Original Article

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Breeding and Selection for Resistance to Sacbrood Virus for *Apis cerana*

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Abstract

South Korea has over 0.38 million of managed honey bee (*Apis cerana*) colonies before 2009 years ago, which produce the highest quantity of honey in the Korea; however, almost colony (90%) were collapsed by Korean Sacbrood Virus (KSBV) in South Korea. Korean Sacbrood Virus (KSBV) is the pathogen of *A. cerana* Sacbrood disease, which poses a serious threat to honeybee *A. cerana*, and tends to cause bee colony and even the whole apiary collapse. Colony collapse of *A. cerana* was first reported on the Pyeong-Chang of the South Korea in 2009. Several scientists and governments has been tried research for cure the sacbrood disease in *A. cerana* colony by medicines and management techniques. Unfortunately, The sacbrood disease dosen`t improve. So, we were developed a better breed of *A. cerana* for resistance of sacbrood virus by selection and than artificial insemination. *A. cerana* breeding technique was first successful applied with *A. cerana* in Korean. Queens was grafted from sacbrood resistance line and than it were growing in sacbrood disease colony that was survived 100%. Altogether selected 18 queens were artificially inseminated and 2,000 drones of *A. cerana* in Korea was used to evaluate amount of semen collection. We are select two scabrood resistance *A. cerana* line (R and H). R line be used for rearing the Queen. Drone was reared in H line colony. The RH hybrid were not infected sacbrood virus even spread sacbrood virus (2×10^6). RH colonies has very excellent hygienic behavior, brood, and sacbrood disease resistance activity.

Key words: Honeybee, *Apis cerana*, Breeding, Resistance, Sacbrood virus, Artificial insemination

INTRODUCTION

Honeybees species in Korea are divided into *Apis mellifera* and *Apis cerana*. These two types of honeybees coexist in the broad category as a most important pollinator on each house crop and preservation of forest resources. Among them, *A. cerana* is one of the ten types of bees species in all over the world; *Apis* family (Family Apidae, Subfamily Apinae, Tribe Apini) (Ruttner, 1988; Arias & Sheppard, 2005) that was settled in Korea. However, most of the *A. cerana* colonies in Korea had lost due to Sacbrood virus (Choi *et al*., 2010).

A. cerana in countries in South-East Asia including Korea is very weak in resisting against Sacbrood virus unlike the *A. mellifera*. Sacbrood virus is a disease occurring on larva. As it firstly occurred in Thailand in 1976, all

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of the infected bee colonies were dead (Barley *et al*., 1982). In Korea, they were diffused in to all over the nation after it was firstly diagnosed in 2009 (Choi *et al*., 2010). As for typical symptoms of Sacbrood virus, internal organs were corrupted and melted down where skin of larva was left. When Sacbrood virus occurred in the beginning, farms raising the *A. cerana* had difficult time in identifying the Sacbrood virus and foulbrood. Therefore, confusion has been added.

Foulbrood has feature as internal organs are melted down in the form of water unlike Sacbrood that there is a difference in symptoms of disease (Ratna *et al*., 2012). In addition, larva infected with Sacbrood virus occurring in Korea has been reported to have tails discolored into black. (Toan *et al*., 2014). After Sacbrood virus occurs, medicine or negative ion was used in Korea making an effort to overcome Sacbrood virus through improvement of bee colony management technique (Choi *et al*., 2013; Ahn *et al*., 2015). Especially, research for the treatment of gene has been conducted in the nation. Using the dsRNA and RNAi techniques, research has been conducted on the treatment of Sacbrood virus. As a result, it was reported that there was an effect of reducing the Sacbrood virus (Liu *et al*., 2010; Zhang *et al*., 2016).

However, they have not been developed to be practically used in the field. Results such as improvement of bee colony management techniques and development of medicines had the death of bee colony reduced by Sacbrood virus by farms except for several fams due to issues of less speedy diffusion of farms and low efficacy of medicines. Therefore, since development of vaccine for treatment of disease by honeybee virus was impossible, it has been discussed as to how selecting the systme resisting against diseases and distributing them through cultivation were of the best option to prevent the occurrence of Sacbrood virus. Honeybee breed has been pro-actively led in *A. mellifera*, and the purpose of sarcoma was the resistance against disease such as foulbrood and chalk brood (U. Riessberger *et al*., 2001; Liu *et al*., 2016) and sand fly (Lattorff *et al*., 2015), and improvement of water tightening ability (Lee *et al*., 2014).

Development of 'Jangwon' (*Apis mellifera*) with outstanding honeybee production seems to be the representative outcome in Korea (Lee *et al*., 2014). Honeybee tends to mate with queen and drone. Therefore, there is much difficulty in stably maintaining the order while researching the breed. In order to reduce the risk of mongrelization, artificial insemination has been usually used for culivating and selecting the systems. Honeybee artificial mating technique has been firstly succeeded by Dr. Watson in Cornel Univesity in America in 1927 and improved by many of the scientists (Cobey, 1983; Cobey & Schley, 2002). Artificial mating technique of *A. cerana* was to inject semen of male bee (*A. cerana*) to queen bee (*A. mellifera*) and experimenting the artificial mating (Ruttner, 1969). Except for this case, there were no cases where artificial insemination was applied for sarcoma.

Artificial insemination technique for *A. cerana* has been introduced by Dr. Woyke in Poland in 1973 in details (Woyke, 1973). Among the countires that raised *A. cerana*, professor Wongsiri in Chulalongkorn University in Thailand used artificial insemination technique to cultivate the cross-breed *A. cerana* indica in Thailand and *A. cerana cerana* in China to cultivate the *A. cerana* with outstanding sand fly removing ability in 1990. However, proceeding artificial insemination on *A. cerana* was difficult more than honeybees in western countries, and the amount of semen was low in male bees that there was difficulty in collecting semen from male bees. Therefore, whether there was focused artificial insemination technique was the most important technique of *A. cerana*.

Therefore, in spite of various effort to treat Sacbrood virus and prevent the diffusion of it, research has been conducted to select the systems resisting against Sacbrood disease of *A. cerana* for preventing the diseases where Sacbrood virus has been diffused and chronic, maintaining the pure line by using artificial insemination technique, and preparing for the cross-breeding of propagation of bee colony.

MATERIALS AND METHODS

Collection of resisting bee colonies against *Apis cerana* Sacbrood virus

Local bees: A collection of colonies in different locations, such as Gumi, Ichon, Gangjin and Tongyeon, in

movable frame hives used for queen and drone rearing and SBV evaluation was established in 2015 and 2016 as experimental population. Ten colonies of each location were collected and assigned in a group. All colonies in the population were inoculated with SBV. In each group, higher brood viability, higher hygienic value and better colony-development were selected as parent colonies to rear queen and drones.

Resistant strain: A resistant strain was selected from collected bees in different locations in Korea. In 2016, survival pupae of different SBV-infected colonies in a same group were collected and emerged in a colony and let to lay drones. Laid drones from survival laying workers were used to artificially inseminate for virgin queens that reared from selected mother colonies of different groups.

Artificial insemination

Mother colonies were selected with their best strait performance to get larvae for queen rearing. The nursering colonies were populous and crowded. Grafted queen cells were rear in SBV infectious nursering colonies to screen for the queen cell that can be survived with the SBV-infection. One day before the date of larval grafting, laying queen of nursering colony is caged and eliminated to make the colony queenless. At the date of grafting, a frame with 25 queen cups is well prepared each cell having 10μl royal jelly. First day old larvae are transferred from the comb of mother colony to every queen cup by using grafting tool Drones of *A. cerana* for semen collection were caught on the entrances of selected father colonies and caged in a plastic box (having supplement food and water supplied) with 200 drones in one batches. Virgin queens were artificially inseminated 6 days after emergence. The Schley's instrument was used to do artificial insemination for the virgin queens. CO2 narcotizing was used during the inseminating process to immobile queens on queen's holder. The process of artificial insemination was applied following the method of Laidlaw (1987). Each virgin queen was instrument ally inseminated 4μL of semen. To stimulate egg laying of artificially inseminated queen, there were twice CO2 treatments, once before and once after the date of artificial insemination.

Rearing of larva in vitro

For the collection of resisting systems of *A. cerana* Sacbrood virus, injecting the Sacbrood virus into the bee colony has a chance to be environmentally influenced, and there might be difficulty in experiment if bee colonies end up dying. Therefore, they were artificially contaminated with Sacbrood virus to verify the resisting ability. As for feed composition for raising larva in indoor space, 5ml of boiled water was cooled down followed by cooling the 6g D-glucose (6%), 6g of D-fructose (6%) and 1g of yeast extract (1%) and placing them on the beaker and adding and mixing 37 ml of warm sterilized water.

In addition, refrigerated royal jelly was melt in the 4°C for a day and left in the room temperature for one or two hours while using the stick magnet with 50g of royal jelly (50%) to mix them well. Immediately afterwards, 100μl of feed was given to 96 well plates. Feeds were preserved for three days in 4°C and supplied hereof. Larva of three to four days was transferred to 96 well plates with feed. At this time, they were warmed in 34°C incubator for 15 minutes to improve the survival rate of larva before transferring. 96 well plates with larva were inserted to desiccator that humidity was set to 95% and raised in incubator in 34°C At this time, warmed liquid was 10% sulfuric acid liquid.

Selection of bee colonies resisting against Sacbrood virus

In order to confirm the resistance against Sacbrood virus of larva raised in indoor space, larva with symptoms of Sacbrood virus were collected and pulverized with 1ml of sterilized water for ten bees and centrifuged with 4,000 rpm for five minutes to separate supernatant including the virus. Separating RNA of virus by using total RNA extraction kit (Promrga), it was used as template for quantifying the virus. Virus quantification was analyzed by using quantitative real time PCR, and Forward primer 5'-GAC CAA GAA GGG AAT CAGC-3', Reverse primer 5'-CAT CTT CTT TAG CAC CAG TAT CCA-3' as a gene specific primer of Sacbrood virus (SBV) were used to react by mixing 10 pmol primer, 1xSYBR green (applied

biosystems), and 1st strand cDNA 1μl so that the final volume of qPCR reaction was set to be 25μl.

With the analysis of Ct value of final result of reaction, virus was quantified (Applied Biosystems, user bulletin #2). Virus solution that was quantitatively analyzed was processed to be 2×10^6 (Inoculation concentration) viruses in the indoor breeding processing area artificially deriving the Sacbrood virus. Larva raised in indoor space in survived bee colonies in the period when larve was converted to pupa was chosen as Sacbrood virus resisting bee colonies while selecting resisting bee colonies by deriving artificial Sacbrood virus in the same manner on the bee colonies raised in bee farms.

SBV diagnosis

Test bee colonies artificially infected with Sacbrood virus was chosen with ten *A. cerana* work bees to investigate the Sacbrood virus, and collected work bees were separated with total RNA by using Trizol. After pulverizing collected work bees with liquid nitrogen, 200μl chloroform and Trizol 1ml were mixed with 100mg of samples and vortexed for 15 seconds and reacted in the room temperature for two to three minutes. After reaction, samples were centrifuged in conditions of 12,000 xg and in 4°C for 15 minutes separating the supernatant to mix it with 500μl isopropanol to react in the room temperature for 10 minutes. Then, they were centrifuged in conditions of 12,000 xg for ten minutes and in 4°C for 15 minutes. Then, pellet was washed with 80% EtOH. Final pellet was melted in DEPC water and used for cDNA synthesis.

cDNA synthesis was reacted in 65°C for five minutes with PrimeScript II 1st strand cDNA Synthesis kit by using oligo dT primer with extracted total RNA as a mold and immediately cooled down in ice and synthesized with cDNA in 42°C for 60 minutes. Synthesized cDNA was used as a mol to amplify the Sacbrood virus (SBV) as a mold, and SBV gene was released in 33 cycles in pre-denature 94°C for 5 minutes, denature 94°C for 1 minute, and annealing 50°C for 1 minute by using Forward primer 5'-GACCAAGAAGGGAATCAGC-3', Reverse primer 5'-CATCTTCTTTAGCACCAGTATCCA-3' while performing final extension in 72°C in five minutes. PCR outcome is the 1% agarose gel that contained 0.5ug/ml ethidium bromide and was confirmed with UV-light.

Quantitative analysis of Northern blotting and gene expression (Real Time PCR: qPCR)

In order to clarify the effect of expanding the life span of work bees in colonies resisting against Sacbrood virus, the amount of expressed vitellogenin gene in the body of work bees was measured. First of all, Northern blotting was separated with total RNA by using Trizol proceeding electrophoresis on formamid gel and transferring them to nitrocellulose membrane for 18 hours and corssed linked with UV. Membrane was proceeded with pre-hybridization in hybridization solution for an hour and hybridized for 18 hours. Probe was exposed to X-ray for 24 hours by labeling the radiation isotope p^{32} on refined PCR outcome after amplifying the vitellogenin gene to PCR and printed by using the development solution and fixer.

In addition, quantification of expressed vitellogenin gene was anlayzed by using quantitative real time PCR, and Forward primer 5'-GTTATCGCTTCTGATATGGCT-3' Reverse primer 5'-GATGGGAAATAGGTACCGAC-3' as a vitellogenin gene specific primer were used to setup the final volume of qPCR as 25μl by mixing 10 pmol primer, 1×SYBR Green (Applied Biosystems), and 1st strand cDNA 1μl and reacting. With the analysis of Ct value of final result of reaction, gene expression was quantified (Applied Biosystems, user bulletin #2).

RESULTS AND DISCUSSION

Selection of types of resistance to Sacbrood virus

Over wintering survival colonies of Gumi (K) in 2015 were evaluated to rear queens in spring 2016. Virgin queens were artificial inseminated with semen of drones from Ichon (I). Survival pupae from SBV-inoculated KxI colonies were gathered in one hive for their emergences. Those worker bees were let to lay eggs in queenless colonies. Semen from drones of laying worker (KxI) were used to artificial inseminate for virgin queen of Gumi (K).

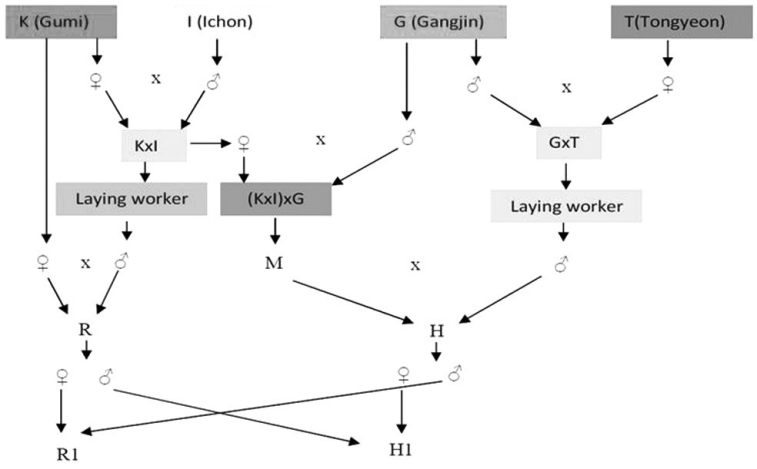

Fig. 1. *Apis cerana* breeding pedigree. Pedigree showing backcross performed using artificial insemination. A representation of a colony of bees as a genetic superfamily. The colony in the figure has four subfamilies.

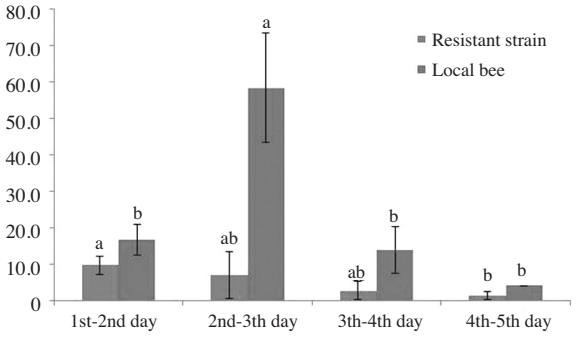

Fig. 2. Percentage of dead larvae at different instar-stages in SBV infected colonies. All colony was treated sacbrood virus (2×10^6/worker), artificially.

Laying queens of this crossbreeding diagram were named R.

Virgin queens reared from selected KxI colonies were artificially inseminated with semen of G drones. Colonies resulted from this crossbreeding diagram were named M. Colonies of Guangjin (G) were evaluated to rear queens in spring 2016. Virgin queens were artificial inseminated with semen of drones in colonies that collected from Tongyeon (T). Survival pupae from SBV-inoculated GxT colonies were gathered in one hive for their emergence, and those worker bees were let to lay unfertilized eggs in 2017. Semen from drones of laying workers (TxG) was used to artificial inseminate with virgin queen of M. The laying queens in those colonies were name H. Virgin queen of H and R were artificially inseminated with drones of T, and H in 2017. The crossbreeding between virgin queens of R and semen of H were called resistant strain. Colonies headed by Ichon and Tongyeong queens were called local bees (Fig. 1).

Raising the bee colony resisting against Sacbrood virus

After artificially infecting each of the bee colonies with Sacbrood virus that has been traditionally conducted in farms as well as RH systems to compare how much the bee colony was raised according to characteristics of Sacbrood virus in RH system, the number of larva showing symptoms of diseases was investigated. As for the larvas that a day or two have passed by after artificially infecting them with virus, 9.7% of larvas in RH systems turned out to be dead, and 16.7% of them turned out to be dead in normal bee colonies. As for resisting systems, 6.9% of larvas that two or three days have passed turned out to be dead. However, 58.3% of them in the non-resisting system turned out to be dead. However, resisting systems tended to have decreasing death rate as age increased.

If five days have passed after artificially infecting bees with virus, non-resisting systems showed 93.1% of death rate. However, resisting systems were normally raised (Fig. 2). In this study, as the 2×10^6 bees with virus concentration have been artificially processed directly to the bee colony, it was confirmed that young larvas in year one or

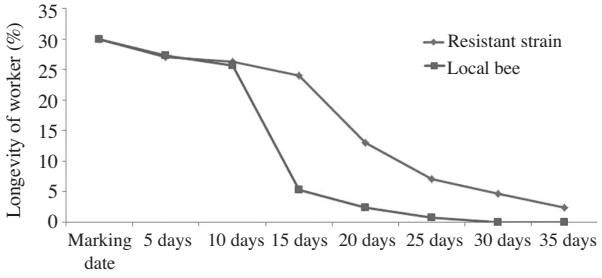

Fig. 3. Comparison of longevity of worker bees between resistance line and normal line. New emerging worker bees were marked on the thorax. Each colonies had 30 marked worker bees and repeated 3 times every 1 weeks. Marking colors were different for each time.

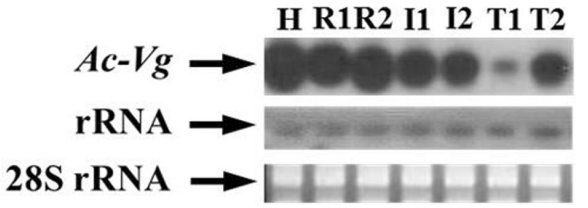

Fig. 4. Transcriptional expression of the vitellogenin gene in different colonies of *A. cerana*. (A) Northern blot analysis of the vitellogenin in *A. cerana*. Total RNAs were isolated from the resistance line H colony (lane 1), resistance line R1 (lane 2), resistance line R2 (lane 3), normal colony I1 (lane 4), normal colony I2 (lane 5), normal colony T1 (lane 6), normal colony T2 (lane 7). The RNAs were separated by 1.0% formaldehyde agarose gel electrophoresis, transferred onto a nylon membrane and hybridized with radiolabled vitellogenin partial gene.

two turned out to be dead in the beginning in resisting system bee colonies. However, as age increased, the death rate decreased due to the resistance that system had, and they were confirmed to be raised in normal bee coloneis. Therefore, it seems that they can stably survive in the bee farms that are full of Sacbrood virus.

Life span of work bees resisting against Sacbrod virus

As for RH, there was no change in the number of bees up to five days with the non-resisting systems (the number of resisting system: 27 bees, non-resisting systems: 25.7 bees). However, there was a difference in the number of survived bees from ten days that 24 bees in resisting systems turned out to survive after 15 days, while only 5.3 of bees in non-resisting systems ended up surviving.

After 30 days have passed as an average number of days as life span of normal work bees, 4.7 bees in resisting system turned out to survive, and all 30 work bees indicated on non-resisting systems ended up dying (Fig. 3). 2.5 work bees in resisting bee colonies ended up surviving that resisting work bees turned out to have a longer life span than non-resisting bee colonies. Therefore, it seems that they are superior over non-resisting systems in terms of raising ability and productivity in bee colonies from an increase of the number of work bees.

In this study, the degree of expanding the life span of work bees in the bee colonies has been measured. In general, life span of work bees tends to be expanded when specific gene is expressed the most. Genes related to the expansion of life span of work bees tend to adjust the life span of work bees when various genes interact with each other. It has been reported that the difference of expression of vitellogenin gene decides the life span of work bees as it can be used as a model for expressing genes in the final form (Nelson, 2007). Therefore, according to the amount of expressed vitellogenin genes between resisting systems and non-resisting systems of Sacbrood virus, they turned out to have more amount of expressed genes compared to I and T as collected systems for R system as a mother line and H system as a father line in resisting systems (Fig. 4). Therefore, it was confirmed that systems resisting against Sacbrood virus had longer life span and were confirmed to be raised as powerful colonies.

Hygenic behavior of the systems against Sacbrood virus

Productivity of outcome and adjustment in environment of honeybees tend to have complicated genetic features including the life span of work bees, spawning ability of queen bees, length, and feed consumption. On the other hand, leaning behavior ability for removing the larva and pupa on the bee colony due to work bees were known to be controlled by u (behaviors for peeling the brood capping by finding the comb cell of dead pupa) and r (biting dead pupa on the comb cell) (Rothenbuhler, 1964, 1974). Therefore, in this study, cleaning ability of systems resisting against Sacbrood systems was compared with the

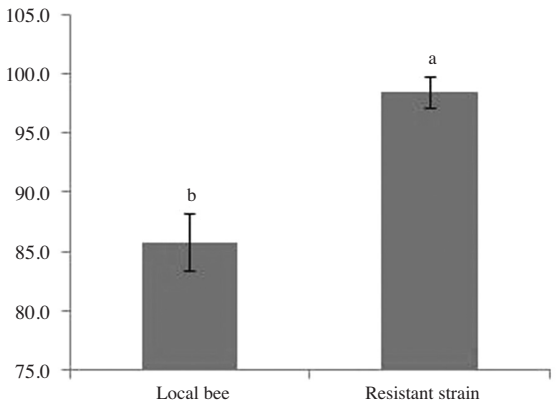

Fig. 5. The hygienic behavior observed (%) from cleaning pupae killed in the nest by pupae pin-killed method: After 12 hours shown the ability of hygienic behavior.

general bee colony.

Pinching 100 cells of systems resisting against virus and normal bee colonies with pin, pupa was made to die and compared with cleaning ability in comb cell. As a result, resisting systems ended up cleaning 93% of dead pupa after 12 hours, but non-resisting systems only cleaned 85.7% of comb cells (Fig. 5). Therefore, Sacbrood virus resisting systems (RH) turned out to have superior ability for removing dead larva and pupa as a source of contamination of disease in the bee colony compared to normal bee colony. Therefore, they have an effective feature for preventing the diffusion of disease through the removal of larva in case of infection of Sacbrood virus in the beginning.

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