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Hygienic Behavior Test of Six Inbred Lines in *Apis mellifera* Through Freeze-killed Brood Method

Hye-Kyung Kim*, Man-young Lee, Myeong-Lyeol Lee, Yong-Soo Choi and Kyu Ho Byeon

National Institute of Agricultural Sciences, RDA, Wanju, Republic of Korea

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Abstract

Honey bees, *Apis mellifera*, perform hygienic behavior, by quickly detecting, uncapping and removing diseased brood from the nest. Hygienic behavior is a complex, genetically based quantitative trait that serves as a key defense mechanism against parasites and diseases, and is considered a potential characteristic associated with resistance to these parasites and pathogens. In this study, six inbreed lines of *A. mellifera*, bred over the course of eight years at the National Academy of Agricultural Science in Korea, were studied to hygienic behavior, a freeze-killed brood aay was conducted at 24 h and 48 h intervals. The results indicate that, after 24 h, two inbred lines were classified as non-hygienic (removal of dead brood <50%), three inbred lines exhibited intermediate behavior, and one inbred line was hygienic (removal of dead brood >90%). However, after 48 h, the removal rate of two intermediate lines was >90%; thus, these lines were classified as hygienic at this interval.

Key words: *Apis mellifera*, Hygienic behavior, Freeze-killing method

INTRODUCTION

Honey bees (*Apis mellifera*) are imprtant insects in agriculture, ecology and research. Honey bees not only produce honey, royal jelly, propolis, and pollen, but also mediate pollination of various plants and crops (Jensen *et al.*, 2005). In Korea, the economic value of honey bees as pollinators is reported to be more than over 6 trillion dollars (Jung, 2008) and the demand for pollination also has increased drastically. Honey bee have been reared in Korea since the early 1900s, and today, the number of bee hives approximates 2,000,000. However, *A. mellifera* faces challenges including a lack of genetic diversity, resulting in reduced populations, and regional limitations. In addition, pesticides, pathogens, and parasites (such as mites) have contributed to their decline. Importantly, because of regional limitations, genetic resources of honey bees in Korea are very restrictive.

Hygienic behavior includes detection of sick or dead brood, subsequent uncapping of the brood cells, and removal of infected larvae or pupae (Rothenbuhler, 1964; Wilson-Rich *et al.*, 2008). First described in honey bee colonies in relation to the brood disease American foulbrood (AFB), hygienic behavior has also been indicated as important for the control of chalkbrood and Varroosis (Spivak, 1996). Further, hygienic behavior is recognized as an important mechanism that contributes to colony resistance against these pathogens and parasites (Boecking and Spivak, 1999; Spivak and Reuter, 2001), and thereby may be relevant in breeding programs for resistance. Originally it was thought to be controlled by simple Mendelian genetics, Rothenbuhler (1964) proposed a two

*Corresponding author. E-mail: hyegyeong@korea.kr

locus model to explain hygienic behavior inheritance. Subsequent studies have determined hygienic behavior could be a more quantitative trait (Gramacho and Spivak 2003; Danka *et al*., 2011). Since then, other studies based on molecular techniques have suggested there may be at least seven quantitative trait loci (QTLs) associated with hygienic behavior (Oxley *et al*., 2010; Lapidge *et al*., 2002; Spötter *et al*., 2012; Tsuruda *et al*., 2012). Recently, Boutin *et al*. (2015) reported that hygienic behavior relies on a limited set of genes linked to different regulation patterns (expression level and biological processes) associated with an over-expression of cytochrome P450 genes. These results suggest that the genetic basis of hygienic behavior is more complex than previously thought.

In this study, we examine hygienic behaviors in six inbred lines of *A. mellifera* at the National Academy of Agricultural Science (NAAC) in Korea and discuss the value of hygienic behavior to colony health.

MATERIALS AND METHODS

Honey bee breeding lines

The honey bee breeding line has been controlled through the production of queen from a selected colony and matting using artificial insemination over 8 years. Detailed information of six inbred lines was summarized in Table 1. A line was *Apis mellifera ligustica* collected in Australia, which have a dark brown color and gentle in its behavior toward beekeepers. But, this strain have susceptible to disease. C was *Apis mellifera ligustica* collected in China, which have light brown color and high honey productivity, but is week to nosema disease and low fertility. D were *Apis mellifera caucasica* which was superior to foraging and winter resistance. E strain was *Apis mellifera carnica* collected from Europe, which gentle and superior foraging. E strain can be physically distinguished by their dusky brown- gray color. These bees are particularly adept at adjusting worker population to nectar availability. But these strains has weaknesses to swarming and robbing and low level of expand worker bee populations. F strain was *Apis mellifera ligustica* collected from USA, which having brown color. This has good egg laying but easy swarming and weaknesses to high food consumption. G strain was *Apis mellifera ligustica* collected from Korea, which having brown color. This is superior to amount of laying eggs and producing of royal jelly and pollen.

Hygienic behavior test using freeze-killed brood assay

Hygienic behavior was tested according to the method of freeze-killed brood assay (Spivak and Reuter, 2001). Experiments were carried out at the experimental apiary of National Academy of Agricultural Science during May to June of 2015 from the xis inbred lines (named lines A, C, D, E, F and G) and control which is not breeding line. Twenty one colonies (three colonies per each inbred line and control) were established in the spring of 2015. Each colony was comprised of 8-frame Langstroth hive body. Selected colonies were of equivalent strength (6-7 frames of bees/brood). All hives were placed in the same apiary to avoid influence of environmental conditions. A 3-inch (7.62cm) diameter PVC cylinder was pressed into a frame of capped brood. This area corresponds to approximately 160 cells. Briefly, 100mL of liquid nitrogen was poured into the circle within the brood area of each hive (7 day old larvae). The section was photographed before the donor

Table 1. Six inbred lines of *Apis mellifera* (A, C, D, E, F and G) maintained at National Academy of Agricultural Science (NAAS), Korea

Iine	Subspecies	Color	Traits	Collection
A	*A. m. ligustica*	Dark-brown	Gentleness	Australia
C	*A. m. ligustica*	Light-brown	High fertility	North-estern China
D	*A. m. caucasica*	Dark-brown	High foraging	North-estern China
E	*A. m. carnica*	Dusky brown-gray	Gentleness	Europe
F	*A. m. ligustica*	Brown	High fertility	USA
G	*A. m. ligustica*	Brown	High fertility	Korea

Table 2. Hygienic evaluation of the honeybee colonies studied. The data showed percentage of capping, removing or uncapping Freeze-killed brood, counted for two days at intervals of 24 h, in seven colonies, A, C, D, E, F, G and control

Inbred line	Hygienic status	24 h (%)			24 h (%)		
		Capped	Removal	Uncapped	Capped	Removal	Uncapped
A	Intermediate	27.2	64.0	8.8	27.0	68.4	4.7
C	Intermediate	10.9	84.8*	4.4	1.9	97.0	1.1
D	Intermediate	5.4	86.0*	8.6	3.1	94.9	2.0
E	Non-hygienic	51.9	37.2	10.9	28.3	67.1	4.7
F	Hygienic	1.8	97.4*	0.8	1.3	98.4	0.3
G	Non-hygienic	46.0	40.5	13.5	35.3	54.0	10.7
Control	-	61.4	32.0	6.6	35.4	64.0	0.6

*P<0.005.

frame was placed in the test colony. This procedure was carried out twice per hive. Hygienic behavior was evaluated by calculating the number of brood removed in a period of 24 h and 48h by comparing both photographs and counting the number of capped brood that had been removed, or cells that had been opened, by the bees during the test period. The percent hygienic behavior for a colony was calculated as follows: number of capped cell and partially removed pupae/(160-number originally uncapped or empty cells) X 100. A colony that uncapped and removed 90% of the dead larvae or more was considered hygienic and a colony that removed less than 60% was considered non-hygienic.

Statistical analysis

In this study, one-way ANOVA (PROC ANOVA; SAS Institute, 1989) were used to compare hygienic behavior among the six inbred lines.

RESULTS AND DISCUSSION

To determine the ability of hygienic behavior of the 6 inbred lines in Apis mellifera, Freeze-killed method was applied (Table 1). The experimental colonies contained 7 day old larvae that have been bred using artificial insemination, thus presumably homozygous for the alleles that regulate the behavior. The experimental colonies were evaluated 3 times in 24 and 48 hours for hygienic behavior on April to July each year. The experiment reveals variation in honey bee sensitivity to dead brood and the ability of honey bees to discriminate between dead and healthy brood. Table 2 shows that the rate of removal (expressed as a percentage) of dead brood in 24 h for the six inbred honey bee lines ranges from 37.2% to 97.4%, the capped rate ranges from 1.8% to 61.4%, and the uncapped rate ranges from 0.8% to 13.5%. The rate of removal in 48 h ranges from 54.0% to 98.4%, the capped rate ranges from 1.3% to 35.4% and the uncapped rate ranges from 0.3% to 10.7%. Based on the data, after 24 h, two inbred lines (E and G lines) are classified as non-hygienic (removal of dead brood < 50%), three inbred lines (A, C, and D) exhibit intermediate behavior, and one inbred line (F) is hygienic (removal of dead brood > 90%). However, the removal rate of lines C and D lines in 48 h more higher (removal of dead brood > 90) than 24 h, thus these lines can be classified as hygienic. Lines C, D and F also display a lower rate of uncapping in 48 h than other populations, although C and D lines are not lower in 24 h.

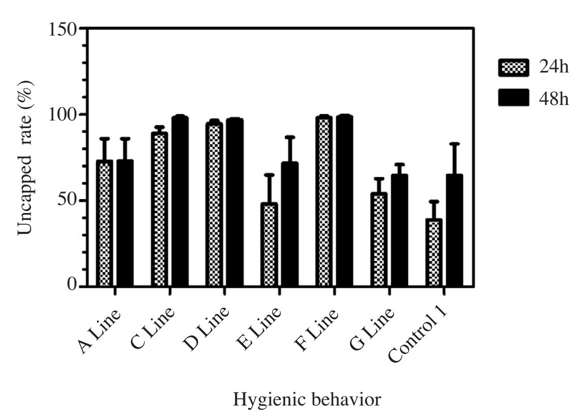

Fig. 1. Hygienic behavior is calculated as a percentage based on the number of dead brood removed and uncapped in 24 and 48 hours, in seven colonies, A, C, D, E, F, G and control. *P<0.005 compared control.

Fig. 1 shows hygienic behavior as a percentage based on the number of dead brood removed and uncapped in 24 h and 48 h in lines A, C, D, E, F, G, and a control. Among the colonies studied, the 24 h removal rates for lines C, D, and F are 84.8%, 86.0%, and 97.4%, respectively. This removal rate of dead brood in lines C, D and F is significantly higher than other lines during the first 24 h ($p<0.005$), even though lines C and D are considered intermediate in their behavior at this interval (Table 2). The removal rates at 48 h for lines C, D, and F are 97.0%, 94.9% and 98.4%, respectively, again significantly higher than other lines during the 48 h interval (Fig. 1, $p<0.005$). Thus, these lines are classified as hygienic at the 48 h interval (Table 2). Spivak (1998) reported that colonies that are resistant to diseases remove at least 95% of killed brood within 48 h.

Our results demonstrate that lines C, D and F have hygienic ability. In a previous study, hygienic behavior was found to be related to olfactory sensitivity (Gramacho and Spivak, 2003). The sensitivity of a colony could be an important factor in detection and removal of dead or diseased brood. A brood removal trait appears to serve as a mechanism to maintain brood and colony health. It is believed to be a multi-genic trait yet only a single strong QTL has been identified (Danka *et al*., 2011). The gene conferring the trait remains unknown. In further studies, it is essential to identify the genetic basis of hygienic behavior associated with the six inbred lines of *A. mellifera* examined here. Knowledge of the genetic basis for hygienic behavior will be valuable in breeding honey bee lines resistant to pathogenic organisms and parasites, which may reverse the present decline of honey bee populations.

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