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Production of Knockout Mutants by CRISPR/Cas9 in the European Honeybee, *Apis mellifera* L.

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The European honeybee (*Apis mellifera* L.) is used as a model organism in studies of the molecular and neural mechanisms underlying social behaviors and/or advanced brain functions. The entire honeybee genome has been sequenced, which has further advanced molecular biologic studies of the honeybee. Functions of genes of interest, however, remain largely to be elucidated in the honeybee due to the lack of effective reverse genetic methods. Moreover, genetically modified honeybees must be maintained under restricted laboratory conditions due to legal restrictions, further complicating the application of reverse genetics to this species. Here we applied CRISPR/Cas9 to the honeybee to develop an effective reverse genetic method. We targeted *major royal jelly protein 1* (*mrjp1*) for genome editing, because this gene is predominantly expressed in adult workers and its mutation is not expected to affect normal development. By injecting sgRNA and Cas9 mRNA into 57 fertilized embryos collected within 3 h after oviposition, we successfully created six queens, one of which produced genome-edited male offspring. Of the 161 males produced, genotyping demonstrated that the genome was edited in 20 males. All of the processes necessary for producing these genome-edited queens and males were performed in the laboratory. Therefore, we developed essential techniques to create knockout honeybees by CRISPR/Cas9. Our findings also suggested that *mrjp1* is dispensable for normal male development, at least till the pupal stage. This new technology could pave the way for future functional analyses of candidate genes involved in honeybee social behaviors.

Key words: honeybee, genome editing, CRISPR/Cas9, knockout, *mrjp1*, apiology

INTRODUCTION

Genome editing has recently developed as the standard method for gene manipulation. One genome-editing tool is the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR/Cas9), first discovered as an adaptive immune system in Bacteria and Archaea (Ishino et al., 1987; Jansen et al., 2002). Because of the potential of the CRISPR/Cas9 system to target almost arbitrary sites on the genome with high mutation rates, as well as the ease with which constructs can be made and applicability for knock-in by homologous recombination, CRISPR/Cas9 has already been applied to a variety of organisms (Bassett et al., 2013; Awata et al., 2015; Markert et al., 2016), and its application to animal species with insufficient effective reverse genetic methods has been highly anticipated.

One such species is the European honeybee *Apis mellifera* L. In addition to being an important pollinator in agriculture, the honeybee is a model animal for studies of the molecular and neural bases underlying social behaviors (Kamikouchi et al., 1998; Rybak et al., 1998; Menzel and Giurfa, 2001; Takeuchi et al., 2001, 2002; Kucharski et al., 2002; Kiya et al., 2007; Sen Sarma et al., 2009; Kaneko et al., 2013). The honeybee is a social insect and colony members exhibit various complex social behaviors. Females differentiate into two castes, and the queens (reproductive caste) are engaged in reproduction, whereas workers (non-reproductive caste) are engaged in various tasks to maintain colony activities (Winston, 1987; Seeley, 1995). Workers' tasks change depending on the workers' age after eclosion, a process known as age-polyethism: while younger workers (nurse bees, 6–12 days after eclosion) clean the nest and care for the brood by secreting royal jelly, older workers forage for nectar and pollen outside the hive (foragers, older than 10 days old) (Winston, 1987; Seeley, 1995). In addition, foragers that return to their hive communicate information regarding the distance and direction of a food source to their nestmates using dance communication (Frisch et al., 1967; Winston, 1987; Seeley, 1995), which makes the honeybee an excellent model animal for studying both social behaviors and advanced brain functions (Rybak et al., 1998; Srinivasan et al., 2000; Esch et al., 2001; Giurfa et al., 2001;

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Menzel and Giurfa, 2001; Kiya et al., 2007). The entire honeybee genome has been sequenced and recently updated, which has sparked molecular biologic studies of the honeybee (Honeybee Genome Sequencing Consortium, 2006; Elsik et al., 2014).

Many studies have aimed to elucidate the molecular and neural mechanisms underlying honeybee social behaviors. Extensive transcriptome analyses to search for candidate genes involved in honeybee social behaviors led to the identification of many genes expressed in a brain area-preferential and/or a task-dependent manner (Kamikouchi et al., 1998; Takeuchi et al., 2001, 2002; Kucharski et al., 2002; Sen Sarma et al., 2009; Kaneko et al., 2013; for Review, see Kubo, 2012). In addition, the genomes of 10 bee species have been compared and genes expanded in complex eusocial species that likely contribute to enable their complex social behaviors have been identified (Kapheim et al., 2015). Due to the lack of effective and easily applicable reverse genetic methods other than RNA interference (RNAi) (Amdam et al., 2003; Hasselmann et al., 2008; Démares et al., 2014), functional analyses of many of these genes have not been performed in the honeybee. The relationships and causations between the candidate genes and honeybee social behaviors remain largely unknown.

Several RNAi experiments have been performed to downregulate genes during embryogenesis or in adult organs, such as brains and fat bodies (Amdam et al., 2003; Hasselmann et al., 2008; Démares et al., 2014). The knockdown efficiency is not always sufficient, however, and thus RNAi may not be suitable for functional analysis of candidate genes related to honeybee social behaviors in general. Robinson et al. (2000) previously reported application of a sperm-mediated transformation method in the honeybee, and more recently, Schulte et al. (2014) reported the first generation of genetically engineered male honeybees (drones) using the transposon *piggyBac*. However, while transgenesis based on the random integration of a transposon into the genome is generally effective to knock in exogenous genes, it is difficult to apply this technique to knockout target genes (Thibault et al., 2004; Xu, 2015). For these reasons, methods to knockout specific gene(s) in the honeybee have been desired.

Here, we aimed to develop an effective gene knockout method in the honeybee and focused on the CRISPR/Cas9 method. One major problem in generating genome-edited honeybees is that in some countries, including Japan, bees must be maintained under restricted laboratory conditions for legal reasons. In addition, many queens must be generated and maintained in the laboratory to obtain queens whose genomes are edited, and therefore a genome-editing method with high efficiency is required. We investigated whether this method could be effectively applied to honeybees.

To elucidate gene functions in adult honeybees using CRISPR/Cas9, target genes must have no essential functions during embryonic, larval, or pupal development. Genome editing of genes involved in development could be lethal prior to adult eclosion, and thus functional studies of these genes cannot be explored in adult honeybees. We focused on the genes for major royal jelly proteins (MRJPs), which are synthesized and secreted as a major component of the royal jelly in the hypopharyngeal gland of nurse bees (Kubo et al., 1996; Ohashi et al., 1997; Schmitzová et al., 1998). As the expression levels of these genes during development are much lower than in adult worker bees (Drapeau et al., 2006), we consider it likely that they are dispensable for development.

In the present study, we produced genome-edited male offspring, in which mutation was occurred in the target position of genome, from a genome-edited queen. We expect that our study will provide essential techniques for future functional analyses of candidate genes involved in honeybee social behaviors.

MATERIALS AND METHODS

Honeybees

European honeybee (*Apis mellifera* L.) colonies were maintained in an apiary at Tamagawa University in Machida, Japan. To maintain colonies in the laboratory, a mosquito net [2.5 m (width) × 2.5 m (depth) × 1.9 m (height)] was set in an insectary with room temperature and relative humidity controlled to ~25°C and ~60%, respectively, under long-day conditions of 16 h light and 8 h dark (hereafter 'flight room'). Queenless colonies were prepared by removing the queen from the colonies and transferred into the flight room one day before introducing larvae and queens from injected embryos. One and two small colonies, each containing three or four comb plates, were used for larval rearing and queen oviposition.

Preparation of single-guide RNA and Cas9 mRNA

Based on the gene sequence for *mrjp1* (GB14888/NC_007080.3) obtained from the NCBI database, we surveyed appropriate genome-editing target sites (N_{20}NGG) which did not have any 'off-targets' in the honeybee genome. We identified a 20-base sequence for a single-guide RNA (sgRNA) target site (+55 to +74; RefSeq. NM_001011579.1) that was followed by an NGG sequence as a protospacer adjacent motif (PAM). Oligonucleotides, taggN_{20} and aaacN'_{20} (N'_{20} is complementary to N_{20}), were synthesized (Fasmac Co., Ltd., Kanagawa, Japan), annealed, and integrated into an in vitro transcription vector pDR274 (Addgene) treated with *Bsa*I-HF (New England Biolabs) using Ligation high ver.2 (Toyobo). *Escherichia coli* DH5α transformed with the resulting plasmid was cultured, and the plasmid was extracted using FastGene Plasmid Mini (Nippon Genetics). The plasmid was treated with *Dra*I (Takara), purified by phenol/chloroform extraction and ethanol precipitation, and the sgRNA was transcribed in vitro using an AmpliScribe T7-Flash Transcription Kit (Epicentre) and the plasmid as the template. Transcripts were purified by phenol/chloroform extraction and ethanol precipitation.

The Cas9 expression vector, pXT7-hCas9 (China Zebrafish Resource Center; Chang et al., 2013), was treated with *Xba*I (Takara), purified by phenol/chloroform extraction and ethanol precipitation, and capped transcribed and poly-adenylated using an mMESSAGE mMACHINE T7 Ultra Transcription kit (Thermo Fisher Scientific). Transcripts were purified using an RNeasy Mini kit (Qiagen). sgRNA and Cas9 mRNA were electrophoresed on 2% agarose gels under denaturing conditions and major bands corresponding to the predicted length were confirmed. RNAs were diluted at 500 ng/μl (sgRNA) and 1.25 μg/μl (Cas9 mRNA) with diethyl pyrocarbonate (Nacalai Tesque, Inc)-treated MilliQ water, and then stored at –80°C until use.

Injection

Injection was performed essentially as described previously (Schulte et al., 2014), with some modifications. Newly laid eggs younger than 3 h after oviposition were collected using a plastic comb box purchased from Kumagaya Honeybee Farm (Saitama,

Japan). This comb box contains many small cells that can be detached from the bottom, which allows for the eggs laid onto the cells to be collected individually. Queens were confined in the boxes one day before injection to familiarize them with the boxes, because they might not lay eggs onto clean plastic cells. In the morning of the injection day, eggs laid during the previous night were removed, and the queens were again confined in the same cages for 3 h. Each cell containing an egg was then detached and set onto oil-based clay in a Petri dish to facilitate the injection. sgRNA and Cas9 mRNA were diluted to 50 ng/μl and 1 μg/μl, respectively, with injection buffer (10 mM HEPES, pH 6.7, containing 130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 25 mM glucose, and 0.16 M sucrose) (Fiala et al., 1999), and loaded into a glass capillary tube (Drummond Precision Calibrated Micropipettes, 10 μl) with its tip pulled and polished to a 35° angle and an outer diameter of ~5 μm. Injection was conducted under a stereoscopic microscope (Olympus SZX10) using an injector (Eppendorf Femtojet). The injection time was 0.2 s, the injection pressure was 700 hPa, and the balance pressure was 50 hPa. We injected constructs into the dorsal posterior side of the eggs around the site where germ cells are generated to increase the likelihood of genome editing in the offspring. Injected embryos were incubated at 34°C in a chamber with saturated $CuSO_4$ solution (relative humidity 98%). To obtain an adequate number of hatched larvae, we repeated the same manipulation for five successive days (days 1–5).

Production of mosaic queens and genome-edited drones

An overview of the experimental procedures to create mosaic queens and genome-edited drones is shown in Supplementary Figure S1 and Supplementary Table S1 online. Each hatched larva (~3 days after oviposition) was transferred onto a drop of artificial food (75% royal jelly, 6.4% glucose/fructose, 0.5% yeast extract, diluted in distilled water) in a Petri dish and maintained at 34°C and relative humidity 98% (days 4–8). This artificial food was modified from Kaftanoglu et al. (2011), in which artificial food was used for *in vitro* rearing of honeybee larvae into queens. Larvae were transferred onto fresh artificial food every day and introduced into the queenless colony in the flight room on the hatching day of embryos injected on day 5 (day 8). For introduction of hatched larvae to the queenless colony, wild-type larvae less than three days old were grafted onto a small amount of artificial food in plastic queen cells and introduced into the queenless colony one day before the hatched larvae were introduced (day 7). The next day, the wild-type larvae in queen cells containing royal jelly supplied by nurse bees were replaced with hatched larvae (day 8). After eight days, capped queen cells were removed and incubated at 34°C until the queens emerged (day 16). Emerged queens were kept with eight workers in an incubator at 34°C.

Queens were anesthetized with CO_2 for 10 min at six and seven days after eclosion. This treatment induced virgin queens to lay unfertilized eggs (Mackensen and Roberts, 1948). We introduced these queens into queenless colonies set in the flight room after transiently anesthetizing all the workers in the hive with CO_2. Workers exhibited more acceptance toward exogenous queens after CO_2 treatment. A queen that was excluded by the workers of a queenless colony was replaced with another queen.

Genotyping analysis

Drone larvae and pupae were sampled from potentially mosaic queen-right colonies set in the flight room, and stored at –80°C until use. Genomic DNA was extracted from drones by the methods previously described by Issa et al. (2013). Pieces of frozen drone larval or pupal bodies excised with fine forceps were placed into the lysis buffer (5 mM Tris-HCl, pH 8.5, containing 100 mM KCl, 2.5 mM $MgCl_2$, 1% (v/v) NP40, 0.4 M sucrose, and 200 μg/ml Proteinase K) and treated at 66°C for 2 h, followed by 92°C for 10 min. Polymerase chain reactions were performed to amplify the genomic region around the sgRNA target site using Ex *Taq* DNA polymerase (Takara) and gene-specific primers for *mrjp1* (ATATTCCATTGCTTCGTTACTCG and TGGATATGAAGAATTTTGGACAAG).

Polymerase chain reaction products derived from the drone genomic DNA were treated with Exonuclease I (Wako) and Shrimp Alkaline Phosphatase (Wako), and the sequences were confirmed by DNA sequencing (Fasmac Co., Ltd., Kanagawa, Japan). DNA sequences were aligned to the reference *mrjp1* sequence obtained from NCBI using alignment software A plasmid Editor (ApE) and the genotypes were confirmed.

RESULTS

Designing the sgRNA target site

We targeted *mrjp1* for genome editing, because MRJP1 is synthesized predominantly in the hypopharyngeal gland of nurse bees (Kubo et al., 1996; Ohashi et al., 1997; Schmitzová et al., 1998) and is thought to have a nonessential role during embryonic, larval, and pupal development. To produce a null mutant of the target gene by CRISPR/Cas9, insertion or deletion (indel), which is caused by Cas9 digestion followed by non-homologous end-joining, must be introduced at an appropriate position in the coding region (CDS) of the target gene to result in a frameshift mutation. Therefore, we determined a genome-editing target site as far upstream within the CDS of *mrjp1* as possible. In addition, because off-target cleavage frequently occurs at other genomic regions to which PAM and the adjacent ~12 bp of the sgRNA target site (seed region) are identical, whereas even a single base mismatch within the sequence decreases the rate of off-target cleavage (Cong et al., 2013; Cho et al., 2014), we excluded candidate sgRNA target sites, including seed regions, that were identical to other genomic regions, and designed an sgRNA target site on the second exon (+55 to +74 of GB14888/NC_007080.3), which contains the initiation codon (Fig. 1).

Production of mosaic queens and genome-edited drones

In the honeybee, fertilized eggs develop into females (queens or workers), whereas unfertilized eggs develop into drones (Winston, 1987). To produce genome-edited honeybees under restricted laboratory conditions, we designed the experimental procedure shown in Fig. 2. First, 'mosaic queens', which have genome-edited germline cells in mosaic, are produced by injecting sgRNA and Cas9 mRNA into fertilized embryos. Transiently treating the queens with CO_2, forces them to lay unfertilized eggs that grow into drones. By analyzing the genotypes of the emerged drones, we can confirm that the genomes of the germline cells of the queen were successfully edited, and mutant drones were created. In the present study, we tried to produce genome-edited drones, because the subsequent procedure after producing genome-edited drones only requires artificial insemination, which is a conventional beekeeping technique. A previous study also reported production of transgenic drones, but not queens or workers, that possess reporter genes in their genomes (Schulte et al., 2014).

An overview of the experimental procedures used in this study is shown in Supplementary Figure S1 and Supplementary Table S1 online. To produce genome-edited honeybees, sgRNA and Cas9 mRNA were injected into 57 fertilized embryos collected from hives within 3 h after ovi-

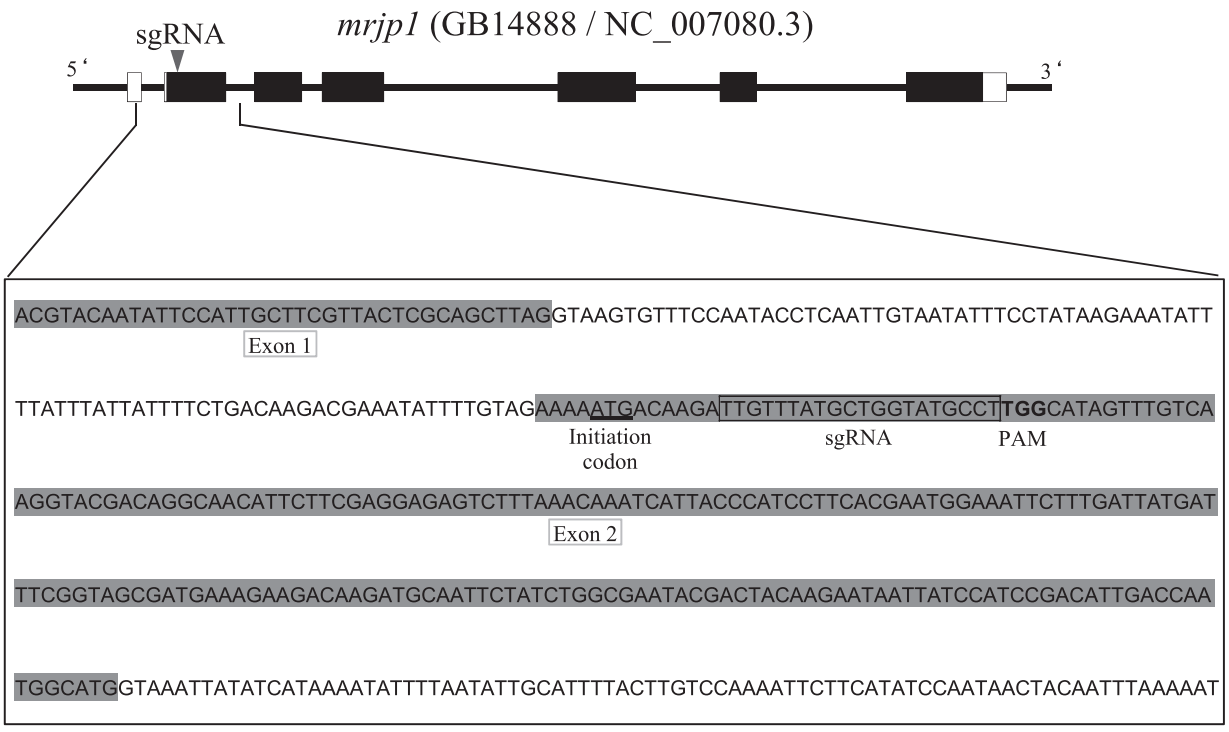

Fig. 1. Position and design of sgRNA target site in *mrjp1* (GB14888 / NC_007080.3). Schematic diagram of the gene structure of *mrjp1* is shown in the upper part. Bars, white boxes, black boxes, and arrowhead indicate introns, UTRs, CDSs, and the position of the sgRNA target site, respectively. Sequences around the sgRNA target site are shown below the gene structure. Letters in gray boxes, underlined, in the black box, and bolded indicate exons, the initiation codon, sgRNA target site, and PAM, respectively.

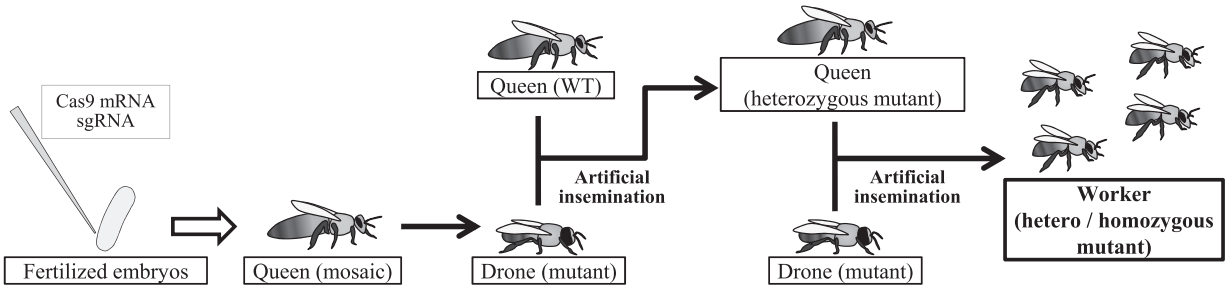

Fig. 2. Processes to create genome-edited honeybee workers. Overview of experimental procedures to create genome-edited honeybee workers by CRISPR/Cas9 is indicated. Queens whose germline cells are genome-edited [Queen (mosaic)] are produced by injecting sgRNA and Cas9 mRNA into fertilized embryos and rearing these embryos into queens. The queens are then induced to lay unfertilized eggs, which grow into drones, by transiently anesthetizing them with CO_2. Genome-edited drones [Drone (mutant)] are selected from among drones produced by these mosaic queens, and sperm is collected from the genome-edited drones. A heterozygous queen is produced from a wild-type queen [Queen (WT)] artificially inseminated with sperm from the genome-edited drones. Heterozygous and homozygous mutant workers [Worker (heterozygous/homozygous mutant)] are produced from the heterozygous queen [Queen (heterozygous mutant)], which is again artificially inseminated with sperm from the genome-edited drones.

position. We injected constructs into the dorsal posterior region of the embryos, where the germ line cells differentiate at a later embryonic stage (Schulte et al., 2014). Among the 23 larvae that hatched (40.4%), 14 were alive and introduced into the queenless colony in the flight room to induce their differentiation into queens (Table 1). Nine larvae died, however, possibly due to damage resulting from manipulation and/or the lack of food soon after hatching, as the timing of hatching differed among individuals (66–93 h after oviposition) (Collins, 2004). After eight days, we retrieved eight capped queen cells from the colony in the flight room and incubated them in an incubator at 34°C. Six queens emerged from these queen cells (42.9% of grafted larvae, Table 1) and were designated Nos. 1–6, according to the order they emerged. We anesthetized these queens with CO_2 for 10 min, on both days 6 and 7 after eclosion, and introduced them to queenless colonies in the flight room after transiently anesthetizing all colony members with CO_2 to reduce their aggressiveness to queens.

Two queens (Nos. 2 and 6) laid unfertilized eggs. Drone

larvae, pupae, and adults hatched from these eggs were sampled and their genomic DNAs were extracted. Genotyping analyses revealed that 20 of 161 offspring derived from one queen (No. 2) had indels around their sgRNA target site, whereas no indels were detected in the offspring of the other queen (No. 6), suggesting that this queen had no genome-edited germline cells (Table 2). We therefore obtained one mosaic queen who produced genome-edited drones of the six queens that emerged from larvae injected with the genome-edited constructs. The proportion of genome-edited germline cells in the mosaic queen ovary was estimated to be 12.4% (Table 2).

Another two queens were attacked by workers when they were introduced into queenless colonies set in the flight room (Nos. 1 and 5), and one queen was discarded a few days after its introduction to the colony (No. 3), possibly because these queens were not functionally mature despite exhibiting a queen-like appearance. Queen No. 4 died in the incubator before its introduction into a queenless colony. The ovaries of this queen, however, were not well developed, indicating that queen No. 4 may also not have been functionally mature.

Confirmation of null mutations in the genome-edited drones

To knockout *mrjp1*, indels occurring in the offspring drones must result in frameshift mutations. Therefore, we checked the pattern of indels detected in the genome-edited drones (Fig. 3 and Supplementary Figure S2 online). We found four types of simple deletions (Types A to D in Fig. 3), five types of mutations with both deletions and insertions (Types E to I), and one base substitution type (Type J). Five types of indels detected in seven genome-edited drones resulted in frameshift mutations (Types B, C, D, H, I in Fig. 3). Two base substitutions that resulted in neither deletion nor insertion at the sgRNA site were detected in five genome-edited drones (Type J in Fig. 3). The two base substitutions, however, resulted in nonsense mutation, in which a triplet codon was changed from UGC to UAG, corresponding to an amino acid substitution from cysteine to a stop codon. Offspring with these substitutions were therefore considered to be null mutants. The remaining four types of indels in eight genome-edited drones did not result in frameshift mutations. Some of the genome-edited drones exhibited the same indel patterns (Types A, C, G, J in Fig. 3), indicating that they derived from the same germline stem cell. Double-strand breaks induced by Cas9 nuclease mainly occur between the third and fourth nucleotides upstream of the PAM sequence (Garneau et al., 2010; Bassett et al., 2013), and this was the case for indels detected in our experiment, as all indels included mutations at this putative cleavage site (Fig. 3, black arrow). On the other hand, we detected a base substitution in the sixth base from the 5′ side of the sgRNA target site. This substitution, which was synonymous (phenylalanine), was detected in 96 of 161 offspring of queen No. 2. Due to its extremely high detection rate, we concluded that the substitution was due to a single nucleotide polymorphism and not to genome editing.

Table 1. Summary of the process to produce mosaic queens. The rates of hatched larvae, emerged queens, and queens with genome-edited offspring denote the proportions of hatched larvae to injected embryos, emerged queens to larvae introduced into the queenless colony, and queens with genome-edited offspring to queens with offspring, respectively.

Number of injected embryos	Hatched larvae	Number of larvae intriduced into colony	Emerged queens	Queens with offspring	Queens with genome edited offspring
57	23 (40.4%)	14	6 (42.9%)	2	1 (50.0%)

Table 2. Numbers and ratios of genome-edited offspring produced from two queens that laid eggs. The rate of genome-edited offspring denotes the proportions of genome-edited offspring to all offspring produced by each queen.

Name of queen	Number of offspring	Genome edited offspring
No.2	161	20 (12.4%)
No.6	67	0 (0.0%)

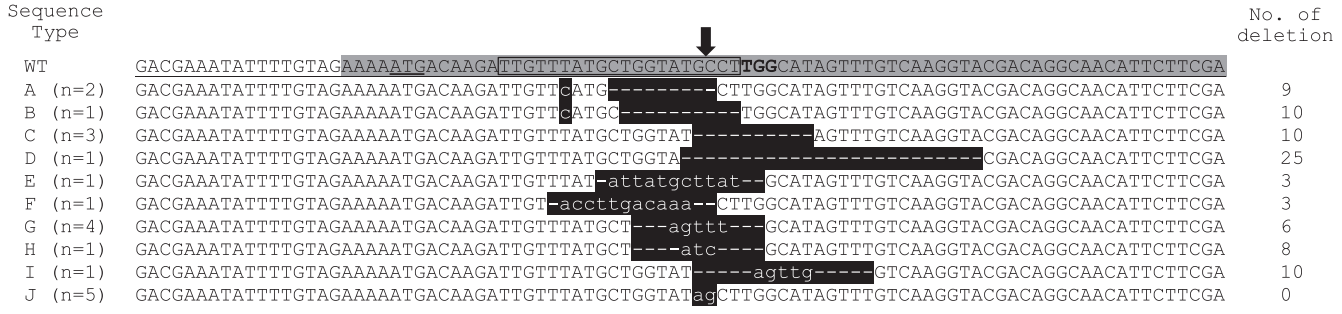

Fig. 3. Variety of indels detected by genotyping analysis of genome-edited drones. The patterns of indels detected in genome-edited drones produced from a mosaic queen (No. 2) are indicated. The sequence shown at the top is the reference sequence (NCBI), which is the wild-type sequence. Letters in gray boxes, underlined, in blank boxes with a black frame, and bolded indicate the second exon of *mrjp1*, initiation codon, sgRNA target site, and PAM, respectively. Black arrow indicates the nucleotide positions where double-strand breaks by Cas9 nuclease are thought to occur. White letters and dashes in the black boxes indicate inserted and deleted nucleotide sequences, respectively. Sequence type and numbers of drones from which each sequence type was detected are shown on the left. The numbers of nucleotide deletions that differed between the genome-edited drones and wild-type (WT) sequences are shown on the right.

DISCUSSION

The present study reports successful genome editing of embryo germline cells to produce genome-edited offspring by application of CRISPR/Cas9 to the honeybee. We produced six queens by injecting sgRNA and Cas9 mRNA into 57 fertilized embryos, and two queens laid eggs under the restricted laboratory conditions (Table 1). One of the two queens produced genome-edited drones, indicating that the germline cells of this queen were genome-edited. Of the 161 drones produced by this queen, 20 were genome-edited (Table 2), an efficiency (12.4%) comparable to that (4–43%) of a previous study using the transposon *piggyBac* (Schulte et al., 2014). Our study makes an important contribution to the development of essential techniques necessary to create knockout honeybees using the CRISPR/Cas9 method. Further studies are needed to confirm the genome-editing efficiency of this method using the same or another sgRNA, however, as our results were obtained from only a limited number of queens and a single sgRNA.

Subsequent procedures after producing genome-edited drones are as follows (Fig. 2). Artificial insemination was used to induce wild-type virgin queens to lay eggs, which were fertilized by sperm from the genome-edited drones. The resulting offspring queens were *mrjp1* heterozygotes. Artificial insemination was then used again to induce the heterozygote queens to lay eggs fertilized by the genome-edited sperm. The resulting offspring workers were either homozygote or heterozygote *mrjp1* mutants. As the beekeeping techniques necessary for these procedures are well established (Büchler et al., 2013; Cobey et al., 2013), we are confident that the remaining processes to create knockout honeybee workers can be accomplished. In the present study, when we collected the genome-edited drones from two hives in the flight room, all of them had been discarded from the hives (Supplementary Table S1 online), possibly evicted by workers for seasonal reasons (drones were produced in December 2015, in the flight room) and/or due to colony conditions. An adequate supply of worker larvae from outside colonies is indispensable for maintaining colony activity, in which mosaic queens lay unfertilized eggs that developed into drones. Seasonal effects, however, complicated this step in our experiment. In Japan, only small numbers of larvae and pupae are maintained in outside hives from autumn to winter. Therefore, to produce an adequate number of sexually mature genome-edited drones, we needed to conduct all of the experimental procedures from spring to early summer.

We confirmed that the genome of all 20 drones, not only live larvae ~3 days of age, but also the dead adults, were edited (Fig. 3 and Supplementary Figure S2 online). The adults appeared to be normal, although we did not perform any behavioral or histochemical analysis of these drones. Nonetheless, our findings strongly suggest that *mrjp1* is dispensable for normal drone development, at least until the pupal stage. MRJP1, also known as royalactin, is a major component of royal jelly responsible for inducing queen differentiation in female larvae at an early stage (Kamakura, 2011). On the other hand, *mrjp1* is also expressed in worker brains (Kucharski et al., 1998; Hojo et al., 2009; Peixoto et al., 2009), suggesting its pleiotropic functions in larvae and adults. To our knowledge, this is the first report of knockout of a specific gene in honeybees, even in drones. Functional analysis of genes in adult honeybees using the CRISPR/Cas9 method requires that the target genes have no function during normal development to avoid embryonic lethality. There are nine genes homologous to *mrjps* in the honeybee that are expressed in different organ-preferential manners, suggesting that each homolog has distinct functions (Klaudiny et al., 1994; Ohashi et al., 1997; Drapeau et al., 2006; Buttstedt et al., 2013). The MRJP family is especially expanded in the genus *Apis* (Kapheim et al., 2015), but the function of this family of proteins in adults is largely unknown. Among them, MRJPs 1–3 are expressed preferentially in the hypopharyngeal gland of nurse bees as food for larvae, drones, and queens (Kubo et al., 1996; Schmitzová et al., 1998; Furusawa et al., 2008). Whether MRJPs 1–3 are involved in the functional physiology and/or behavior of the worker honeybees, however, is unknown; e.g., do nurse bees lacking *mrjp1* expression still engage in nursing, or do they skip this task and become prematurely engaged in foraging? To address this question, it is necessary to conduct further extended experimental procedures to produce genome-edited workers (Fig. 2) during the summer season.

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