

Interspecific Hybridization of the Bumblebees *Bombus ignitus* and *B. terrestris*

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The large bumblebee, *Bombus terrestris*, indigenous to Europe and used extensively for high-value crop pollination, has been artificially introduced in several parts of the world. Here we show the interspecific hybridization between bumblebee species, *B. terrestris* and *B. ignitus*, under laboratory conditions. The mating and oviposition percentages of the interspecific hybridization of a *B. terrestris* queen with a *B. ignitus* male were higher than those of the intraspecific mating of *B. ignitus*. Furthermore, the competitive copulation experiment indicated that the mating of *B. ignitus* males with *B. terrestris* queens was 1.8-fold more frequent than with *B. ignitus* queens. The interspecific hybridization of a *B. ignitus* queen with a *B. terrestris* male produced either *B. ignitus* workers or the *B. ignitus* male phenotype, and the hybridization of a *B. terrestris* queen with a *B. ignitus* male produced *B. terrestris* males. Genetic tests using a portion of the mitochondrial COI gene for the parent and hybrid phenotypes indicated that mitochondrial DNA in the interspecific hybridization was maternally inherited. Our results indicated that interspecific hybridization occurred between *B. ignitus* and *B. terrestris*, which suggests that the hybridization will have a negative impact of competition and genetic pollution of native bumblebees.

Key words: *Bombus ignitus*, *B. terrestris*, Bumblebee, Interspecific hybridization, Pollinator, Mitochondrial DNA

Introduction

The introduction of bumblebees into greenhouses for pollination has become widespread in recent years and demand increases annually. Bumblebees provide farmers the opportunity to decrease their pollination labor costs and promise a good crop yield, both in quantity and in quality (reviewed by Velthuis and van Doorn, 2006). They are more adept than honeybees in cloudy weather and in small areas such as a greenhouse. Bumblebees also tend to devote themselves mainly to the crops within the greenhouse, whereas honeybees are apt to escape en masse to the outside. Bumblebees are particularly effective at pollinating Solanaceae, including the tomato and eggplant.

The large bumblebee, *Bombus terrestris*, which is indigenous to Europe, has been artificially introduced in several parts of the world. Since 1988, *B. terrestris* has been available commercially in portable boxes for crop pollination (Mitsuhashi, 2000). Colonies of *B. terrestris* have been imported into many countries, including Korea, Japan, China, Taiwan, Mexico, Chile, Argentina, Uruguay, South Africa, Morocco, and Tunisia (Dafni, 1998). There has been some anxiety associated with the introduction of *B. terrestris* into greenhouses because it is highly invasive (Hingston *et al.*, 2002), could possibly escape from greenhouses and could have negative effects through competition (Ono and Wada, 1996; Velthuis and van Doorn, 2006) or genetic contamination by hybridization (Ono, 1997; Velthuis and van Doorn, 2006) with native bumblebees. The competitive displacement of native pollinators and the invasion of native vegetation by *B. terrestris* have already been recorded in Tasmania (Semmens *et al.*, 1993; Hingston and McQuillan, 1998, 1999; Hingston *et al.*, 2002). In Israel, there has been a decline in the numbers of honeybees and solitary bees with the range expansion of *B. terrestris* (Dafni and Shimida, 1996). *B. terrestris* has also colonized Japan, where it

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escaped from greenhouses in 1996 after its introduction in 1991 (Iwasaki, 1995; Goka, 1998; Washitani, 1998). For this reason, the governments of Canada and the USA prohibit the introduction of foreign bumblebee species, and at present a native bumblebee *B. impatiens* is used for commercial pollination in North America (Velthuis and van Doorn, 2006). In Korea, *B. terrestris* was first introduced in 1994 and in early May 2002 to 2004, *B. terrestris* overwintering queens were caught in several regions (Yoon H. J. personal communication). We are studying the artificial year-round mass rearing of *B. ignitus*, a Korean native bumblebee, because this species is the most reliable native bumblebee for crop pollination (Yoon *et al.*, 1999, 2002, 2003, 2004).

In the present study, we investigated the interspecific hybridization between the two bumblebee species, *B. ignitus* and *B. terrestris*, under laboratory conditions. The objectives of this study were to answer the following questions: (1) Do the two species hybridize? (2) If they do hybridize, does the bumblebee produce viable offspring capable of reproduction? We obtained offspring from an interspecific hybridization of *B. ignitus* and *B. terrestris* and performed a genetic test using a portion of the mitochondrial COI gene for the parent and hybrid phenotype.

Materials and Methods

Experimental animals

Experimental insects were 2nd-6th generation queens obtained from *Bombus ignitus* and *B. terrestris* colonies which were year-round reared in a controlled climate room (28°C, 65% relative humidity, and continuous darkness) at the Department of Agricultural Biology, National Academy of Agricultural Science, Republic of Korea.

Indoor rearing

We followed a basic colony-rearing technique described previously (Yoon *et al.*, 2002). The queens were reared in three types of cardboard (1.5 mm thick) boxes for nest initiation (10.5×14.5×6.5 cm: small box), colony foundation (21.0×21.0×15.0 cm: medium box), and colony maturation (24.0×27.0×18.0 cm: large box). Each box had a wire net window on its lid for ventilation. The sizes of these windows were 5.5×6.5 cm, 7.0×14.0 cm and 10.0×20.0 cm, respectively. Queens were first confined individually in small boxes for colony initiation and remained there until oviposition. When the adults emerged from the first brood, the nest was transferred to a medium box for colony foundation and was left there until the number of workers reached 50. The nest was then moved to the large box for further colony development. Sugar solution and

pollen dough were provided *ad libitum*. The pollen dough was made from sugar solution and fresh pollen collected from an apiary (v:v = 1:1).

Hybridization and colony development of *Bombus* spp.

To verify the interspecific hybridization of *Bombus* spp., experimental regimes of *B. ignitus* queen×*B. terrestris* male or *B. terrestris* queen×*B. ignitus* male were provided. Bumblebees used for these experiments were taken from reared colonies. Newly emerged virgin queens were collected directly from the nest and maintained separately in cages until the copulation experiments took place. This isolated the queen from the males' odour and from any potential nest copulations. The unmated males were taken from the nest before the experiment and introduced into the cage. Like the queen, they were isolated from the odour of their future partner (the queen). For the mating experiments, thirty 6-7 day-old virgin queens and thirty 10-11 day-old males were introduced into a wooden mating arena (55×65×40 cm), which was covered with wire mesh. The mating experiment started at 09:00 and ended at 17:00, and environmental conditions of the mating room were as follows: intensity of illumination, over 2,000 Lux; UV, 11-13 $\mu\text{W}/\text{cm}^2$; temperature, 23-24°C; humidity: 60-65%. If copulation occurred, the mated queen and the male were moved in a small cardboard box and were monitored during the duration of mating. To investigate the competitive copulation between a *B. ignitus* queen and a *B. terrestris* queen, 30 *B. ignitus* queens and 30 *B. terrestris* queens were simultaneously put in one cage with 60 *B. ignitus* males. Two days after mating, in both experiments, queens were narcosised with 99% CO₂ for 30 min for two consecutive days in a flask to stimulate diapause (Yoon *et al.*, 2003) and reared to investigate egg-laying characteristics, colony development, and emergence of the next generation. Mating duration was recorded as the time (min) when the queen first engaged in a copula with a male until the time the copula ended. The actual copulation can be easily observed because the male, while freely hanging behind the queen, grasps and firmly holds the female outer genitalia. The developmental ability of each colony was estimated by the percentage of oviposition, colony foundation, and progeny-queen foundation. Colony foundation indicates when more than 50 workers emerged in a colony. Preoviposition period was measured as days until the first oviposition. Queens that did not oviposit within 60 days were excluded from counts of the number of oviposited colonies.

Mitochondrial DNA isolation, PCR, and sequencing

A single bumblebee sample was frozen in liquid nitrogen and homogenized in 20 volumes of chilled sucrose-TE

buffer (0.03 M Tris, 0.25 M sucrose, 0.01 M EDTA, pH 7.6) to break down the cells. The homogenate was transferred to a chilled 1.5 ml disposable centrifuge tube and centrifuged at 1,000× g for 2 min at 4°C to pellet nuclear and cellular debris. The resulting supernatant went through another round of centrifugation, at 12,000× g for 10 min at 4°C, to pellet the mitochondria. Mitochondrial DNA was isolated from the pellets using the Wizard™ Genomic DNA Purification Kit according to manufacturer's instructions (Promega, USA).

For the amplification of a portion of the mitochondrial COI gene, a pair of primers was designed based on Cha *et al.* (2007) and Simon *et al.* (1994): CI-J-1751, 5'-GGATCACCTGATATAGCATTCCC-3' and CI-N-2191, 5'-CCCGGTAAAATTTAAATATAAACTTC-3'. After an initial denaturation step at 94°C for 5 min, a 40-cycle amplification (94°C for 30 s, 50°C for 40 s, and 72°C for 45 s) was conducted. The PCR product was purified using a PCR Purification Kit (QIAGEN, Germany). The COI gene fragments were directly sequenced from PCR products using the ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit with an ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems, USA). All fragments were sequenced from both strands. Sequence alignment was performed using CLUSTAL X (ver. 1.8; Thompson *et al.*, 1997).

Statistical analysis

Statistical analysis was done using Chi-square tests and One-way ANOVA (MINITAB Release 13 for Windows, 2000). The Chi-square test was used to compare the percentage of mating, oviposition, and death within one month in hybridization of *Bombus* spp. Tukey's pairwise comparison test (One-way ANOVA) was used to examine the mating durations and preoviposition period in hybridization of *Bombus* spp.

Results

Interspecific hybridization of *B. ignitus* and *B. terrestris*

We investigated whether interspecific hybridization can occur between the Korean native bumblebee *B. ignitus*

and the imported European bumblebee *B. terrestris* (Table 1). Compared to the mating percentage of *B. terrestris* (93.3%), interspecific hybridization of *B. terrestris* queens with *B. ignitus* males led to a decrease in mating percentage (56.7%). This indicates that the interspecific hybridization percentage is lower than the intraspecific mating percentage. A similar effect was seen in the interspecific hybridization of *B. ignitus* queens with *B. terrestris* males (26.7%). The intraspecific mating percentage of *B. terrestris* is 2.5-fold higher than that of *B. ignitus* (36.7%). Interestingly, the mating percentage of interspecific hybridization of *B. terrestris* queens with *B. ignitus* males is 20% higher than the intraspecific mating of *B. ignitus*. There was a statistically significant difference in the mating percentages of intraspecific and interspecific crosses of *B. ignitus* and *B. terrestris* (Chi-square test: $\chi^2 = 31.34$, d.f. = 3, $P = 0.0001$).

The mating duration of intraspecific and interspecific crosses of *B. ignitus* and *B. terrestris* is about 22-25 min (Table 1). The mating duration of intraspecific mating of *B. ignitus* tended to be a little shorter than that of intraspecific and interspecific mating of *B. terrestris*, although there was no statistical difference in mating duration of the *Bombus* spp. (One-way ANOVA test, $F = 0.631$, d.f. = 5, $P = 0.418$).

We wanted to confirm whether the frequency of interspecific hybridization of *B. terrestris* queens with *B. ignitus* males is higher compared to intraspecific matings of *B. ignitus* queens. To test this, *B. ignitus* queens and *B. terrestris* queens were simultaneously put in one cage with *B. ignitus* males. We found that the mating percentage of interspecific hybridization of *B. terrestris* queens with *B. ignitus* males was 36.7%, which is 1.8-fold higher than that of the intraspecific matings of *B. ignitus* queens (Fig. 1). This result indicates a strong preference for *B. ignitus* males and *B. terrestris* queens to mate, though this preference was not statistically significant (Chi-square test: $\chi^2 = 2.052$, d.f. = 1, $P = 0.0001$).

Queens of mated *Bombus* spp., after being treated with carbon dioxide to stimulate diapause (Yoon *et al.*, 2003), were reared to ascertain whether they oviposit. We surveyed oviposition percentages and the preoviposition period of intraspecific and interspecific mated *Bombus*

Table 1. Mating percentage and mating duration of *Bombus* spp.

Surveyed items	n	<i>B. ignitus</i> queen × <i>B. ignitus</i> male		<i>B. ignitus</i> queen × <i>B. terrestris</i> male		<i>B. terrestris</i> queen × <i>B. terrestris</i> male		<i>B. terrestris</i> queen × <i>B. ignitus</i> male	
		n		n		n		n	
Mating (%)	30	36.7	30	26.7	30	93.3	30	56.7	
Mating duration (min)	11	21.8 ± 10.3	8	24.9 ± 7.5	28	25.3 ± 9.9	17	25.4 ± 7.8	

The statistical analysis: mating percentage; Chi-square test: $\chi^2 = 31.339$, d.f. = 3, $P = 0.0001$, mating duration; Tukey's pairwise comparison test, $F = 0.631$, d.f. = 5, $P = 0.418$.

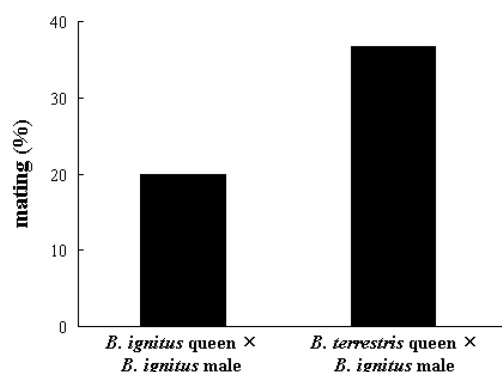


Fig. 1. Competitive copulation of the *B. ignitus* queen and *B. terrestris* queen. Thirty *B. ignitus* queens and 30 *B. terrestris* queens were put in one cage with 60 *B. ignitus* males. The statistical analysis: mating percentage; Chi-square test: $\chi^2 = 2.052$, d.f. = 1, $P = 0.152$.

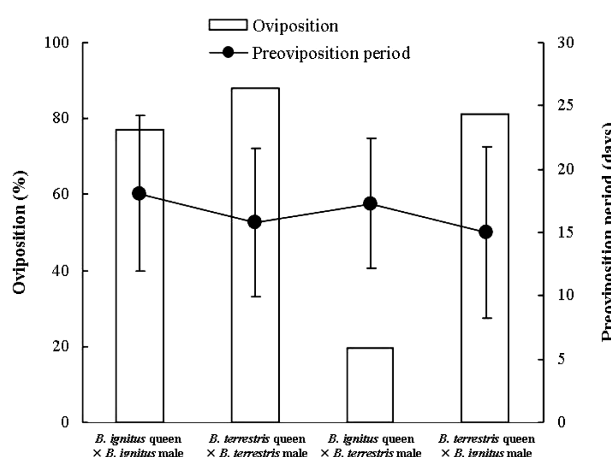


Fig. 2. Oviposition percentage and preoviposition period of hybridized queens. Queens were reared after 2-day CO₂ treatment to simulate diapause. For the statistical analysis, a Chi-square test was used: $\chi^2 = 43.181$, d.f. = 3, $P = 0.0001$ for the percentage of oviposition.

spp. (Fig. 2). The oviposition percentage is highest in *B. terrestris* queen × *B. terrestris* male (88.0%), followed by

B. terrestris queen × *B. ignitus* male (81.3%), *B. ignitus* queen × *B. ignitus* male (76.9%), and *B. ignitus* queen × *B. terrestris* male (19.4%). Notably, the oviposition percentage of interspecific hybridized *B. terrestris* queens with *B. ignitus* males is slightly higher than that of intraspecific-mated *B. ignitus* queens and is also 4-fold higher than that of mated *B. ignitus* queens with *B. terrestris* males (One-way ANOVA: $F = 24.135$, d.f. = 1, $P = 0.0001$). There was a statistically significant difference in the oviposition percentage for the two *Bombus* spp. (Chi-square: $\chi^2 = 39.844$, d.f. = 3, $P = 0.0001$). However, there was no significant difference in preoviposition period for the *Bombus* spp., which was 15–18 days.

Colony development in interspecific hybridized *Bombus* spp.

We investigated the percentage of death within 1 month post-rearing, the percentage of adult emergence, and the percentage of colony foundation for colony development (Table 2). The percentage of death within 1 month after rearing was 4.0% and 6.3% of intraspecific mated *B. terrestris* queens and interspecific hybridized *B. terrestris* queens, respectively. Compared to intraspecific matings of *B. ignitus*, 11.5% of which died, 74.2% of interspecific hybridized *B. ignitus* queens with *B. terrestris* males died within 1 month after rearing. The death percentage in intraspecific matings of *B. ignitus* was 2.9-fold higher than that of intraspecific matings of *B. terrestris* (Chi-square: $\chi^2 = 1.002$, d.f. = 1, $P = 0.317$). Furthermore, the percentage of death of interspecific hybridized *B. ignitus* queens with *B. terrestris* males was 11.8-fold higher than that of hybridized *B. terrestris* queens with *B. ignitus* males (Chi-square: $\chi^2 = 30.369$, d.f. = 1, $P = 0.0001$). The percentage of death within 1 month after rearing was significantly affected by interspecific hybridization in the maternal line of *B. ignitus* (Chi-square: $\chi^2 = 53.769$, d.f. = 3, $P = 0.0001$).

In adult emergence, hybridized *B. ignitus* queens with *B. terrestris* males produced *B. ignitus* worker and *B. ignitus* males, although the percentage of worker emergence was low (3.2%) (Table 2). In contrast, hybridized *B. ter-*

Table 2. Colony development of hybridized *Bombus* spp.

<i>Bombus</i> spp.	n	Death within 1 month after rearing (%)	Worker emergence (%)	Male emergence (%)	Progeny-queen production (%)	Colony foundation (%)
<i>B. ignitus</i> queen × <i>B. ignitus</i> male	26	11.5	46.2	57.7	11.5	15.4
<i>B. terrestris</i> queen × <i>B. terrestris</i> male	25	4.0	72.0	76.0	32.0	40.0
<i>B. ignitus</i> queen × <i>B. terrestris</i> male	31	74.2	3.2	12.9	0.0	0.0
<i>B. terrestris</i> queen × <i>B. ignitus</i> male	32	6.3	0.0	31.3	0.0	0.0

For the statistical analysis, Chi-square test was used: $\chi^2 = 53.769$, d.f. = 3, $P = 0.0001$ for the percentage of death within 1 month.

restris queens with *B. ignitus* males only produced males (31.2%) (Table 2). Hybrid phenotypes were easily differentiated by morphological characteristics as either coming from *B. terrestris* or *B. ignitus* (data not shown). Based on the morphological characteristics, hybrid phenotype was

dependent on the maternal line. Our results show that interspecific hybridized queens of *B. ignitus* or *B. terrestris* produced hybrids, but did not form a colony. We also determined that a *B. terrestris* male produced by interspecific hybridization can copulate with a *B. terrestris* vir-

A

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P-Bi-Q      TTGGATCATTAATAATTTTATTGTAACATTTTTAATAATAAAAAATTTTCATTAAATTATG
P-Bt-M      -----C-T-----T-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      ATCAAATTAATTTATTTTCATGATCAGTATGTATTACAGTTATTTTACTAATTTTATCAT
P-Bt-M      -----A-----T-----C-----T-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      TACCAGTTTTAGCCGGAGCTATACTATATTATTATTGATCGAAATTTAATACTTCAT
P-Bt-M      -----A-----T--A-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      TCTTTGATCCTATACGAGGAGGGATCCAATTTTATCAACATTTATTTTGATTTTTTTG
P-Bt-M      -T-----C--A--G-----A--C-----C-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      GACATCCAGAAGTTTATATTTTAATTTTACCAGGATTTGGATTAATTTCTCAAATTATTA
P-Bt-M      -C--C-----A-----C-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      TAAATGAAAGAGGTAAAAAGAACTTTTGAAATTTAAGAATAATTTATGCTATATTAG
P-Bt-M      -----C-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      GTATTGGATTTTTAGGATTTATGTTTGAGCACATCACATATTCACGTGGATTAGATG
P-Bt-M      -A-----T-----T-----T-----T-----A-----C-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      TTGACACACGAGCATATTTTACATCAGCTACAATAATTATTGCCGTACCTACAGGAATTA
P-Bt-M      ---T-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      AAGTTTTTAGATGATTAGCTACATATCATGGTTCAAAAATAAATTTAATATTACAATTA
P-Bt-M      -----A-----C-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      TTTGATCAATTGGATTTATTTAATATTTACAATTGGAGGATTAACGGTGAATACTTT
P-Bt-M      -C-----C-----
H-Bi-W      -----
H-Bi-M      -----

P-Bi-Q      CTAATTCATCAATTGATATTATTTTACATGATACCTATTATGTAGTTGGTCATTTTCATT
P-Bt-M      -----C-----A--C-----A-----C-----
H-Bi-W      -----
H-Bi-M      -----

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Fig. 3. Alignment of partial sequences (660 bp) of the mitochondrial COI gene of the parent and hybrid. Dots represent identical nucleotide sequences. (A) COI gene sequences of *B. ignitus* worker (H-Bi-W) and *B. ignitus* male (H-Bi-M) phenotype produced from a hybridized *B. ignitus* queen (P-Bi-Q) with a *B. terrestris* male (P-Bt-M). (B) COI gene sequences of a *B. terrestris* male (H-Bt-M) phenotype produced from a hybridized *B. terrestris* queen (P-Bt-Q) with a *B. ignitus* male (P-Bi-M).

B

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P-Bt-Q      TTGGATCATTAAATTTTATCGTTACTATTTTATTAATAAAAAATTTTCATTAAATTATG
P-Bi-M      -----T--A-----A-----
H-Bt-M      -----

P-Bt-Q      ATCAAATTAATTTATTTTCATGATCAGTATGTATTACAGTAATTTTATTAATTCATCTT
P-Bi-M      -----T-----C-----T-----A-
H-Bt-M      -----

P-Bt-Q      TACCAGTATTAGCCGGTGCAATTACTATATTATTATTTGATCGAAATTTAATACTTCAT
P-Bi-M      -----T-----A--T-----
H-Bt-M      -----

P-Bt-Q      TTTTGGACCAATAGGAGGAGAGACCAATCCTTTATCAACATTTATTTTGATTTTTTG
P-Bi-M      -C-----T--T---C-----G--T---T-----
H-Bt-M      -----

P-Bt-Q      GCCACCCAGAAGTATATATTTTAATTTTACCAGGATTGGATTAATCTCTCAAATTATTA
P-Bi-M      -ACAT-----T-----T-----
H-Bt-M      -----

P-Bt-Q      TAAATGAAAGAGGTAAAAAGAAACCTTGGAAATTTAAGAATAATTTATGCTATATTAG
P-Bi-M      -----T-----
H-Bt-M      -----

P-Bt-Q      GAATTGGATTTTTAGGTTTTATTGTTTGAGCTCATCATATATTTACTGTAGGATTAGACG
P-Bi-M      -T-----A-----A-----C-----C-----T-----T-
H-Bt-M      -----

P-Bt-Q      TTGATACACGAGCATATTTTACATCAGCTACAATAATTATTGCCGTACCTACAGGAATTA
P-Bi-M      ---C-----
H-Bt-M      -----

P-Bt-Q      AAGTTTTTAGATGATTAGCAACATATCATGGTTCAAAAATAAATTTCAATATTACAATTA
P-Bi-M      -----T-----T-----
H-Bt-M      -----

P-Bt-Q      TCTGATCAATTGGATTCATTTTAATATTTACAATTGGAGGATTAACTGGTGAATACTTT
P-Bi-M      -T-----T-----
H-Bt-M      -----

P-Bt-Q      CTAATTCATCAATCGATATTATTTTACATGATACATACTATGTAGTAGGTCATTTTCACT
P-Bi-M      -----T-----C--T-----T-----T-
H-Bt-M      -----

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Fig. 3. Continued.

gin queen, which then oviposits, but does not initiate a nest (data not shown).

Finally, we tested whether the mitochondrial DNA in the interspecific hybridization of bumblebees was maternally inherited. Fig. 3 shows partial sequences (660 bp) of the mitochondrial COI gene of the parents and hybrids from interspecific crosses of *B. ignitus* and *B. terrestris*. In the cross with a *B. ignitus* queen hybridized to a *B. terrestris* male, the COI gene sequences of *B. ignitus* worker and *B. ignitus* male phenotypes were identical to those of *B. ignitus* queen (Fig. 3A). Likewise, the COI gene sequences of *B. terrestris* male phenotype produced from a *B. terrestris* queen hybridized with *B. ignitus* male were identical to those of *B. terrestris* queen (Fig. 3B). These results show that the mitochondrial DNA was maternally inherited.

Discussion

B. terrestris, a bumblebee valued for its crop pollination abilities, has been artificially introduced in several parts of the world. Introduction of the bumblebee to greenhouses in many countries has generated some anxiety, as foreign bumblebees that have escaped from a greenhouse may have some negative effects on the native bumblebee populations (Ono and Wada, 1996) or cause genetic contamination by hybridization with native bumblebees (Ono, 1997). We wanted to investigate possible competition or genetic contamination of the native bumblebee, *B. ignitus*, by interspecific hybridization with the imported *B. terrestris*. Our study focused on the interspecific hybridization between bumblebee species under laboratory conditions.

We showed that mating, survival and oviposition per-

centages of interspecific hybridized *B. terrestris* queens with *B. ignitus* males are higher compared to the intraspecific mating of *B. ignitus*. As we have shown in the competitive copulation experiment, hybridization of *B. ignitus* males with *B. terrestris* queens is much more frequent (1.8-fold) than with *B. ignitus* queens. These observations indicate that *B. ignitus* males mate with *B. terrestris* queens, as previously reported (Ono, 1997), and suggests that the mating behavior of *B. terrestris* queens may potentially interfere with intraspecific *B. ignitus* matings. Furthermore, our results indicate that *B. terrestris* queens have more successful matings with *B. ignitus* males than *B. ignitus* queens have with their own males. Although this effect may be due to the establishment of *B. terrestris* in the laboratory compared to *B. ignitus* (Velthuis and van Doorn, 2006), our finding suggests that *B. terrestris* queens will have a competitive impact on the mating of *B. ignitus* queens. Consequently, this could have a negative effect, through competition, on intraspecific matings of *B. ignitus*.

On the other hand, mating duration has been recorded in a number of bumblebee species (Duvoisin *et al.*, 1999; Brown *et al.*, 2002). Our observations show that mating durations are approximately 22 min in *B. ignitus* and about 25 min in *B. terrestris*. The hybridization of *B. ignitus* with a *B. terrestris* queen or male has a longer mean mating duration (about 25 min) than the intraspecific mating of *B. ignitus* (about 22 min). This indicates that, in a hybridization of *B. ignitus* and *B. terrestris*, the mating duration shows a tendency toward the duration associated with *B. terrestris*. In addition, the intraspecific mating rate of *B. terrestris* was 2.5-fold higher than that of *B. ignitus*. Considering the mating ability of *B. terrestris*, it appears more likely that *B. terrestris* will be a significant competitor of *B. ignitus* and could be superior to *B. ignitus* in competition for nest sites and food resources (Hingston and McQuillan, 1999; Hingston *et al.*, 2002; Matsumura *et al.*, 2004; Velthuis and van Doorn, 2006).

Interspecific hybridization of *Bombus* spp. has already been reported. A previous study has shown that *B. terrestris* mated with *B. ignitus*, *B. hypocrita hypocrita* and *B. hypocrita sapporoensis* with no hybrid female obtained (Ono, 1997). In contrast, Mitsuhashi and Ono (1996) reported that a *B. hypocrita sapporoensis* queen hybridized with a *B. terrestris* male produced hybrid workers and new queens, and the hybrid queen copulated with a male *B. terrestris* but did not initiate a nest. Recently, a study has shown that interspecific mating of *B. terrestris* and *B. hypocrita sapporoensis* results in inviable hybrids (Kanbe *et al.*, 2008). These previous studies were similar to our observation that interspecific hybridized queens of *B. ignitus* or *B. terrestris* produced hybrids but did not cre-

ate a colony, and when a hybrid male *B. terrestris* copulated with a virgin queen of *B. terrestris*, the queen oviposited but did not initiate a nest. However, although we did not show the hybrid worker's oviposition, a previous report observed that the hybrid workers oviposited unfertilized eggs, which developed into adult second-generation males (Mitsuhashi and Ono, 1996). Taken together, our results show that hybridized *B. ignitus* queens with *B. terrestris* males produce *B. ignitus* workers and *B. ignitus* males; however, hybridized *B. terrestris* queens with *B. ignitus* males only produce *B. terrestris* males, although there is no evidence yet to explain this. Based on the morphological characteristics, hybrid phenotypes were dependent on the maternal line. Furthermore, genetic tests using the mitochondrial COI gene showed that the mitochondrial DNA in the interspecific hybridization of bumblebees is maternally inherited, as shown previously in animals (Birky, 2001).

In conclusion, we have shown the interspecific hybridization of *B. ignitus* and *B. terrestris*. Our study indicates the competitive impact on intraspecific matings of naturally-occurring *B. ignitus* by demonstrating that *B. ignitus* males mate favorably with *B. terrestris* queens. In addition to the interspecies hybridization of *Bombus* spp., we suggest that such interspecies hybridization could cause genetic contamination of the native bumblebees. Further study of interspecies hybridization and its development will ultimately provide important information on the ecological impact to native bumblebees.

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