number of haplotypes. On the other hand, no ITS2 sequence type was found in more than one

Table 2. Summery of ITS2 size and G+C contents in other insects

Order	Family	Specise	ITS2 size (bp)	G+C Contents (%)	Source
Hymenoptera	Apidae	*Melipona beecheii*	1728	53.1	De la Rúa *et al.* (2007)
		Melipona yucatanica	1789	52.9	De la Rúa *et al.* (2007)
		Bombus ardens	1971 - 1984	51	Oh *et al.* (2009)
		Bombus ignitus	2034 - 2052	49.6	This study
	Formicidae	*Strumigenys* spp.	659 - 945	55.2-64.1	Hung et al. (2004)
	Ichneumonidae	*Diadegma* group	613 - 700	53.3	Wagener *et al.* (2006)
	Trichogrammatidae	*Trichogramma minutum*, *T. platneri*	416 - 420	-	Stouthamer *et al.* (2000)
		T. aurosum	450	-	Samara *et al.* (2008)
	Agaonidae	*Galoglychia* group	346 - 554	-	Erasmus *et al.* (2007)
Coleoptera	Chrysomelidae	*Timarcha*	486 - 574	46.7	Gómez-Zurita *et al.* (2000)
Diptera	Culicidae	*Anopheles maculipennis*	280	54.1	Marinucci *et al.* (1999)
		Anopheles sacharovi	300	49.4	Marinucci *et al.* (1999)
		Anopheles punctulatus group	549 - 656	65.0-70.9	Beebe *et al.* (1999)
		Anopheles rivulorum	380	-	Hackett *et al.* (2000)
		Anopheles stephenis	466	-	Alam *et al.* (2008)
	Psychodidae	*Phlebotomus* group	241 - 291	23.3	Muccio *et al.* (2000)
	Simuliidae	*S. inthanonense*	247	26.7	Thanwisai *et al.* (2006)
		S. choochotei	308	18.5	Thanwisai *et al.* (2006)
Siphonaptera	Pulicidae	*Ctenocephalides felis*	500	-	Vobis *et al.* (2004)
Odonata	Calopterygidae	*Calopteryx* group	211 - 212	-	Weekers *et al.* (2001)

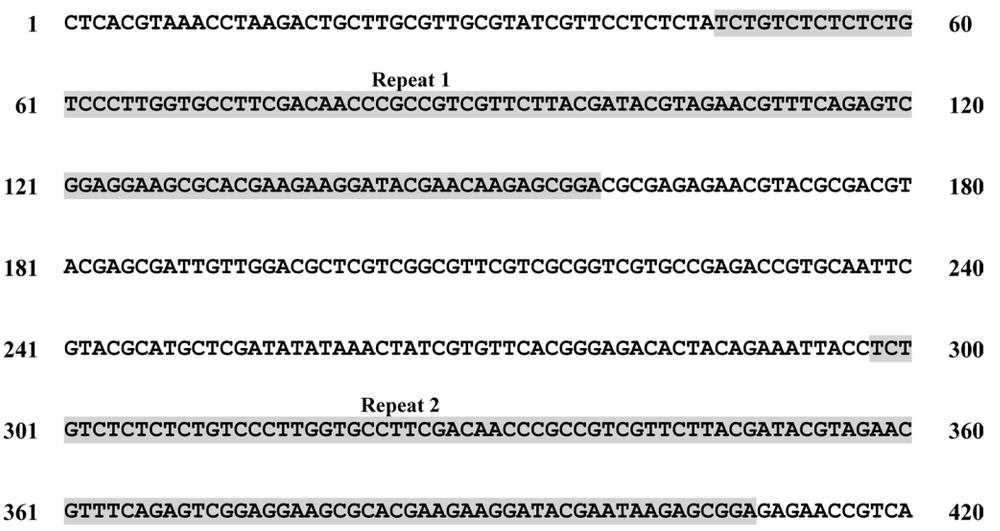
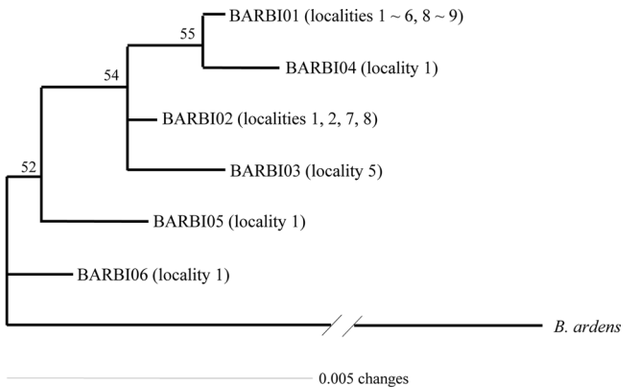


Fig. 2. Beginning sequences of *Bumbus ignitus* ITS2 rDNA, which contained two repeat units.

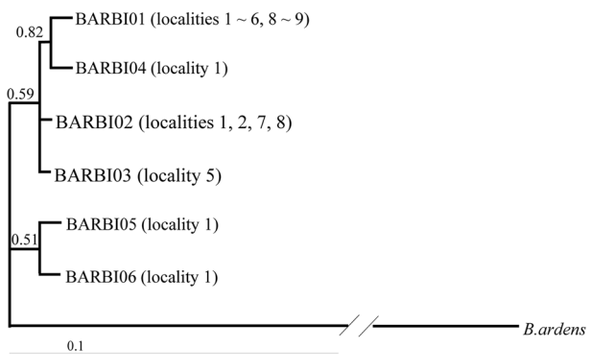
Table 3. Pairwise comparisons among six haplotypes obtained from mitochondrial COI gene sequence of *Bombus ignitus*

	1	2	3	4	5	6
1. BARBI01	-	0.00152	0.00304	0.00152	0.00456	0.00456
2. BARBI02	1	-	0.00152	0.00304	0.00304	0.00304
3. BARBI03	2	1	-	0.00456	0.00456	0.00456
4. BARBI04	1	2	3	-	0.00608	0.00608
5. BARBI05	3	2	3	4	-	0.00304
6. BARBI06	3	2	3	4	2	-

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



(A)



(B)

Fig. 3. Phylogenetic trees obtained from six mitochondrial COI haplotypes of the *Bombus ignitus*. (A) Tree acquired via the MP method incorporated in the PAUP ver. 4.0b10 software (Swofford, 2002). The numbers on the branches represent bootstrap values of 1,000 replications. (B) Tree obtained via the Bayesian Inference phylogram. Numbers at each node specify the BPP. Within-parentheses indicate the locality number, from which the particular sequence type was obtained. *Bombus ardens* was used as an outgroup in order to root tree. The scale bar indicates the number of substitutions per site.

locality, except for the sequence type ITSBI01, which was found in five localities as a single individual at each locality (1, 2, 5, 7, and 8) (Table 1).

Phylogenetic relationships

Phylogenetic analysis was conducted to determine the relationships among the six haplotypes of *B. ignitus* obtained in this study (Fig. 3). All haplotypes were weakly associated or unresolved, owing principally to low genetic divergence among them. One exception was the clustering between BIBAR01 and BIBAR04 in the BI analysis with slightly higher node support (BPP = 0.82), but this group was very weakly supported in the MP analysis as 55%.

Previously, Tokoro *et al*. (2010) sequenced 1,048-bp of the COI gene from *B. ignitus* collected from a Korean and Chinese locality and several Japanese localities. They obtained a total of 15 haplotypes, which consisted of six obtained from both Korea and China and nine from Japan and found that Japanese haplotypes were different from both South Korean and Chinese haplotypes using the MP and Neighbor-Joining method. In order to prepare a total dataset for phylogenetic analysis, the current six haplotypes obtained in this study were combined and aligned with those of Tokoro *et al*. (2010), resulting in 13 new haplotypes with 463 bp in length. These included five from this study (BIBAR01, BIBAR02 = BIBAR03, BIBAR04, BIBAR05, and BIBAR06) and eight from Tokoro *et al*. (2010) (F1 = F2 = F3, F4, F5 = F6, J1 = J2 = J3 = J5, J4, J6, J7 = J8, J9). Phylogenetic analysis by MP and BI methods demonstrated that the haplotypes found in South Korea and China formed one relatively inclusive group (0.92 by BI and 52% by MP), whereas those found in Japan did not form a monophyletic group (data not shown). This

Table 4. Within-locality diversity estimates of *Bombus ignitus* obtain from COI and ITS2 sequence

Locality	SS[a]		NH[b]		H[c]		NP[d]		MSD[e] (%)		MPD[f]		p[g]	
	COI	ITS2	COI	ITS2	COI	ITS2	COI	ITS2	COI	ITS2	COI	ITS2	COI	ITS2
1. Jeongseon	34	34	5	34	0.2745	1.00	5	101	0.45	0.74	0.50446	8.42	0.00077	0.00420
2. Youngheungdo	12	12	2	12	0.1667	1.00	1	27	0.15	0.44	0.16667	5.30	0.00025	0.00265
4. Busan	15	15	1	15	-	1.00	-	38	-	0.54	-	6.44	-	0.00321
8. Muju	14	14	2	14	0.1429	1.00	1	51	0.15	0.54	0.14286	9.25	0.00022	0.00462
9. Ulleungdo	8	8	1	8	-	1.00	-	25	-	0.39	-	7.00	-	0.00349

a) Sample size
b) Number of haplotypes
c) Haplotype diversity
d) Number of polymorphic sites
e) Maximum sequence divergence
f) Mean number of pairwise differences
g) Nucleotide diversity

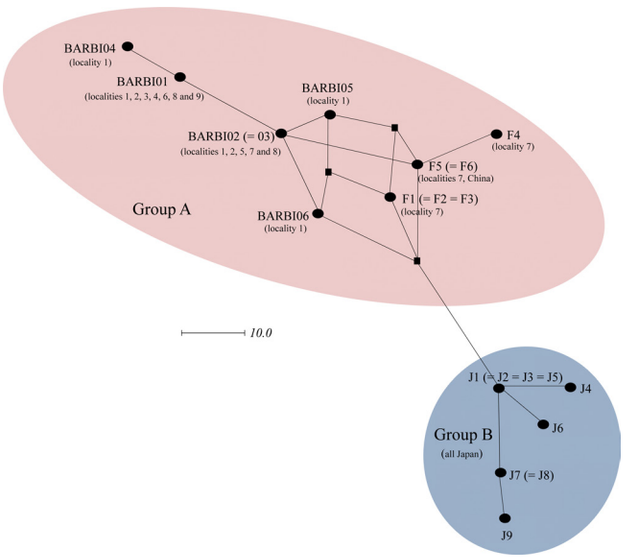


Fig. 4. Median-Joining networks indicating the relationship among COI haplotypes obtained in this study and those of Tokoro *et al*. (2010). Haplotype in group A were found in Korean and Chinese localities, whereas those in group B were found solely in Japan. Identical haplotypes generated from the selection of overlapping 463 nucleotides between this study and that of Tokoro *et al*. (2010) are indicated next to the haplotype names within parentheses. Locality numbers and names are presented under the haplotype type names within parentheses. The branch lengths represent the amount of character-state changes occurring on that branch. Circular dots represent haplotypes found in this study, whereas rectangles indicate the hypothetical ones that were not found in this study.

was likely due to the short sequence length (463 bp) employed in this study.

In order to further understand the relationships among *B. ignitus* haplotypes, the COI-based median-joining network was prepared using the haplotypes obtained in this study and those of Tokoro *et al*. (2010). Among the five haplotypes found in this study (BIBAR01, BIBAR02 = BIBAR03, BIBAR04, BIBAR05, and BIBAR06) the haplotypes BIBAR01 and BIBAR04 were somewhat distantly related to others, but these were grouped together with other haplotypes found in the South Korea and China with some complexity, due to the presence of more than one most parsimonious connection among haplotypes, forming one distinct group (termed group A) (Fig. 4). On the other hand, the haplotypes found solely in Japan (J1 = J2 = J3 = J5, J4, J6, J7 = J8, J9) formed a somewhat distant independent group (termed group B) that was derived from a single founder, J1 (Fig. 4).

Phylogenetic analyses among the 88 ITS2 rDNA sequence types of *B. ignitus* have shown largely unresolved multi-branches, but a few sequence types were grouped together, although the nodal support for the grouping was relatively low (Fig. 5). MP-based analysis has shown that the majority of the sequence types were weakly associated or unresolved, but two groups were exceptional, with bootstrap values of either 85% or 99% (Fig. 5A). Similarly, BI-based analysis also resulted in largely unresolved or weakly associated groups (Fig. 5B). In an attempt to further understand the relationships among *B. ignitus* sequence types, an ITS2-based median-joining network was prepared (Fig. 6). In contrast to the MP- and BI-based

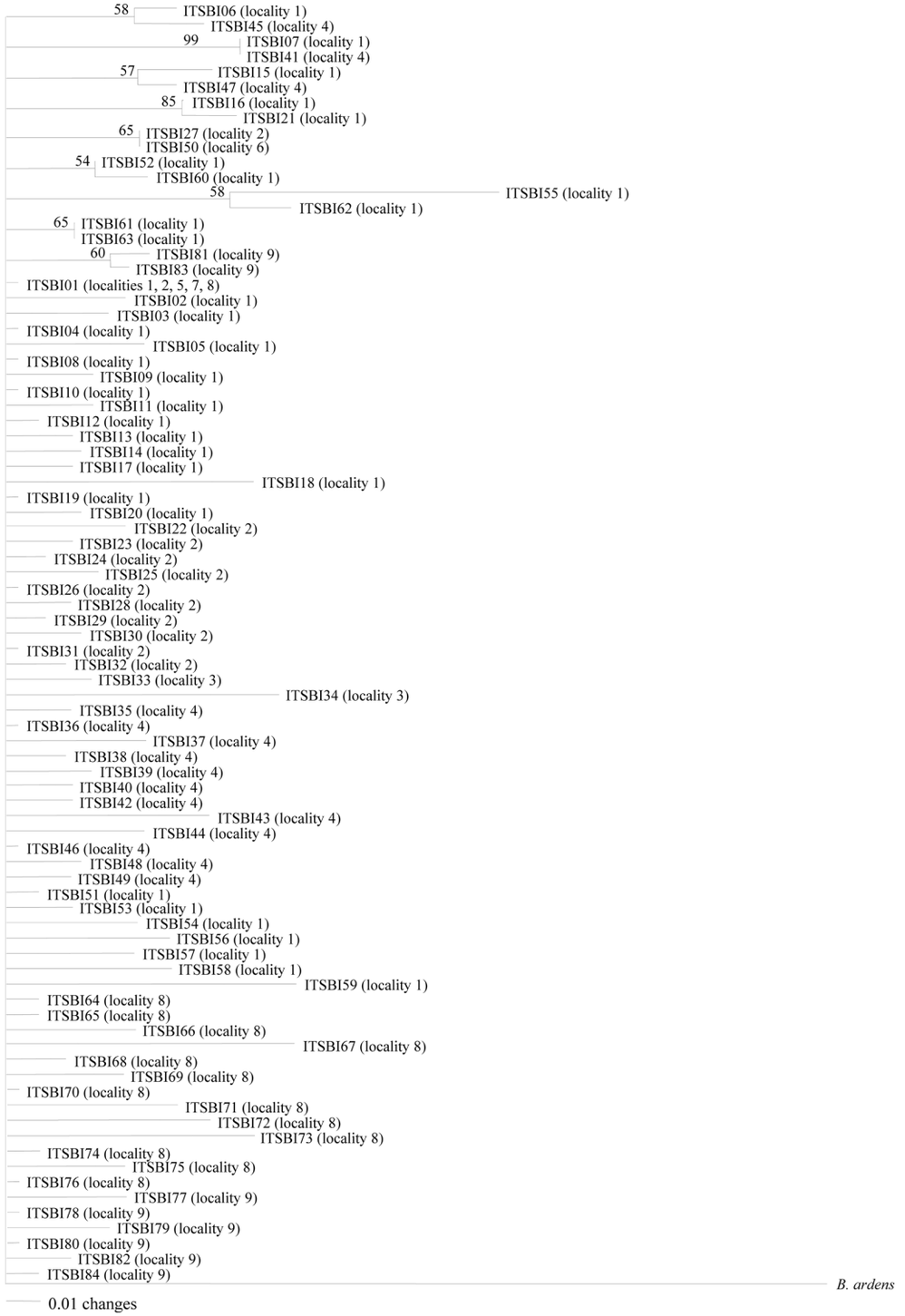


Fig. 5. Phylogenetic trees obtained from 84ITS2 sequence types of the *Bombus ignitus*. (A) Tree acquired via the MP method incorporated in the PAUP ver. 4.0b10 software (Swofford, 2002). The numbers on the branches represent bootstrap values of 1,000 replications. (B) Tree obtained via the Bayesian Inference phylogram. Numbers at each node specify the BPP. Within-parentheses indicate the locality number, from which the particular sequence type was obtained. *Bombus ardens* was used as an outgroup in order to root tree. The scale bar indicates the number of substitutions per site.

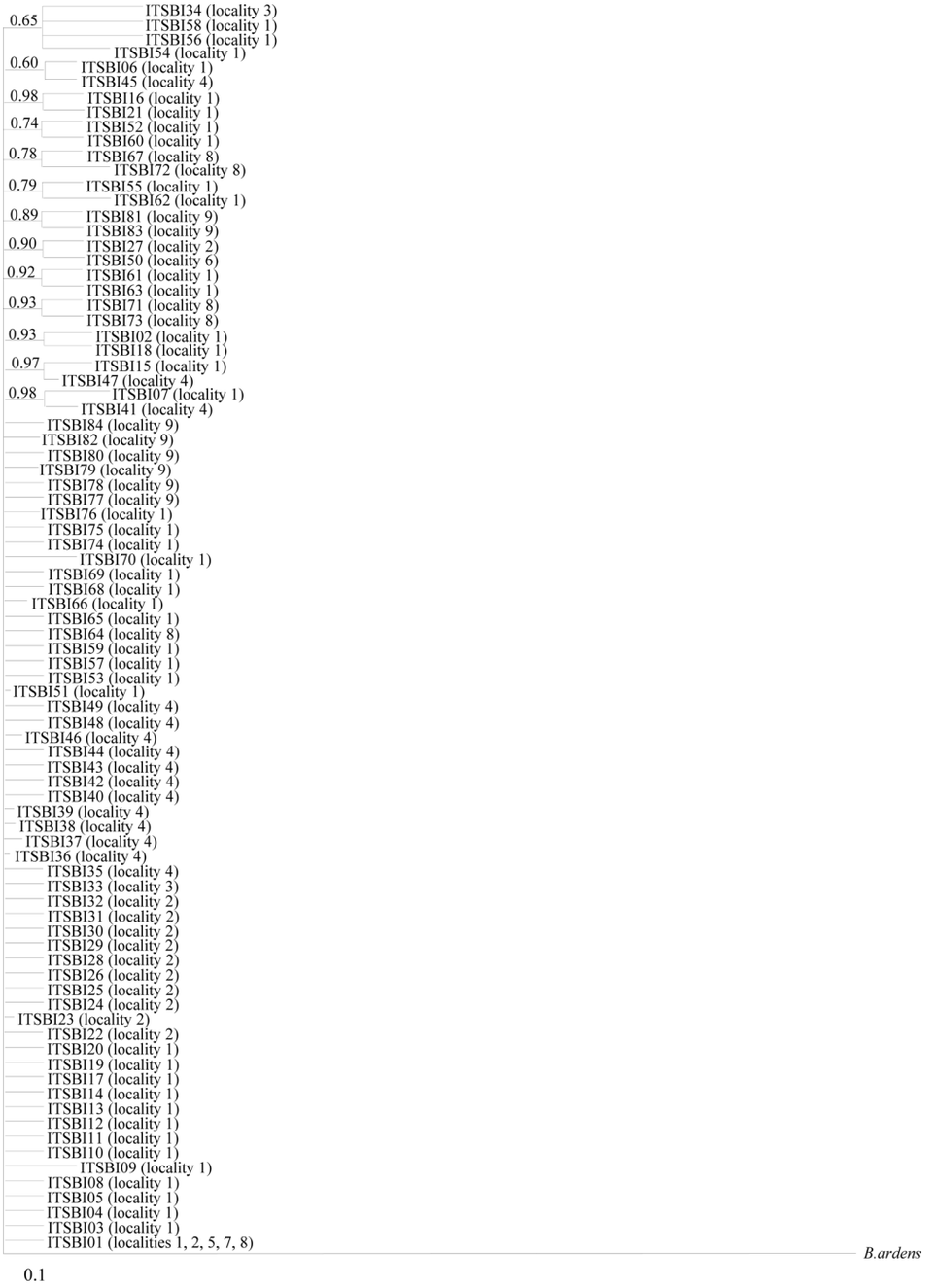


Fig. 5. Continued

phylogenies, the network resulted in a larger number of inclusive groups, providing several star phylogenies, wherein sequence types were composed of centered ones and their derived ones. In any star phylogeny, the derived sequence types were composed of several sequence types that were originated from a diverse locality. Thus, the clustering pattern of the sequence types derived from these localities may reflect non-locality-based clustering, indicating gene flow.

Population genetic estimates

Within-locality diversity was estimated in terms of haplotype

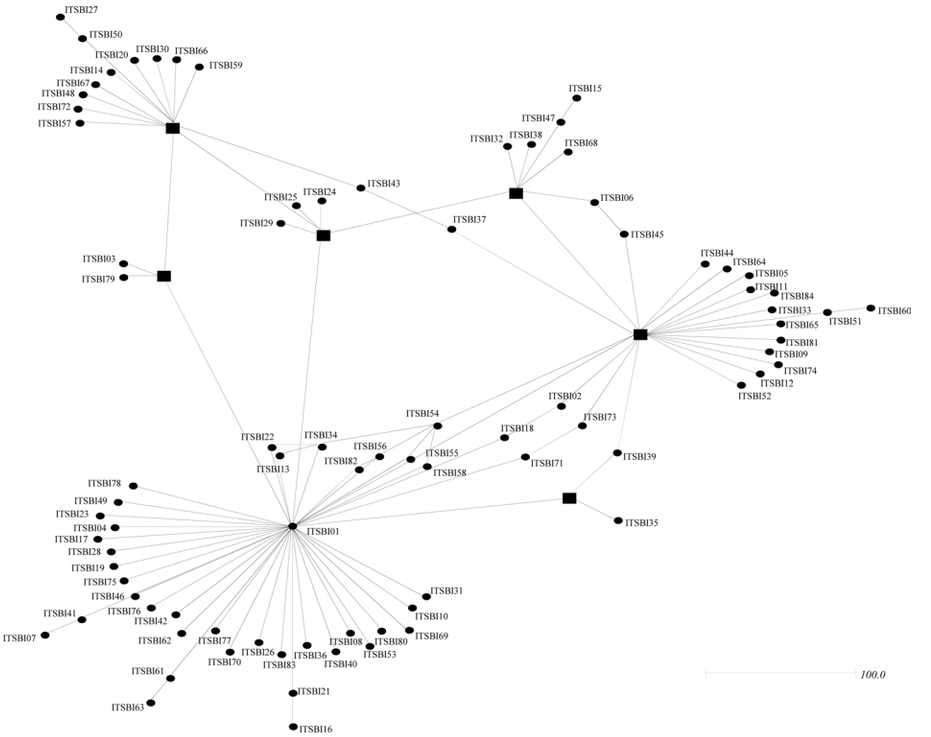


Fig. 6. Median-Joining networks indicating the relationship among 84 ITS2 sequence types. The branch lengths represent the amount of character-state changes occurring on that branch. Circular dots represent sequence types found in this study, whereas rectangles indicate the hypothetical ones that were not found in this study.

diversity (*H*), maximum sequence divergence (MSD), mean number of pairwise differences (MPD), and nucleotide diversity (π) for both COI and ITS2 sequences from the localities with a sample size and haplotype number of ≥ two. Consequently, the three and five localities for the COI and ITS2 sequences, respectively, were subjected to analysis (Table 4). In the case of the COI gene, all three localities displayed substantially low estimates of π, ranging from 0.000767 (locality 1; Jeongseon) to 0.000217 (locality 8; Muju). Considering the maximum difference between the highest and lowest estimates only accounted for about 3.5-fold, no single particular locality could be attributed to a center for genetic diversity. Similarly, the ITS2 sequences also provided only 1.75-fold of the maximum difference between the highest and lowest estimates of π, ranging from 0.004615 (locality 8; Muju) to 0.002645 (locality 2; Youngheungdo). Thus, the ITS2 data also indicated that no single particular locality could be attributed to a center for genetic diversity either. On the other hand, the values of π in the ITS2 were about five to 20-fold higher than those of the COI gene sequence.

Both COI and ITS2-based genetic distance (F_{ST}) revealed no statistically significant F_{ST} in any case (Online Resources 9 and 10). Consistent with the F_{ST} data, the estimate of per-generation migration rates (*Nm*) showed an overall high gene flow between pairs of populations both in the COI and ITS2 sequences (Tables 5 and 6). To better understand the nature of the genetic relationships between the *B. ignitus* populations, the hierarchical relationships among localities were analyzed

Table 5. Fixation indices (F_{ST}) and migration rate (Nm) between pairs of populations of *Bombus ignitus* obtain from mitochondrial COI gene

	1. Jeongseon	2. Youngheungdo	8. Muju
1. Jeongseon		F_{ST} = -0.04075 *Nm* = inf	F_{ST} = -0.03099 *Nm* = inf
2. Youngheungdo			F_{ST} = -0.08279 *Nm* = inf
8. Muju			

* $p < 0.05$.
inf, infinite.

Table 6. Fixation indices (F_{ST}) and migration rate (Nm) between pairs of populations of *Bombus ignitus* obtain from ITS2 sequence

	2. Youngheungdo	4. Busan	8. Muju	9. Ulleungdo
1. Jeongseon	F_{ST} = 0.0058 *Nm* = 85.2274	F_{ST} = -0.0011 *Nm* = inf	F_{ST} = 0.0120 *Nm* = 41.0159	F_{ST} = 0.0026 *Nm* = 189.7444
2. Youngheungdo		F_{ST} = 0.0224 *Nm* = 21.7906	F_{ST} = 0.0120 *Nm* = 41.1283	F_{ST} = 0.0483 *Nm* = 9.8571
4. Busan			F_{ST} = -0.0001 *Nm* = inf	F_{ST} = 0.0207 *Nm* = 23.6980
8. Muju				F_{ST} = 0.0133 *Nm* = 37.1765

* $p < 0.05$.
inf, infinite.

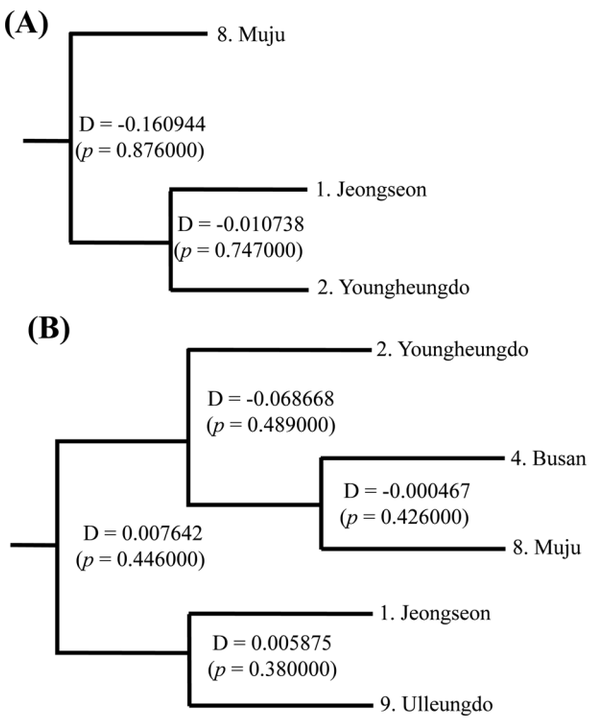


Fig. 7. Hierarchical relationships among localities analyzed using the Holsinger and Mason-Gamer method (1996). The dendrogram obtained from (A) COI and (B) ITS2. The value at each node is the distance (D) between its two daughter nodes and the *p* value is the significance of differentiation (based on 10,000 random resamplings).

(Fig. 7). The COI-based analysis demonstrated that there was a close relationship between Jeongseon (locality 1) and Youngheungdo (locality 2), although this relationship was not statistically significant ($p > 0.05$). In the case of the ITS2-based analysis, Busan and Muju (localities 4 and 8) formed into one group, and Jeongseon and Ulleungdo (localities 1 and 9) formed into another group, and the Busan + Muju group clustered together with the Youngheungdo (locality 2), forming (Youngheungdo + (Busan + Muju)). Nevertheless, separation into these groups was not statistically supported ($p > 0.05$). Thus, hierarchical analyses also indicated non-geographic distance-based population clustering.

Discussion

Major characteristic of each molecule

Other available bee species present in South Korea have a COI sequence divergence of 0.3% for the within-generic bumblebee *B. ardens* (Kim *et al*., 2009) and 0.76% for the mason bee *Osmia cornifrons* (Kim *et al*., 2008), indicating that the sequence divergence was < 1%. Thus, the maximum sequence divergence of the COI gene found as 0.61% in *B. ignitus* was moderate when compared with other bee species occurring in South Korea.

To the best of our knowledge, the *B. ignitus* ITS2 sequence is the longest ever reported in insects, which ranged between 2,034 ~ 2,052 bp. Previously, two within-familial species, *Melipona*, were reported to harbor a ITS2 with lengths of 1,728 bp and 1,789 bp, respectively (De La Rúa *et al*., 2007) and the length of the within-generic species, *B. ardens* ITS2 sequence was reported to be 1,971 ~ 1,984 bp (Oh *et al*., 2009), but our *B. ignitus* was at least 50 bp longer than those of the *B. ardens* ITS2 sequences. Except for the Apidae family, the length of the ITS2 in other hymenopteran and other ordinal groups were

far less than 1,000 bp in most cases (Table 2). Similarly to *B. ignitus*, the *B. ardens* ITS2 sequences was 114-bp in length and contained four repeat units, repeated twice at the beginning region within ~430 bp of ITS2, but no such repeat sequence was detected in the ITS2 regions of any other insect, including two within-familial *Melipona* species (De La Rúa *et al*., 2007). Thus, this repeat sequence of the *Bombus* ITS2 seems to be a molecular synapomorphic characteristic for the genus, although more *Bombus* species should be analyzed.

The sequence type diversity of ITS2 was also noteworthy in that nearly all individuals accounted for a different sequence type (Table 1). One of the possible reasons for such high diversity in sequence type is a higher evolutionary ratio of ITS2 since this ITS2 may have been relatively free of structural and functional constrains (Tang *et al*., 1996).

Relationships between South Korean and Japanese *B. ignitus*

Previously, Shao *et al*. (2004) sequenced 450 bp of mitochondrial cytochrome *b* gene from 16 individuals collected from three localities in China, South Korea, and Japan, and no sequence variation was observed among them. However, further variable microsatellite analysis from 124 individuals showed statistically significant genetic differentiation of the Chinese and South Korean populations from the Japanese populations, which was possibly due to the geographical isolation of Japan (Shao *et al*., 2004). Tokoro *et al*. (2010) also found that the genetics of Japanese populations were differentiated from South Korean and Chinese populations when a 1,048 bp of the COI gene was sequenced. Our median-joining network using the data from Tokoro *et al*. (2010) and the current study also suggested that there was a genetic differentiation between mainland populations and Japan (Fig. 7). Collectively, this data indicate that the *B. ignitus* in South Korea and China are genetically a large group, but those in Japan is roughly separable into another group. This result reinforces the finding of Tokoro *et al*. (2010), even though limited sequence information was utilized. It is likely that the geographic connection between the Korean peninsula and mainland China facilitated gene glow, but the geographically isolated Japanese populations may have had little or no chance for gene flow during continental separation at about 110,000 ~ 280,000 (*Kawamura, 2006*).

Genetic relationships among South Korean populations

Our F_{ST} and Nm analyses on the basis of both COI and ITS2 indicate that *B. ignitus* populations occurring in South Korea were not genetically differentiated from each other (Tables 5 and 6). Furthermore, the hierarchical relationship data based on both the COI and ITS2 indicate that all *B. ignitus* populations in South Korea were genetically interrelated (Fig. 7). Consistent with the population genetic perspective, the phylogenetic analyses (Figs. 3 and 5) and the median-joining networks (Figs. 4 and 6) among South Korean populations also indicated the importance of gene flow in the *B. ignitus*.

Although no precise report on the dispersal distance of *B. ignitus* is available it has been known that the within-generic species, *B. terrestris*, *B. lucorum*, and *B. lapidaries* are capable of dispersal for several thousand kilometers (Mikkola, 1978). Furthermore, it has been theorized that a small number of migrants may be sufficient to prevent drift for genetically homogeneous populations (Hartl and Clark, 1989). Considering these findings, the *B. ignitus* populations in South Korea seem to be a single panmictic population, regardless of geographic distance.

Summarized, we collected a total of 88 individuals of *B. ignitus* from nine South Korean localities and sequenced 658 bp of the mitochondrial COI gene and 2,034 ~ 2,052-bp long complete ITS2 rDNA. The sequence analysis of *B. ignitus* COI provided a moderate to low magnitude of sequence divergence, whereas that of ITS2 indicated higher variability, presenting a total of 84 sequence types. In particular, the lengths of ITS2 were the longest among known insects. Considering the F_{ST} and *Nm* estimates, along with phylogenetic analyses, it appears that the geographic populations of *B. ignitus* have a high ratio of gene flow, which indicates the potential/actual dispersal ability of the species without long-term zoogeographic barriers. This was likely due to high dispersal ability.

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