

Genetic variation and phylogenetic relationships of commercial populations of *Bombus ignitus* (Hymenoptera, Apidae) with wild populations in Eastern Asia

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Abstract

The bumblebee, *Bombus ignitus* (Hymenoptera, Apidae), plays a vital role in pollination in Northeast Asia, including Korea, China, Japan, and Far East Russia. Understanding the genetic makeup of the population can aid in its conservation. This study explores the DNA barcode region of cytochrome C oxidase subunit I (COI) of *B. ignitus* in commercial populations from Korea and Japan. The results reveal low intraspecific genetic diversity among commercially reared populations, with a maximum sequence divergence of 0.3%. Analysis of a 458-bp region of the COI gene, including 384 previously reported sequences, identified 20 haplotypes with the highest sequence divergence of 2.01% in East Asia. Commercial populations show a genetic similarity primarily with the Japanese population. Cross-mating with native populations could result in competition and genetic contamination, leading to reduced fitness and sensitivity to future environmental conditions. Morphological similarities make monitoring of such effects challenging. This study provides a basis for further research on population studies, conservation, and commercialization of local populations of *B. ignitus* for better pollination services while minimizing risks of reducing genetic diversity and increasing competition between native and introduced populations.

Keywords

Bombus ignitus, Bumblebee, COI, wild populations, commercial population

Introduction

Bumblebees, play a critical role in pollinating many agricultural crops (Grixti et al. 2009; Hou et al. 2009). *Bombus ignitus* is an important pollinator species throughout its distributional range, including Korea (Yoon et al. 1999), China (An et al. 2010; Zhang et al. 2018) and Japan (Asada and Ono 2000) where it is used commercially in pollination of greenhouse plants (Cooley and Vallejo-Marin 2021). It plays a significant role in the pollination of various crops such as tomatoes, strawberries, and blueberries (Toni et al. 2020). The decline of *B. ignitus* populations could have adverse effects on agricultural production and ecosystem health in Korea, highlighting the need for conservation measures. However, *B. ignitus* populations in Korea are threatened by various factors, including habitat loss, fragmentation, and pesticide exposure (Carvell et al. 2006; Wu et al., 2010). Furthermore, the introduction of non-native bumblebees such as *B. terrestris* for pollination purposes has further exacerbated the decline of the species through competition for food, spread of diseases, and hybridization with native populations (Dimou et al. 2008; Whittington et al. 2004; Ings et al. 2005; Kanbe et al. 2008; Tsuchida et al. 2019; Keum et al. 2020). These negative impacts have led some countries to ban the introduction of foreign bumblebee species and promote the use of local pollinators instead (Velthuis and Doorn 2006).

Maintaining genetic diversity in local populations is essential for survival and fitness in different environmental conditions, such as climate changes and food availability (Kawecki and Ebert 2004). Inbreeding can occur in populations with lower genetic diversity, leading to inbreeding depression and reduced fitness, including lower reproductive rates, slower growth rates, and less flexibility in adapting to different environmental conditions (Zayed and Packer 2005). Failure to adapt could ultimately lead to extinction. Therefore, knowledge of genetic diversity and geographic relationships of *B. ignitus* is crucial for long-term conservation, artificial selection, and the development of effective conservation strategies (Lee et al. 2006; Goulson et al. 2008; Zayed 2009; Whitehorn et al. 2009; Goka 2010; Habel et al. 2014).

Mitochondrial DNA is inherited maternally and characterized by a relatively fast mutation rate. It exhibits high genetic variation between related species and low intraspecific variation (Moritz et al. 1987). Among mitochondrial genes, Cytochrome oxidase subunit I (COI) has emerged as the most interesting and widely used gene due to its lower mutation rates and high incidence of nucleotide substitution at the third codon position compared to other protein-coding genes (Yi et al. 2002; McClellan 2000). Prior studies have demonstrated that Japanese populations of *B. ignitus* form a distinct clade that is genetically divergent from Korean and Chinese populations, using COI and microsatellites (Shao et al. 2004; Tokoro et al. 2010; Oh et al. 2013). Additionally, Han et al. (2018) used DNA barcoding part of COI to determine that the original stocks of commercially bred *B. ignitus* in Belgium were from Japan. This study aims to assess the genetic diversity of commercial populations of *B. ignitus* in Korea and Japan and explore the phylogenetic relationships between these populations and wild East Asian populations of *B. ignitus* using both newly collected samples and previously deposited sequences in GenBank. Understanding the genetic diversity and

relationships between populations, is helpful to develop strategies for conservation, artificial selection and improving the fitness of *B. ignitus* populations in the face of environmental changes.

Materials and methods

Sample collection

In this study 35 worker bees (one individual from each colony) were collected from three different commercial populations of *B. ignitus* in South Korea and Japan. All populations were acquired from Biobest (Belgium) between 2017 and 2018, as presented in Table 1. Voucher specimens were stored at -20 °C in 100% ethanol until DNA extraction. All analyzed samples are stored in ethanol and deposited in the insect collection of Andong National University, South Korea.

DNA extraction, primer, PCR, and sequencing

Total DNA was extracted from the hind leg of each of the 35 samples using the DNeasy Blood and Tissue kit (Qiagen, Germany). We used Polymerase Chain Reaction (PCR) to amplify a 658-bp region of the COI gene that corresponds to the “DNA Barcode” region (Herbert et al. 2003). The universal primer set LCO-1490 (5'-GGT-CAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGT-GACCAAAAAATCA-3') (Flomer et al. 1994) was used in the PCR reaction, and AccuPower PCR PreMix (Bioneer, Daejeon, Korea) was used as the PCR master mix.

Thermocycler conditions consisted of initial denaturation step for 5 minutes at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 52 °C for 30 seconds, and extension at 72 °C for 30 seconds, and a final extension step for 5 minutes at 72 °C. Sequencing was performed commercially by BIONICS (Seoul, South Korea). All sequences were generated in both directions by Sanger sequencing.

Sequence analysis and genetic diversity estimates

The consensus sequence was assembled from forward and reverse sequences using BIOEDIT v7.0.5.2 (Hall 1999) and all sequences were aligned in MEGA7 (Kumar et al. 2016) using ClustalW. COI sequences of this study have been archived on the GenBank under accession numbers [MN022949–MN022985](#) and [MN423343–MN423360](#) (Table 1, Suppl. material 1: appendix S1).

The within-locality diversity estimates in terms of haplotype diversity (H), mean number of pairwise differences (MPD), and nucleotide diversity (π) which reflect genetic diversity within each locality were analyzed for commercially reared populations of *B. ignitus* based on 658 bp of COI sequences using DNAsp v5 (Librado and Rozas 2009). Because commercial populations are isolated from each other and even from wild populations, we did not calculate the pairwise fixation indices and migration rates.

Table 1. Sampling information of the *B. ignitus* and GenBank accession numbers.

Locality	No. of samples	Accession No.
Commercial – Korea A* (BIKOA1–BIKOA15)	15	MN022949-58, MN022981-85
Commercial – Korea B* (BIKOB1–BIKOB10)	10	MN022959-68
Commercial – Japan** (BIJA01–BIJA10)	10	MN022969-76, MW080642-3

* Commercial stocks imported from a Belgian company and kept in Korea. ** Commercial stocks imported from a Belgian company and kept in Japan.

Network construction

In order to evaluate the phylogenetic relationship between commercial and wild populations of *B. ignitus*, all previously reported *B. ignitus* COI sequences in the nucleotide database of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) were also included in our analysis. Only sequences that overlapped part of the gene by 458 bp were selected for analysis. To construct the most reliable haplotype network, all frequencies of the sequences in Tokoro et al. (2010) were included (Suppl. material 1: appendix S2). The final dataset included 419 sequences, consisting of 35 from the present study and 384 from GenBank (Suppl. material 1: appendix S1).

Since *B. terrestris* is closely related to *B. ignitus* (Cameron et al., 2007), this species was used as an outgroup to root the tree. The evolutionary distances between haplotypes were calculated using Kimura's 2-parameter model (Kimura 1980) implemented in MEGA7 (Kumar et al. 2016). Bayesian analyses were conducted in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2011), using the HKY+G mutation model which were selected by jModeltest 2.1.10 (Darriba et al. 2012). The analyses were performed for 5×10^6 generations, with four chains each. Trees were sampled every 500 generations with 25% burn-in. A haplotype network was constructed using median-joining method (Bandelt et al. 1999) in Network software version 5.0.0.1 to infer the relationships among haplotypes and their geographical distribution.

Results

Sequence analysis and genetic diversity estimates

The Commercial-Japan population was found to have no genetic diversity, as all ten individuals possessed the same haplotype (H9). Although most individuals of Commercial-Korea A and Commercial-Korea B possessed H9 haplotype, two more haplotypes occurred. In terms of π and HD, all three localities displayed low estimates and the highest nucleotide diversity ($\pi = 0.00058$) and haplotype diversity (HD = 0.362) were found in Commercial-Korea A population and the estimates of Commercial-Korea B ($\pi = 0.00031$) was as low as nearly half of that obtained from Commercial-Korea A (Table 2).

Table 2. Within locality diversity estimates in commercially reared populations of *B. ignitus* based on 658-bp partial COI gene.

Locality	N	NH	HF	HD	NP	MPD	π
Japan	10	1	H9, 1.0	–	–	–	–
Korea A	15	3	H5, 0.067; H9, 0.8; H11, 0.13	0.362	2	0.3809	0.00058
Korea B	10	2	H9, 0.9; H5, 0.1	0.2	1	0.2	0.00031

– Zero estimates were obtained either by one haplotype or by in individual from corresponding locality; N: number of sampled individuals; NH: Number of haplotypes; HF: Haplotype frequency; HD: Haplotype diversity; NP: Number of polymorphic sites; MPD: Mean number of pairwise differences; π : Nucleotide diversity

Genetic variation based on 458-bp of COI gene

A total of 20 haplotypes was obtained from 419 studied sequences (Suppl. material 1: appendix S3). The most divergent haplotypes (H18 versus H2) differed by 9 substitutions (2.01%) across the 458-bp sequence region (Suppl. material 1: appendix S3). The highest within country genetic distances were found in China (8 substitutions) followed by 4 substitutions in Korean and Japanese and 2 within Belgian commercial haplotypes. Sequence alignments revealed 17 variable sites; of these, 13 substitutions (76.5%) were transitions (A \leftrightarrow G, C \leftrightarrow T), whereas four (23.5%) were transversions (three T \leftrightarrow A switch and one G \leftrightarrow C switch) (Suppl. material 1: appendix S2, S3).

Phylogenetic and Haplotype analysis

Upon analyzing a 458 bp segment of the COI gene, eight haplotypes from South Korea were identified. Five haplotypes were exclusive to Korean populations, while six were exclusive to Japanese populations. Haplotypes 1 and 4 were shared between Korean and Chinese wild populations, and haplotype 5 was shared between Korean populations and two Belgian commercially reared populations (Table 2, Suppl. material 1: appendix S2). Additionally, four haplotypes were exclusive to Chinese populations, and one haplotype was shared between Japanese and Belgian populations (Fig. 1).

The phylogenetic tree demonstrated that all *B. ignitus* sequences obtained from Korea are grouped together with haplotype 5 from Belgium (commercial) and four Chinese haplotypes forming a separate clade (posterior probability = 50). The other two Chinese haplotypes (H2 and H3) are clustered with haplotype 8 from Japan (posterior probability = 72). Haplotypes from China belong to two different clades. Only a few sub-clusters are well supported and the phylogenetic relationships of the rest of haplotypes remained unresolved (Fig. 2).

Discussion

The results of the study indicate that the Belgian commercial population of *B. ignitus* has low genetic diversity, which is likely the result of repeated inbreeding for

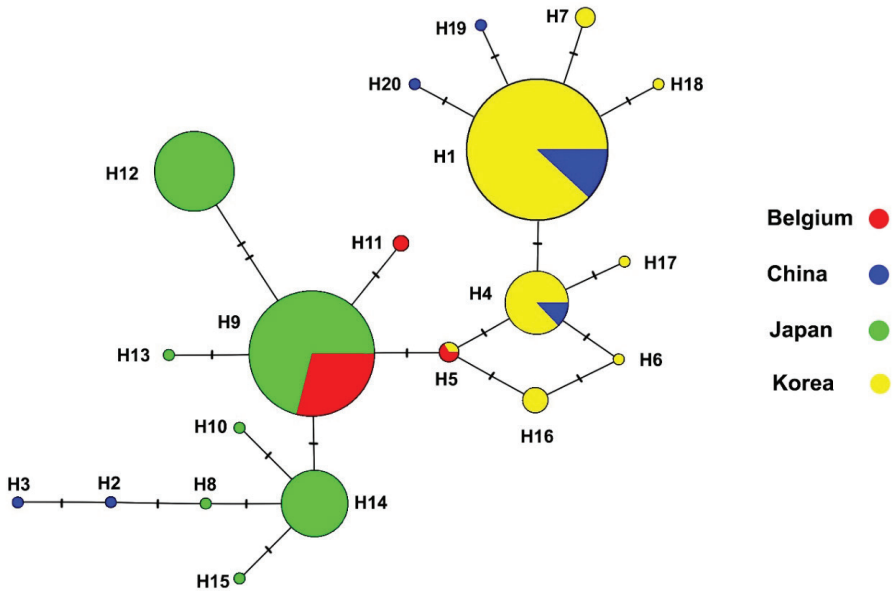


Figure 1. Median joining haplotype network of *B. ignitus* based on 458-bp partial COI gene. Each circle represents one haplotype and the size is proportional to its frequency among the studied sequences. Small black dashes represent mutational steps (Belgium = commercial population).

commercial purposes. (Hedrick and Kalinowski 2000; Cole 2003). The dominant haplotypes in Korea and China are H1 and H4, which differ in one nucleotide substitution, and other Korean haplotypes are derived from these two haplotypes (Fig. 1). These two haplotypes are also the most frequent haplotypes in China. The most widespread haplotypes with highest frequency are likely the oldest ones and appear in the center of haplotype network while recently derived haplotypes distributed in the restricted localities forming a star-shape phylogeny (Watterson and Guesse 1977). Thus Haplotype 1 is the most widespread haplotype in Korea and China and Haplotype 9 is the oldest and most widespread haplotype in Japan and Belgium commercially reared populations (Fig. 1) which suggests that the commercial populations have close relatedness to Japanese wild populations. Han et al. (2018) also revealed that the Japanese sequence from Honshu made one cluster with Belgian sequences.

We found that haplotype 5 is shared between Belgian (commercial) and Korean populations and it is phylogenetically closely related to Korean haplotypes. Among the three commercial populations studied here, the Commercial-Korea A population showed the highest haplotype and nucleotide diversity (Table 2). Since high genetic diversity is known to play a crucial role in enabling populations to adapt to new environmental conditions (Suaraz and Tsutsui 2008; Handley et al. 2011), this population appears to be particularly well-suited for utilization in variable environments for the purpose of pollinating agricultural products. However, it is important to consider the potential risks associated with widespread introduction of alien populations of *Bombus ignitus*

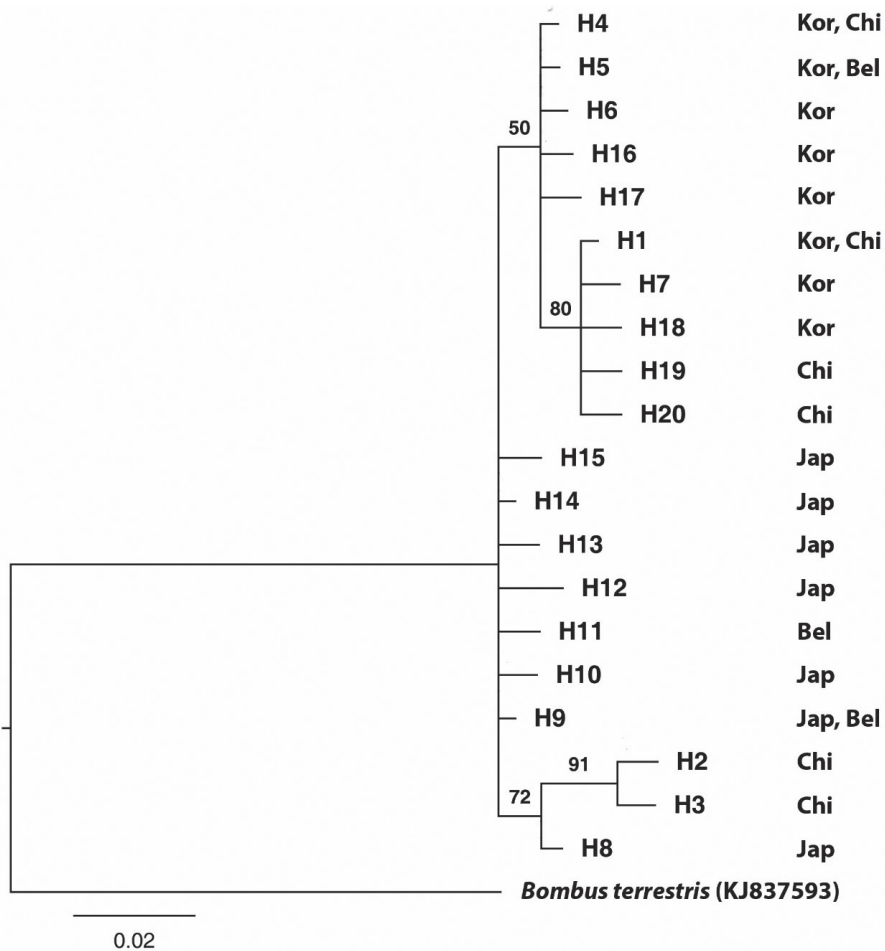


Figure 2. Bayesian inference of 458-bp partial COI gene of the haplotypes of *B. ignitus*. Numbers represent posterior probability. Bel: Belgium (commercial), Chi: China, Jap: Japan, Kor: Korea.

originated from Japan, due to the potential risk of their escape from greenhouses. These include the loss of genetic variation within native populations, decrease in adaptation rate, and disruption of population structure of local populations. Additionally, releasing alien populations can result in competition for food and the spread of diseases and parasites, which may reduce the size of local populations (Dimou et al. 2008). Hybridization between alien and native populations is also a major concern, as it can alter genetic diversity, viability, and productivity of the populations. Furthermore, this phenomenon can reduce individual fitness, negatively impacting the ability of populations to adapt to future environmental changes (Rhymer and Simberloff 1996; Laikre et al. 2010).

The importation of the European bumblebee, *B. terrestris*, to Japan as a pollinator for tomato production has resulted in negative impacts on native bumblebee populations. The species is currently widespread in Hokkaido and has interfered repro-

ductively with native bumblebees, leading to declines in populations of *B. hypocrita* (Inoue et al. 2008; Kondo et al. 2009). As a result of these risks, Williams et al. (2012) proposed preventing the movement of *B. patagiatus* from China into Japan and *B. hypocrita* from Japan into China for pollination services. At the population level, the ongoing release of introduced bees could increase the dominance of the alien population in relation to local bumblebee communities. Additionally, due to the morphological similarities of the female castes of *B. ignitus* and *B. ardens* (Mohamadzade Namin et al., 2021) and similarities of alien populations of *B. ignitus* with native ones, monitoring the adverse effects of the introduction on local populations can be challenging. Therefore, it is essential to carefully weigh the potential risks and benefits of introducing alien bumblebees for pollination services, and to implement measures to minimize negative impacts on native bumblebee populations. While steps are taken to minimize the impact of insecticide spraying on bumblebees, releasing alien populations can have significant negative ecological and genetic consequences. Thus, it is important to thoroughly evaluate the potential risks and benefits before introducing alien populations and to take measures to minimize negative impacts on native populations. This can include monitoring of the introduced populations and implementing strategies to prevent hybridization with native populations, as well as developing alternative solutions for pollination services that do not rely on the introduction of alien species.

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Supplementary material I

Information for COI sequences of *Bombus ignitus* from this study and NCBI-Genbank database

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Data type: phylogenetic (.docx file)

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