

Interspecific Hybridization of the Korean Native Bumblebee *Bombus hypocrita sapporoensis* and the European Bumblebee *B. terrestris*

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The large bumblebee *Bombus terrestris*, indigenous to Europe and adjacent areas and used extensively for high-value crop pollination, has been artificially introduced to several parts of the world. Here we show the occurrence of interspecific hybridization between the bumblebee species *B. hypocrita sapporoensis* and *B. terrestris* under laboratory conditions. The mating and oviposition percentages resulting from the interspecific hybridization of a *B. terrestris* queen with a *B. h. sapporoensis* male were higher than those resulting from the intraspecific mating of *B. h. sapporoensis*. Furthermore, a competitive copulation experiment indicated that the mating of *B. h. sapporoensis* males with *B. terrestris* queens was 1.2-fold more frequent than the mating of these males with *B. h. sapporoensis* queens. The interspecific hybridization of a *B. terrestris* queen with a *B. h. sapporoensis* male produced either *B. terrestris* workers or the *B. terrestris* male phenotype, and the hybridization of a *B. h. sapporoensis* queen with a *B. terrestris* male produced *B. h. sapporoensis* males. Our results indicated that interspecific hybridization occurred between *B. h. sapporoensis* and *B. terrestris*. These results suggest that such hybridization will have a negative competitive impact and will cause genetic contamination of native bumblebees.

Key words: *Bombus hypocrita sapporoensis*, *B. terrestris*, Bumblebee, Interspecific hybridization, Pollinator

Introduction

Pollination is an ecosystem service because wild pollinators, in particular wild bees, contribute significantly to the pollination of a large array of crops (Morandin and Winston, 2005; Greenleaf and Kremen, 2006; Winfree *et al.*, 2007). Commercially managed bees are also available for pollination services and are used in large commercial fields, small gardens, or enclosures such as greenhouses and screen houses (Free, 1993; Dag and Kammer, 2001). Bumblebees are important pollinators for wild flora and agricultural production. The introduction of bumblebees into greenhouses for pollination has become widespread in recent years, and demand increases annually. Bumblebees give farmers the opportunity to decrease their pollination labor costs and offer a good crop yield, both in quantity and in quality (Velthuis and van Doorn, 2006). Bumblebees are more effective pollinators than honeybees in cloudy weather and in small areas, such as greenhouses. Bumblebees also tend to devote themselves primarily 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* is naturally distributed in Europe and adjacent territories, including England, most of Scotland, the north coast of Africa, southern Scandinavia, major Mediterranean islands, and some Atlantic islands (Madeira and the Canary Islands) (Estoup *et al.*, 1996; Chittka *et al.*, 2004; Velthuis and van Doorn, 2006). Since 1987, *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.*

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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; Inoue *et al.*, 2008; Inoue and Yokoyama, 2010), genetic contamination by hybridization (Rhymer and Simberloff, 1996; Ono, 1997; Kanbe *et al.*, 2008) with native bumblebees, or through naturalization (Donovan and Wier 1978; Dafni and Shimida, 1996; Ruz and Herrera 2001, Hingston *et al.*, 2002). The competitive displacement of native pollinators and the invasion of native vegetation by *B. terrestris* have already been recorded in Tasmania, Australia (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 associated with the range expansion of *B. terrestris* (Dafni and Shimida, 1996). *B. terrestris* has also colonized Japan, where it escaped from greenhouses in Monbetsu in the Hidaka region of Hokkaido in 1996 after its introduction in 1991 (Iwasaki, 1995; Goka, 1998; Washitani, 1998; Inoue and Yokoyama, 2010). 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). *B. terrestris* was designated an invasive alien species under the "Invasive Alien Species Act" in 2006 in Japan. According to this law, farmers wishing to use *B. terrestris* for pollination must be issued a permit by the responsible minister (Yoneda *et al.*, 2008). In Korea, *B. terrestris* was first introduced in 1994. From early May 2002 through 2010, overwintering queens of *B. terrestris* were caught in several regions (Yoon *et al.*, 2009, 2010). We are studying the artificial year-round mass rearing of *B. ignitus* and *B. hypocrita sapporoensis*, Korean native bumblebees, because those species are the most reliable native bumblebees for crop pollination (Yoon *et al.*, 1999, 2002, 2004, 2010).

In the present study, we investigated the possible competition or genetic contamination of the native bumblebee, *B. h. sapporoensis*, resulting from hybridization with the imported *B. terrestris*. Our study focused on hybridization between these bumblebee species under laboratory conditions.

Materials and Methods

Experimental animals

The experimental animals were 2nd–10th generation queens obtained from *Bombus hypocrita sapporoensis* and *B. terrestris* colonies reared year-round in a con-

trolled- 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. The 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. h. sapporoensis* queen×*B. terrestris* male or *B. terrestris* queen×*B. h. sapporoensis* male were used. The 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 procedure isolated the queen from the males' odor 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 odor of their future partner (the queen). For the mating experiments, thirty 6- to 7-day-old virgin queens and ninety 10- to 11-day-old males were introduced into a wooden mating arena (55×65×40 cm) covered with wire mesh. The mating experiment started at 09:00 and ended at 17:00. The environmental conditions used in 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; and humidity, 60-65%. If copulation occurred, the mated queen and the male were moved to a small cardboard box and were monitored during the duration of mating. To investigate competition for copulations when *B. h. sapporoensis* queens and *B. terrestris* queens were placed with *B. h. sapporoensis* males and *B. terrestris* males, 10 *B. h. sapporoensis* queens and 10 *B. ter-*

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

Surveyed items	n	<i>B.h. sapporoensis</i>		<i>B.h. sapporoensis</i>		<i>B. terrestris</i> queen × <i>B. terrestris</i> male		<i>B. terrestris</i> queen × <i>B.h. sapporoensis</i> male	
		queen × <i>B.h. sapporoensis</i> male	n	queen × <i>B. terrestris</i> male	n	n	n	n	
Mating (%)	30	36.7	30	13.3	30	86.7	30	43.3	
Mating duration (min)	52	29.5 ± 9.6 abc	6	35.2 ± 7.7ab	45	34.5 ± 10.8a	27	24.8 ± 6.0bc	

Statistical analysis: mating percentage, Chi-square test: $\chi^2 = 34.074$, $df = 3$, $P = 0.0001$; mating duration, Tukey's pairwise comparison test, $F = 6.68$, $df = 3$, 126 , $P = 0.0001$.

restris queens were simultaneously put in one cage with 40 *B. h. sapporoensis* males and 40 *B. terrestris* males. Competition for copulations between *B. h. sapporoensis* males and *B. terrestris* males was also investigated. Sixty males of each species were placed simultaneously in one cage with 60 *B. h. sapporoensis* queens and 60 *B. terrestris* queens. In both experiments, the queens were narcotized two days after mating 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 was indicated by the emergence of more than 50 workers from a colony. The preoviposition period was measured as the number of days until the first oviposition. Queens that did not oviposit within 60 days were excluded from the counts of the number of oviposited colonies.

Statistical analysis

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

Results and Discussion

Hybridization of *B. h. sapporoensis* and *B. terrestris*

We investigated possible hybridization between the

Korean native bumblebee *B. h. sapporoensis* and the imported European bumblebee *B. terrestris* (Table 1). The mating percentage for *B. terrestris* (86.7%) was greater than the mating percentage of *B. terrestris* queens placed with *B. h. sapporoensis* males (43.3%). The interspecific mating percentage was lower than the intraspecific mating percentage. A similar effect was seen in the interspecific mating of *B. h. sapporoensis* queens with *B. terrestris* males (13.3%). The intraspecific mating percentage of *B. terrestris* was 2.4-fold higher than that of *B. h. sapporoensis* (36.7%). Interestingly, the mating percentage for *B. terrestris* queens with *B. h. sapporoensis* males was 6.6% higher than that for the intraspecific mating of *B. ignitus*. There was a statistically significant difference in the mating percentages of intraspecific and interspecific crosses of *B. h. sapporoensis* and *B. terrestris* (Chi-square test: $\chi^2 = 34.074$, $d.f. = 3$, $P = 0.0001$).

As we have shown in the competitive copulation experiment, the mating of *B. h. sapporoensis* males with *B. terrestris* queens is much more frequent (1.2-fold) than with *B. h. sapporoensis* queens. These observations indicate that *B. h. sapporoensis* males mate with *B. terrestris* queens and suggest that the mating behavior of *B. terrestris* queens may potentially interfere with intraspecific *B. h. sapporoensis* matings. Similar results were reported in the interspecific hybridization of *B. ignitus* and *B. terrestris* (Ono, 1997; Yoon *et al.*, 2009). Furthermore, our results indicate that *B. terrestris* queens have more successful matings with *B. h. sapporoensis* males than *B. h. sapporoensis* queens have with their own males. Although this effect may result from the rearing of *B. terrestris* in the laboratory compared with *B. h. sapporoensis*, our finding suggests that *B. terrestris* queens will have a competitive impact on the mating of *B. h. sapporoensis* queens. Consequently, competition could have a negative effect on the intraspecific matings of *B. h. sapporoensis*. In addition, the intraspecific mating rate of *B. terrestris* was 2.4-fold higher than that of *B. h. sapporoensis*. In view of the mating ability of *B. terrestris*, it is probable that *B. terrestris* will compete significantly with *B. h. sapporoensis* and could be superior to *B. h. sapporoensis* 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).

The mating duration in intraspecific and interspecific crosses of *B. h. sapporoensis* and *B. terrestris* was also investigated. The mating duration in intraspecific and interspecific crosses of *B. h. sapporoensis* and *B. terrestris* is about 25-35 min (Table 1). The mating duration in the intraspecific mating of *B. h. sapporoensis* tended to be 5 min shorter than that of the intraspecific mating of *B. terrestris*. The hybridization of a *B. h. sapporoensis* queen and *B. terrestris* with a *B. terrestris* male has a longer mean mating duration (34.5-35.2 min) than that of a *B. h. sapporoensis* queen and *B. terrestris* with a *B. h. sapporoensis* male (24.8-29.5 min). There was a statistical difference in the mating duration of the *Bombus* spp. (one-way ANOVA, $F = 6.68$, $d.f. = 3$, 126 , $P = 0.0001$). This result indicates that in the hybridization of *B. h. sapporoensis* and *B. terrestris*, the mating duration shows a tendency toward the duration associated with *B. terrestris*. A similar result was seen in the interspecific hybridization of *B. ignitus* and *B. terrestris* (Yoon *et al.*, 2009). The mating duration has been recorded for a number of bumblebee species (Duvoisin *et al.*, 1999; Brown *et al.*, 2002).

We sought to determine whether the frequency of interspecific hybridization of *B. terrestris* queens with *B. h. sapporoensis* males and *B. h. sapporoensis* queens with *B. terrestris* males is higher than the frequency of intraspecific mating of *B. h. sapporoensis* and *B. terrestris* queens. To investigate this question, *B. h. sapporoensis* queens and *B. terrestris* queens were placed simultaneously in one cage with *B. h. sapporoensis* males and *B. terrestris* males. We found that the mating percentage for the interspecific mating of *B. terrestris* queens with *B. h. sapporoensis* males was 40.0%, 1.3-fold higher than that of the intraspecific mating of *B. h. sapporoensis* queens, though there was no statistically significant difference (Chi-square test: $\chi^2 = 0.220$, $d.f. = 1$, $P = 0.639$) (Fig. 1A). However, the mating percentage for the interspecific hybridization of *B. h. sapporoensis* queens with *B. terrestris* males was 40.0%, 2.3-fold lower than that of the intraspecific mating of *B. terrestris* queens (Chi-square test: $\chi^2 = 5.495$, $d.f. = 1$, $P = 0.019$) (Fig. 1B). The mating percentage for the interspecific mating of *B. h. sapporoensis* queens with *B. terrestris* males was 26.7%, 2.7-fold higher than that of the intraspecific matings of *B. h. sapporoensis* queens (Chi-square test: $\chi^2 = 2.783$, $d.f. = 1$, $P = 0.095$) (Fig. 2A). Like the mating percentage for the interspecific mating of *B. h. sapporoensis* queens with *B. terrestris*, the mating percentage for the interspecific mating of *B. terrestris* queens with *B. h. sapporoensis* males was 16.7%, 5.0-fold lower than that of the intraspecific

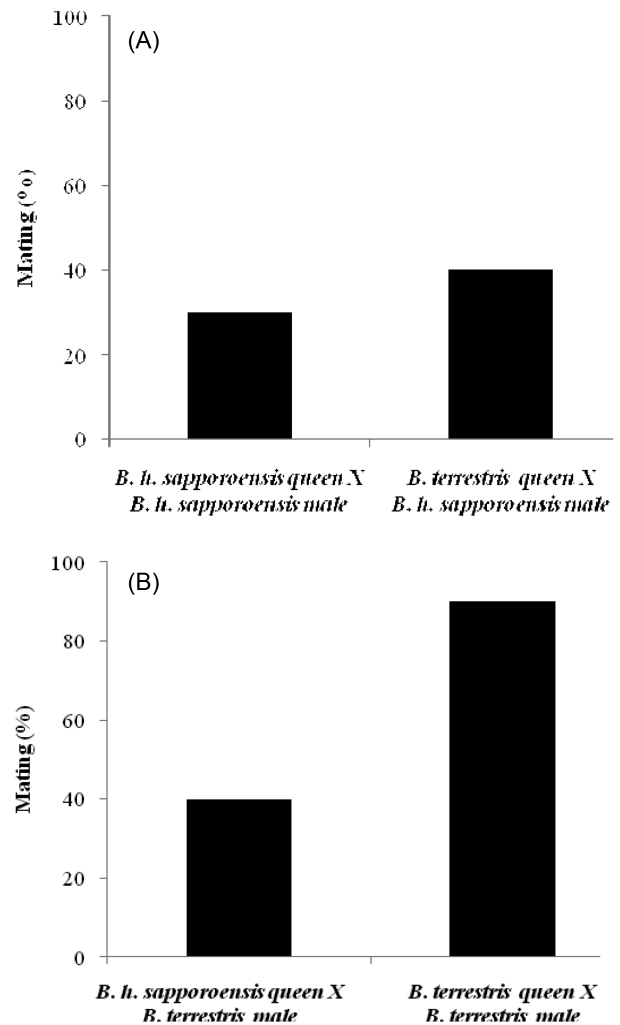


Fig. 1. Competitive copulation of *B. h. sapporoensis* queens and *B. terrestris* queens with *B. h. sapporoensis* males (A) and *B. terrestris* males (B). Ten queens of each species were simultaneously put in one cage with 40 males. Statistical analysis: mating percentage of *B. h. sapporoensis* queens and *B. terrestris* queens with *B. terrestris* males, Chi-square test $\chi^2 = 5.495$, $d.f. = 1$, $P = 0.019$.

matings of *B. terrestris* queens (Chi-square test: $\chi^2 = 29.433$, $d.f. = 1$, $P = 0.0001$) (Fig. 2B). The above results indicate that the *B. terrestris* queen and male are superior to the *B. h. sapporoensis* queen and male in mating. Kanbe *et al.* (2008) reported that nearly 30% of the queens of the Japanese native species *B. h. sapporoensis* and *B. h. hypocrita* were estimated to copulate with *B. terrestris* males in the field and suggested that indigenous bumblebees could suffer from genetic deterioration through the production of hybrids with the introduced species.

Queens of mated *Bombus* spp., after treatment with carbon dioxide to stimulate diapause (Yoon *et al.*, 2003),

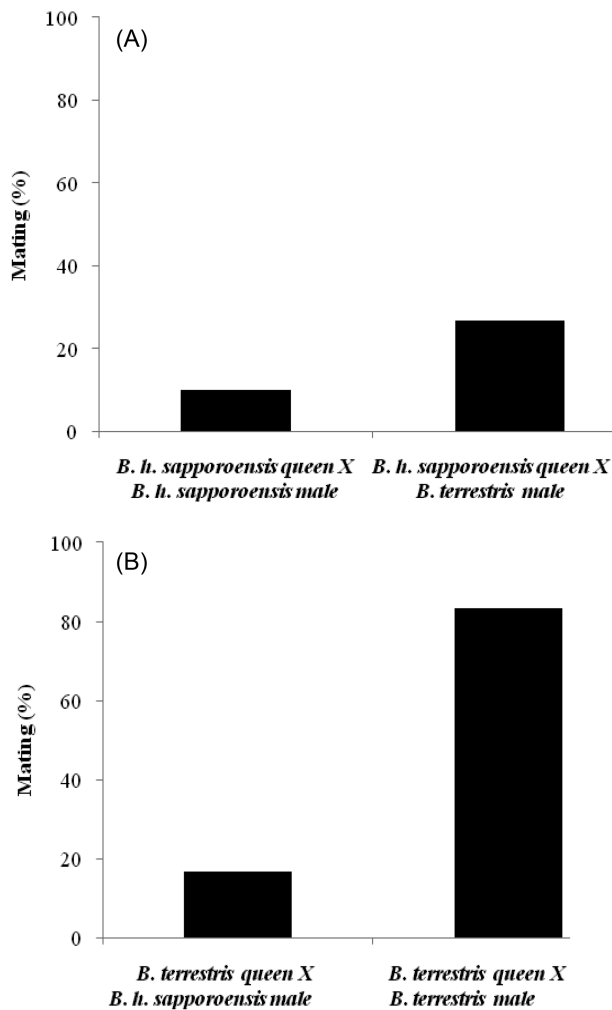


Fig. 2. Competitive copulation of *B. h. sapporoensis* males and *B. terrestris* males with *B. h. sapporoensis* queens (A) and *B. terrestris* queens (B). Sixty males of each species were simultaneously put in one cage with 60 queens. Statistical analysis: mating percentage of the *B. h. sapporoensis* male and *B. terrestris* male with *B. terrestris* queen, Chi-square test $\chi^2 = 29.433$, d.f. = 1, $P = 0.001$.

were reared to determine the presence of oviposition. We surveyed oviposition percentages and the preoviposition periods of intraspecifically and interspecifically mated *Bombus* spp. (Fig. 3). The oviposition percentage was highest in the *B. terrestris* queen \times *B. terrestris* male cross (90.0%), followed by the *B. terrestris* queen \times *B. h. sapporoensis* (80.0%) cross, the *B. h. sapporoensis* queen \times *B. h. sapporoensis* male (66.7%) cross, and the *B. h. sapporoensis* queen \times *B. terrestris* male cross (20.0%). It is noteworthy that the oviposition percentage of *B. terrestris* queens mated with *B. h. sapporoensis* males was 1.2-fold higher than that of intraspecifically mated *B. h. sapporoensis* queens and was 4-fold higher than that of *B. h.*

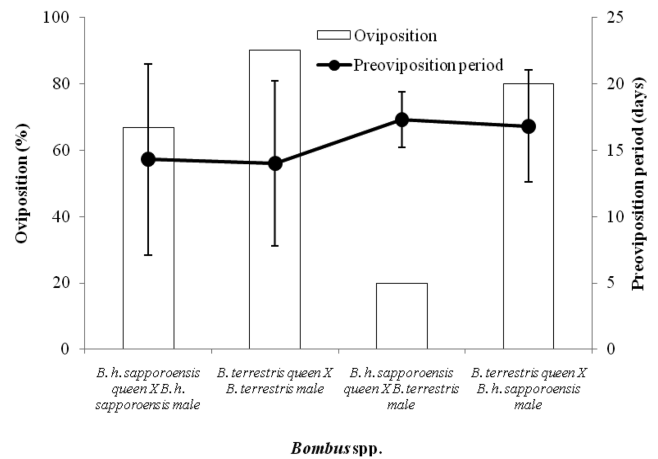


Fig. 3. Oviposition percentage and preoviposition period of hybridized queens. Queens were reared after CO₂ treatment to simulate diapause at 2 days after mating. For the statistical analysis, a Chi-square test was used: $\chi^2 = 37.511$, d.f. = 3, $P = 0.0001$ for the percentage of oviposition.

sapporoensis queens mated to *B. terrestris* males. There was a statistically significant difference in the oviposition percentages of the two *Bombus* spp. (Chi-square: $\chi^2 = 37.511$, d.f. = 3, $P = 0.0001$). However, there was no significant difference in the preoviposition period (14–17 days) for the *Bombus* spp.

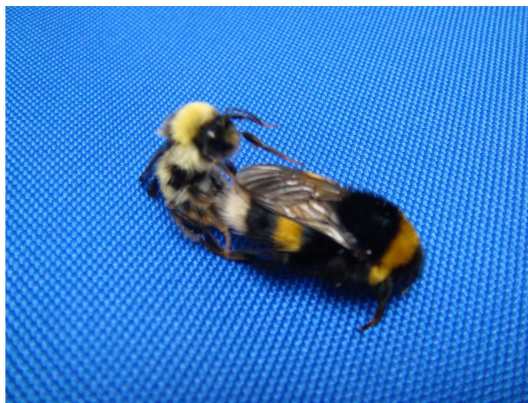
Colony development in hybridized *Bombus* spp.

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 6.6% and 9.1% for intraspecifically mated *B. terrestris* queens and interspecifically mated *B. terrestris* queens, respectively. Compared with the progeny from intraspecific matings of *B. h. sapporoensis*, 13.3% of which died within the month, 70.0% of the progeny from *B. h. sapporoensis* queens mated with *B. terrestris* males died within 1 month after rearing. The percentage of death in intraspecific matings of *B. h. sapporoensis* was 2.0-fold higher than that in intraspecific matings of *B. terrestris* (Chi-square: $\chi^2 = 0.741$, d.f. = 1, $P = 0.389$). Furthermore, the percentage of death of the progeny of *B. h. sapporoensis* queens mated with *B. terrestris* males was 5.3-fold higher than that of *B. terrestris* queens mated with *B. h. sapporoensis* males (Chi-square: $\chi^2 = 24.721$, 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. h. sapporoensis* (Chi-square: $\chi^2 = 45.138$, d.f. = 3, $P = 0.0001$). A similar result was seen in the interspecific mating of *B. ignitus* and *B.*

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.h. sapporoensis</i> queen × <i>B.h. sapporoensis</i> male	30	13.3	43.3	56.7	10.0	13.3
<i>B. terrestris</i> queen × <i>B. terrestris</i> male	30	6.6	76.7	83.3	33.3	43.3
<i>B.h. sapporoensis</i> queen × <i>B. terrestris</i> male	30	70.0	0.0	16.7	0.0	0.0
<i>B. terrestris</i> queen × <i>B.h. sapporoensis</i> male	33	9.1	3.0	36.4	0.0	0.0

Statistical analysis: Chi-square test $\chi^2 = 45.128$, $df = 3$, $P = 0.0001$ for the percentage of death within 1 month.



(A)



(B)

Fig. 4. Copulation of the *B. terrestris* queen with a *B. h. sapporoensis* male (A) and colony produced from a *B. terrestris* queen hybridized with a *B. h. sapporoensis* male (B).

terrestris (Yoon *et al.*, 2009).

The results for adult emergence showed that *B. terrestris* queens hybridized with *B. h. sapporoensis* males produced workers resembling *B. terrestris* workers and males

resembling *B. terrestris* males, although the percentage of worker emergence was low (3.0%) (Table 2, Fig. 4A and B). In contrast, *B. h. sapporoensis* queens hybridized with *B. terrestris* males only produced males (16.7%) (Table 2). The phenotypes of the hybrids were easily differentiated by morphological characteristics and their similarities with *B. terrestris* or *B. h. sapporoensis* identified (data not shown). These morphological characteristics showed that the hybrid phenotype was dependent on the maternal line. Our results show that interspecifically hybridized queens of *B. ignitus* or *B. terrestris* produced hybrids but did not form a colony. The mating, survival and oviposition percentages of *B. terrestris* queens hybridized with *B. ignitus* males were all higher than the corresponding values for the intraspecific mating of *B. ignitus*.

The 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. h. sapporoensis* and that no hybrid females were obtained (Ono, 1997). In contrast, Mitsuata and Ono (1996) reported that a *B. h. sapporoensis* queen hybridized with a *B. terrestris* male produced hybrid workers and new queens and that the hybrid queen copulated with a male *B. terrestris* but did not initiate a nest. A recent study has shown that the interspecific mating of *B. terrestris* and *B. h. sapporoensis* produces inviable hybrids (Kanbe *et al.*, 2008). The results of these previous studies are consistent with our observation that hybridized queens of *B. h. sapporoensis* or *B. terrestris* produced hybrids and that if a hybrid male *B. terrestris* copulated with a virgin queen of *B. terrestris*, the queen oviposited but did not initiate a nest. We did not observe oviposition by hybrid workers, but a previous study found that the hybrid workers oviposited unfertilized eggs, which developed into adult second-generation males (Mitsuata and Ono, 1996).

We have shown that hybridization can occur between *B. h. sapporoensis* and *B. terrestris*. Our study identified the competitive effect of *B. terrestris* on intraspecific matings of naturally occurring *B. h. sapporoensis* by demonstrating that *B. h. sapporoensis* males mate preferentially with *B. terrestris* queens. In addition, we hypothesized that such interspecies hybridization could cause genetic contamination of the native bumblebees. Further studies of interspecific hybridization and the development of any progeny produced will ultimately provide important information about the ecological impacts of introduced *Bombus* spp. on native bumblebees.

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