Targeted gene disruption by use of CRISPR/Cas9 ribonucleoprotein complexes in the water flea *Daphnia pulex*

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Short title: Cas9 RNP-induced mutagenesis in *D. pulex*

Key words: Branchiopoda, *Daphnia pulex*, *Distal-less*, CRISPR/Cas9, Cas9 RNPs, gene disruption, knockout, targeted mutagenesis, gene manipulation, genome editing

ABSTRACT—

The microcrustacean *Daphnia pulex* is an important model for environmental, ecological, evolutionary, and developmental genomics because its adaptive life history displays plasticity in response to environmental changes. Even though the whole genome sequence is available and omics data has actively accumulated for this species, the available tools for analyzing gene function have thus far been limited to RNAi (RNA interference) and TALEN (the transcription activator-like effector nuclease) systems. The development of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) system is thus expected to further increase the genetic tractability of *D. pulex* and to advance the understanding of this species. In this study, we developed a genome editing system for *D. pulex* using CRISPR/Cas9 ribonucleoprotein complexes (Cas9 RNPs). We first assembled a CRISPR single-guide RNA (sgRNA) specific to the *Distal-less* gene (*Dll*), which encodes a homeodomain transcription factor essential for distal limb development in invertebrates and vertebrates. Then, we injected Cas9 RNPs into eggs and evaluated its activity *in vivo* by a T7 endonuclease I assay. Injected embryos showed defective formation of the second antenna and disordered development of appendages, and indel mutations were detected in *Dll* loci, indicating that this technique successfully knocked out the target gene.

INTRODUCTION

The water flea *Daphnia pulex* has been studied for centuries because of its importance in aquatic food chains (Dodson & Hanazato, 1995), as an environmental indicator organism for aquatic toxicology (Iguchi et al., 2007), and for its adaptive responses to environmental changes (Tollrian & Harvell, 1999). These characteristics make it a significant model for environmental, ecological, evolutionary, and developmental genomics research. The complete genome of *D. pulex* sequenced in 2011 (Colbourne et al., 2011) opened a door to challenging research in these fields, and recent omics analyses have profiled a large number of putative factors involved in several characteristics of its unique life history (cf. Dircksen et al., 2011; Spanier et al., 2017). The recent establishment of two gene manipulation techniques in this species, RNA interference (RNAi) (Hiruta et al., 2013) and transcription activator-like effector nuclease (TALEN) (Hiruta et al., 2014), enable researchers to use gene silencing and disruption to characterize the function of genes in *D. pulex*. However, the development of another major genome editing tool, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) system, has been eagerly anticipated. This is because the two major genome editing tools, TALEN and CRISPR/Cas9, have different characteristics including the ease of design and construction, source mechanism of DNA binding, and mechanism of inducing DNA damage, making their application complementary (Sakuma & Woltjen, 2014).

CRISPR are DNA sequences that function as an acquired immunity system in bacteria and archaea, and thus essential components of a microbial nuclease system involved in immune defense against invading phages and plasmids (Wiedenheft et al., 2012). Cas9, the Cas derived from *Streptococcus pyogenes*, forms an active nuclease referred to as Cas9 ribonucleoprotein complexes (Cas9 RNPs) when complexed with transactivating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) which are transcribed from the CRISPR sequence (Jinek et al., 2012). These RNA-guided endonucleases (RGENs) enable targeted genome engineering in diverse organisms (reviewed in Doudna & Charpentier, 2014; Zhang et al., 2014; Sternberg & Doudna, 2015). The basic mechanism is as follows: Cas9 nuclease is generally guided by a chimeric single-guide RNA (sgRNA), a hybrid of tracrRNA and crRNA, and binds to a targeted genomic sequence next to the protospacer adjacent motif (PAM). Binding specificity of RGENs is determined by both sgRNA-DNA base pairing and the PAM sequence (5′-NGG). Cas9 RNPs then induce DNA double-strand breaks (DSBs) that can be repaired by the error-prone non-homologous end joining (NHEJ) pathways to cause insertion and/or deletion mutations at targeted genomic loci. CRISPR/Cas9-mediated gene targeting has been applied to a large number of vertebrates and invertebrates (reviewed in Sakuma & Woltjen, 2014; Wang et al., 2016), since the first application of CRISPR/Cas9 in mammalian cells was reported at the beginning of 2013 (Cong et al., 2013; Mali et al., 2013). There are three major types of methods to introduce CRISPR/Cas9 based on the properties of the

material: 1) introduction of Cas9 and sgRNA plasmid DNA as a “vector”, 2) introduction of Cas9 mRNA and *in vitro* transcribed (IVT) sgRNA as “RNA”, 3) introduction of Cas9 protein and IVT sgRNA as “Cas9 RNPs”. In the third method, since CRISPR reagents are introduced as a complex of Cas9 protein and IVT sgRNA into cells, they are expected to immediately migrate to the nucleus and cleave the target sequence. There is also no need to consider the promoter and codon usage, and cleavage activity is high (Yu et al., 2016). In addition, the risk of nonspecific insertion is the lowest among these methods because neither DNA nor Cas9 mRNA is used (Kim et al., 2014; Liang et al., 2015). Ever since delivery of Cas9 RNPs was first reported in the nematode *Caenorhabditis elegans* (Cho et al., 2013), it has been used as a rapid and highly efficient tool for the analysis of gene function in many organisms such as zebrafish *Danio rerio* (Sung et al., 2014), tropical clawed frog *Xenopus tropicalis* (Shigeta et al., 2016), fruit-fly *Drosophila melanogaster* (Lee et al., 2014) and mouse *Mus musculus* (Sung et al., 2014). In this study, we chose Cas9 RNPs, the third method as described above, to edit the *D. pulex* genome. We expected that the CRISPR/Cas9 system would work successfully in *D. pulex* because it worked in a congener, *D. magna* (Nakanishi et al., 2014; Kumagai et al., 2017).

The homeobox gene *Distal-less* (*Dll*) and its homologs, *Dlx* genes, are well known for their evolutionary conserved role in distal limb development throughout the animal kingdom (Panganiban & Rubenstein, 2002). According to previous studies on arthropods (cf. Beermann

et al., 2001; Liubicich et al., 2009), reduction of Dll activity caused defects of distal appendage segments in daphnid species (Kato et al., 2011; Hiruta et al., 2013, 2014). Due to its easily recognizable phenotype, the *Dll* gene has been used as target gene to establish gene manipulation techniques in *D. pulex* (Hiruta et al., 2013, 2014). In addition, the results of *Dll* RNAi and *Dll* TALEN in *D. pulex* (Hiruta et al., 2013, 2014) provide possible comparative data regarding its phenotype. It is for these reasons that we selected this endogenous developmental gene as a target for proof-of-principle CRISPR/Cas9 in *D. pulex*.

The goal of this study was to develop a targeted gene disruption system by CRISPR/Cas9-mediated artificial DSBs in *D. pulex*. A CRISPR/Cas9 target site was designed and assembled, and then Cas9 RNPs were microinjected into embryos to successfully induce insertion and/or deletion mutations.

RESULTS

Construction and evaluation of *Dll* CRISPR sgRNAs

We first designed three sgRNAs, *Dll*_T1, *Dll*_T2 and *Dll*_T3, in the first exon of the *Dll* gene, which was successfully disrupted by Platinum TALENs in our previous study (Hiruta et al., 2014) (Fig. 1). After constructing these sgRNAs, a solution containing 500 ng/μl of sgRNA and 600 ng/μl of Cas9 protein was injected into eggs just after ovulation, and the genome modification efficiencies *in vivo* were evaluated by a T7 endonuclease I

(T7EI) assay. We found that the signal intensity of homoduplexes in the CRISPR/Cas9-injected embryos was weaker than that in uninjected embryos (Fig. 2A), indicating that the CRISPR/Cas9-mediated indels were induced at the target locus. Furthermore, multiple fast-migrating bands (heteroduplexes), which were selectively digested at the mismatched site, were detected in *Dll*_T1- and *Dll*_T2-injected embryos, and a slightly separated band in *Dll*_T3-injected embryos (Fig. 2A, arrowheads).

Efficiency and effectiveness of Cas9 RNPs in inducing mutations

Results from CRISPR/Cas9 experiments are summarized in Table 1. Only Cas9- or sgRNA-injected embryos showed lower viability than uninjected embryos, suggesting damage caused by microinjection and/or toxicity of injection reagents. Compared to only Cas9- or sgRNA-injected embryos, the low viability of the embryos injected with *Dll* Cas9 RNPs may be partly attributed to the toxicity of Cas9 RNPs as well as the embryonic lethality of *Dll* knockout mutants. This was also supported from results which showed that a lower concentration of Cas9 RNPs increased embryo viability (Table 1 and Fig. 2B, C).

To investigate the dose dependence of CRISPR/Cas9-induced visible mutation, we injected varying amounts of Cas9 protein and *Dll*_T3 sgRNAs. We calculated the visible abnormality rate at each concentration from Table 1 by dividing the number of observed juveniles having shortened second antennae by the number of total juveniles. Serial dilutions

of Cas9 protein showed that 150 and 300 ng/μl Cas9 protein induced abnormality with significantly lower efficiency (1.45% and 6.98%, respectively) than 600 ng/μl (42.86%) (Fig. 2B). Similar results were obtained with sgRNAs, as shown by a significantly lower efficiency of abnormality with 125 and 250 ng/μl sgRNAs (2.94% and 11.43%, respectively) when compared to 500 ng/μl (42.86%) (Fig. 2C). We concluded that both Cas9 protein and sgRNAs induce visible abnormalities (shortened second antennae) in a dose-dependent manner.

Detection of mutations and their sequences

To investigate successful genome modifications of the F_0 founder line, the genomic region surrounding the CRISPR target was amplified and subcloned. We collected five first-instar juveniles having shortened second antennae and numbered them #1 to #5 for each *Dll* sgRNAs, and sequenced sixteen clones for each sample. Sequencing analysis revealed that CRISPR/Cas9 predominantly induced small indel mutations ranging in size from 1–19 bp (Fig. 3A), which is similar to results obtained in other arthropods (Lee et al., 2014; Sun et al., 2017). In *Dll*_T1-, *Dll*_T2- and *Dll*_T3-injected F_0 founders, three, two and nine types of mutant sequences were detected, respectively. All but one type of mutation caused a frame shift that generated a stop codon in the first or second exon, and subsequently a truncated protein that ranged from 83 to 157 amino acids without a homeodomain, which is a functional DNA-binding domain that is highly conserved among other arthropods. All the sequenced

clones had altered sequences except for three mutant lines (Fig. 3A; in *Dll*_T1_#5, *Dll*_T2_#4, and *Dll*_T2_#5, 57.14% [8 out of 14], 56.25% [9 out of 16], and 53.33% [8 out of 15] sequenced clones were mutated, respectively), indicating *Dll* sgRNAs induced mutations with high efficiencies.

To investigate whether these mutations were heritable, we then cloned and sequenced the target genomic region from F_1 progenies of *Dll*_T3-injected F_0 founders. The mutants with severe defects in the second antenna and appendage development failed to molt and died before becoming adults, and the DNA sequencing by using a part of live animal was impossible in *D. pulex* due to its small body size. We therefore randomly chose and raised eight different F_0 founder lines having a normal appearance and numbered them #1 to #8. Then we collected parthenogenetically produced F_1 progeny of each founder line, and sequenced sixteen clones for each sample; subsequently, DNA sequences of the F_0 founders were also determined. DNA sequencing revealed that 1) the wild type sequence and mutated sequence with a 3-nucleotide deletion were detected in the two out of eight F_1 lines (Fig. 3B, #1, 2; same mutation was found in corresponding F_0 founders [data not shown]), and 2) the only wild type sequence was found in the other F_0 founders and their progenies. The mutations we detected did not cause a frame shift and growth defects. In summary, F_1 progenies #1 and #2 had monoallelic mutation genotype and normal phenotype.

Phenotype of *Dll* CRISPR/Cas9 in *D. pulex*

All examined mutants of *Dll*_T1, *Dll*_T2, and *Dll*_T3 showed the same phenotype. Hence, we hereafter describe the phenotype of *Dll*_T3-injected F_0 founders as a representative of all mutants. Similar to the RNAi knockdown and TALEN knockout phenotype of *Dll* (Hiruta et al., 2013, 2014), *Dll*_T3-injected F_0 founders displayed various degrees of defects in second antennae, appendages, the ocellus, the abdominal claw, and abdominal setae, all tissues in which *Dll* is normally expressed (Figs. 4 and S1). In the second antennae, the degree of the segment truncation was variably detected, ranging from severe (peduncle alone, lacking dorsal and ventral rami) to mild (peduncle and deficient rami) (Fig. 4C, D). The first to fifth thoracic limbs, including each exopodite, were shortened (Figs. 4E, F and S1A–F). Moreover, loss of the ocellus and abdominal setae (Fig. S1G, H, and I, J, arrowhead), and a minimized abdominal claw (Fig. S1I, J, arrow), was observed. The mutants with severe defects in the second antenna and appendage development failed to molt, and died before becoming adults, whereas mutants with mild or no phenotypic defects were viable and fertile (data not shown). The lethality of *Dll* knockout mutants was consistent with previous studies on insects (Cohen & Jürgens, 1989; Beermann et al., 2001).

DISCUSSION

In this study, all the designed sgRNAs (Fig. 1) induced targeted somatic mutations

(Fig. 3A) that were quickly and easily evaluated from embryos 48 h after injection by a T7EI assay (Fig. 2A). Moreover, both the Cas9 protein and sgRNAs caused a dose-dependent increase in frequency of visible abnormality (Fig. 2B, C), suggesting Cas9 RNPs induce mutations in a dose-dependent manner. Mutation analysis revealed that indel mutations were induced at regions specific to the target sequences (Fig. 3A) and were inherited in the next generation (Fig. 3B). Common to both the RNAi knockdown and TALEN knockout phenotype of *Dll* (Hiruta et al., 2013, 2014), the phenotype of Cas9 RNPs-injected juveniles displayed various degrees of defects in tissues where *Dll* is normally expressed. Based on these results, we concluded that the CRISPR/Cas9 system via delivery of Cas9 RNPs worked in *D. pulex* to induce heritable mutations into the endogenous genes as was the case with TALEN (Hiruta et al., 2014). Selection of the most appropriate method, CRISPR/Cas9 presented in this study or platinum TALENs, should be based on the intended applications, but either may be used to widen and deepen gene functional analysis in *D. pulex*.

When CRISPR/Cas9 was introduced into mouse zygotes, the occurrence of somatic mosaicism depended on the timing of cell division (cleavage) after injection (Yen et al., 2014). In *D. pulex*, however, the probability of somatic mosaicism is considered to be low since cleavage proceeds without cytokinesis during and for a while after the injection period, and injected reagents can easily diffuse throughout the embryo (Hiruta et al., 2010, 2013), suggesting that mutations can be induced at very early developmental stages. In this study, we

detected only one out of fifteen F_0 mutant lines had three types of indel mutations (Fig. 3A, #1 in *Dll*_T3), indicating somatic mosaicism seemed to occur at low frequency as expected. In this regard, however, further study will be need to clarify the incidence of somatic mosaicism in *D. pulex* because it is uncertain as to whether or not ten F_0 mutant lines having two types of sequences (two distinct indel mutations or one wild type sequence and one indel mutation) had somatic mosaicism. It is also considered that mutant lines having a single germline mutation, and not a mosaic germline mutation, can be established depending on the type of gene. Indeed, our results demonstrated that F_1 progeny from the same F_0 founder had the same mutation genotype (Fig. 3B), indicating that the F_0 founder carried a single germline mutation. However, whether this result is a general tendency or not cannot be concluded until examining the case of other genes.

It is unclear how specific the CRISPR/Cas9 system is in *D. pulex*. Previous studies pointed out that non-specific mutagenesis (off-target effect) of CRISPR/Cas9 occurred at a high frequency in human cells (Fu et al., 2013; Pattanayak et al., 2013), but had a lower off-target effect in mice and zebrafish (Hruscha et al., 2013; Yang et al., 2013). It is difficult to predict the frequency and location of off-target effects of the CRISPR/Cas9 system due to the varying degrees of tolerance for mismatches depending on their position along the sgRNA-DNA interface (Hsu et al., 2013). Techniques to minimize the likelihood of off-target effects have improved in recent years (e.g. Double nicking method (Ran et al., 2013);

FokI-dCas9 system (Guilinger et al., 2014)) and the delivery of CRISPR/Cas9 via Cas9 RNPs is an advanced method. Almost all Cas9 protein is cleared from the cell 48 h after introduction of Cas9 RNPs in human cell lines, but continues to exist even 72 h after introduction of the Cas9 vector (Liang et al., 2015). A low frequency of off-target effects by using Cas9 RNPs was observed in human cells (Kim et al., 2014), indicating a minimized off-target risk posed by remnants and following expression of the Cas9 gene. When all evidence is considered, it is suggested that the resultant phenotype in this study is unlikely to have resulted from an off-target effect for the following reasons: 1) the results of all the designed sgRNAs were consistent with each other; 2) the resultant phenotype induced by CRISPR/Cas9 was consistent with that by RNAi and TALEN (Hiruta et al., 2013, 2014).

Daphnia pulex has only a 200-megabase genome but possesses as many as about 31,000 genes, 36% of which have no detectable homologs with other animal species. It is suspected that a large number of genes allow *D. pulex* to respond to its changing environment (Colbourne et al., 2011). So far, however, little is known about which genes are important for *D. pulex* to cope with environmental stresses. One reason may be that they have many tandem gene clusters in their genome (Mayer et al., 2010; Colbourne et al., 2011). To cite one example, RECQ2, which suppresses homologous recombination, is present in seven copies and might be expressed differently in sexual reproduction and parthenogenesis in response to environmental cues (Schurko et al., 2009). In order to analyze the function of each gene

within tandem clusters, the CRISPR/Cas9 system can be used to induce multiple gene knockouts by introducing multiple sgRNAs simultaneously as well as monitor the expression of each gene by knock-in fluorescent proteins such as GFP.

EXPERIMENTAL PROCEDURES

Daphnia strain and culture conditions

The *Daphnia pulex* strain [West Trenton (WTN6), collected in May 2006 by Sarah Schaak] was obtained from the Center for Genomics and Bioinformatics (Indiana University, USA). The strains were raised parthenogenetically in M4 culture medium (M4), which was prepared using MilliQ water (Elendt & Bias, 1990), at 18°C under artificial light conditions of 14 h light and 10 h dark to maintain reproduction. M4 was not changed for the first week, but was changed every four days after the second week when the strains matured. A 0.01-ml suspension of 10^9 cells/ml of *Chlorella vulgaris* was added every day to each culture (20–25 individuals/L).

CRISPR sgRNA design and assembly

The *Dll* sequence of *D. pulex* WTN6 strain used in this study was PCR amplified from cDNA fragments, and then cloned and sequenced, as described in a previous study (Hiruta et al., 2014). The first exon sequences of the *Dll* gene were scanned for potential CRISPR target

sites, which were identified using the Invitrogen GeneArt CRISPR Search and Design tool at https://www.thermofisher.com/crisprdesign. The top three recommended CRISPR target sequences and optimized sgRNA design were chosen and named as *Dll*_T1, *Dll*_T2, and *Dll*_T3, respectively (Fig. 1). The target oligonucleotide primers were designed for PCR assembly of the sgRNA DNA template (Table S1). The Invitrogen GeneArt Precision gRNA Synthesis Kit (A29377, Thermo Fisher Scientific, MA, Waltham, USA) was used to generate the full-length sgRNA DNA template according to the manufacturer's instructions.

Generation of sgRNA by *in vitro* transcription

The sgRNA DNA templates were transcribed *in vitro* using the TranscriptAid Enzyme Mix (Box 1 of the above kit) according to the manufacturer's protocol. The resultant sgRNAs were purified using the gRNA Clean Up Kit (Box 2 of the above kit) and finally resuspended in RNase-free water, and stored at –20°C until use.

Injection of Cas9 RNPs into embryos

Invitrogen GeneArt Platinum Cas9 Nuclease (B25641; Thermo Fisher Scientific) was used as Cas9 protein. The Cas9 protein and sgRNAs were diluted into injection buffer ($T_{10}E_{0.1}$; 10 mM TrisHCl, 0.1 mM EDTA, pH7.5) to a final concentration of 600 and 500 ng/μl, respectively, for microinjection just before use. When mixed with sgRNAs, the Cas9

protein forms a very stable ribonucleoprotein (RNP) complex. The Cas9 RNPs were injected into embryos as described previously (Hiruta et al., 2013). Briefly, embryos were collected from a brood chamber just after ovulation by *D. pulex* at least two weeks old and placed in ice-cold M4 medium containing 60 mM sucrose (M4-sucrose). The Cas9 RNPs were injected through a glass needle with a microinjector (FemtoJet 4i, Eppendorf, Hamburg, Germany) and a micromanipulator (MN-153, NARISHIGE, Tokyo, Japan). Finally, injected embryos were transferred into 2% agar in a 6-well plate with M4-sucrose and incubated at 18°C.

T7EI assay

To detect CRISPR/Cas9-induced indel mutations, a T7EI assay that can recognize heteroduplex DNAs and cleave DNA at single base pair mismatches was conducted. Twelve embryos were collected 48 h after injection with *Dll*_T1, *Dll*_T2 and *Dll*_T3. Genomic DNA was isolated from CRISPR/Cas9-injected and uninjected embryos using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Table S1 shows the primers used to amplify the region containing the *Dll* target site from genomic DNA for the T7EI assay and mutation analysis. PCR was performed using TaKaRa Ex Taq (TaKaRa Bio, Tokyo, Japan). Amplification conditions were: 98°C for 2 min; 45 cycles of 98°C for 10 s, 56°C for 30 s, 72°C for 30 s; 72°C for 2 min. T7 endonuclease I (New England Biolabs, Beverly, MA, USA) was added to PCR fragments and incubated at 37°C for 60 min. The samples were

electrophoresed on 2% agarose gels.

Mutation analysis

To confirm the presence of CRISPR/Cas9-mediated mutations, genomic DNA of each individual was isolated separately from: 1) five F_0 first-instar juveniles injected with *Dll*_T1, *Dll*_T2 or *Dll*_T3, which had a defect in second antennae; 2) F_1 progenies of eight different *Dll*_T3-injected F_0 founders which had a normal appearance; and 3) the eight F_0 founders. The target genomic region was amplified with TaKaRa PrimeSTAR GXL (TaKaRa Bio) using a set of primers designed from the *Dll* sequence (Table S1). Amplification conditions were: 35 cycles of 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s. After adding a 3′ dA overhang, the resulting fragments were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The sixteen clones for each sample were sequenced using Sanger techniques that included the Big Dye terminator Ver. 3.1 (Thermo Fisher Scientific) on an ABI 3730 Genetic Analyser DNA sequencer (Applied Biosystems Japan Ltd, Tokyo, Japan).

To observe morphological changes, first instar juveniles were examined under a stereomicroscope (M165 FC; Leica Microsystems GmbH, Wetzlar, Germany) and photographed with a digital camera (DS-5M-L1; Nikon, Tokyo, Japan), and then fixed in ethanol and dissected with a pair of needles. The specimens were mounted in glycerin,

observed by Nomarski differential interference contrast microscopy (BX50-DIC; Olympus, Tokyo, Japan), and photographed with a digital camera (DS-Fi1; Nikon). The terminology used herein for morphology follows that used by Stachowitsch (1992).

Statistical analysis

Abnormality rates were analyzed with a chi-square test for ratio difference followed by Tukey's WSD (wholly significant difference) pairwise multiple comparisons using Microsoft Excel 2013.

ACKNOWLEDGEMENTS

Daphnia pulex genomic sequence data were produced by The Center for Genomics and Bioinformatics at Indiana University and distributed via wFleaBase in collaboration with the Daphnia Genomics Consortium https://wiki.cgb.indiana.edu/display/DGC/Home. Our work benefits from, and contributes to, the Daphnia Genomics Consortium. This work was supported by a JSPS Research Fellowship for Young Scientists to CH (No. JP12J04065), JSPS KAKENHI Grant Numbers JP26840115, JP16K18587 to CH, JP15H04396 to TI, NIBB Cooperative Research Program (15-308) to CH, and The Research Council of Norway project NFR-221455 (EDRISK) to KET and TI. This work was carried out in accordance with the regulation on genetic recombination experimentation at Hokkaido University (approval

number: 2017-021).

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FIGURE LEGENDS

Fig. 1. Schematic illustration of the sgRNA targeting sequences on the first exon of the *Dll* gene in *D. pulex*.

Three sgRNAs, *Dll*_T1, *Dll*_T2 and *Dll*_T3, were designed in the first exon of the *Dll* gene. The 20-bp target sequence of sgRNAs is indicated in an orange box, adjacent to the NGG

protospacer adjacent motif (PAM) sequence in a blue box. The gray box indicates the left and right recognition sequence of previously described platinum TALENs (Hiruta et al., 2014). Underline indicates the ATG start codon.

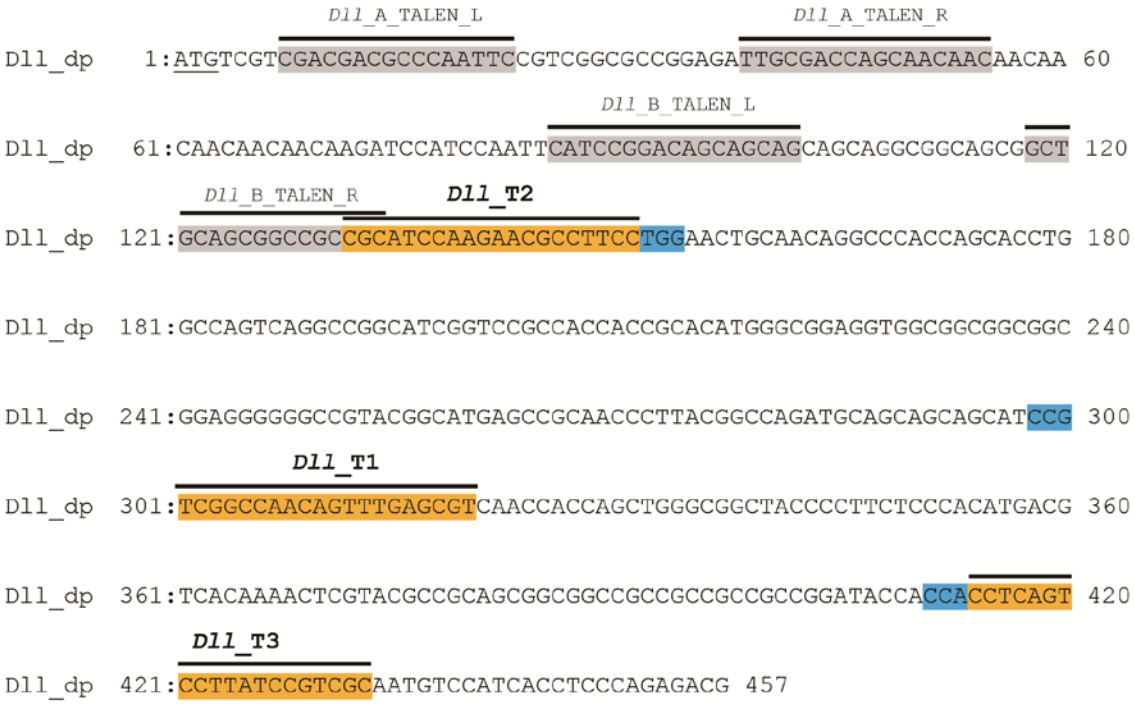

Fig. 2. Evaluation of efficiency and effectiveness of Cas9 RNPs.

(A) Mutations in *Dll* were detected by the T7EI assay. Arrowheads indicate the cleavage products generated in the T7EI assay of F_0 founders. +, injected samples; -, uninjected samples (control). **(B,C)** Visible abnormality rates at each injected Cas9 protein concentration with 500 ng/μl of sgRNAs **(B)** and at each injected sgRNA concentration with 600 ng/μl of Cas9 protein **(C)**. The visible abnormality rate was calculated from Table 1 by dividing the number of observed juveniles having shortened second antennae by the number of total juveniles. The different lower-case letters at the top of the columns indicate statistically significant differences ($P<0.05$; chi-square test followed by Tukey's WSD test).

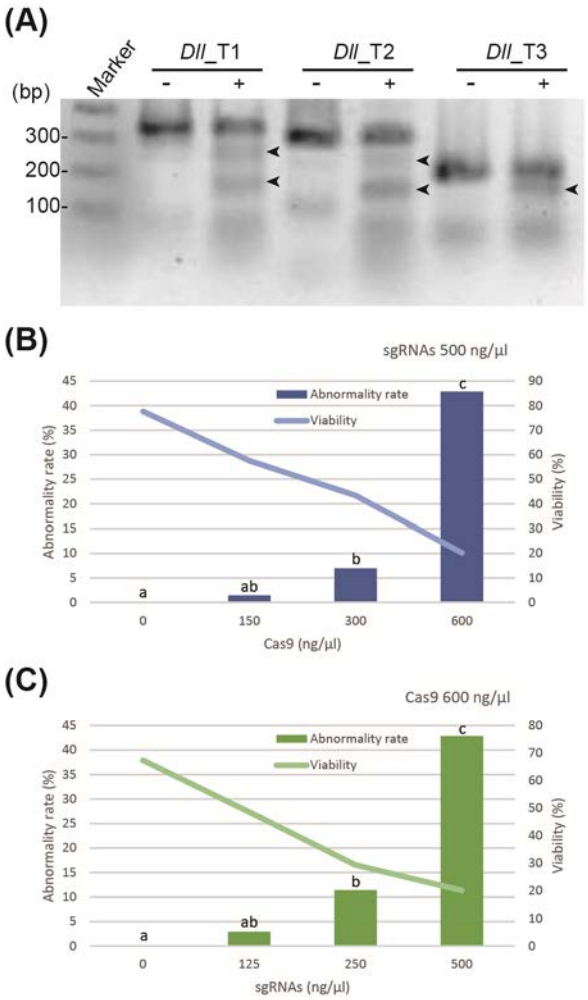

Fig. 3. Detection of CRISPR/Cas9-induced mutation sequences.

(A) Subcloned sequences observed in *Dll*_T1-, *Dll*_T2-, and *Dll*_T3-injected F_0 founders. The wild type (WT) sequence is shown at the top. The subsequent rows indicate sequences of five mutant lines (#1–5).The sgRNA targeting sequence and PAM are indicated in orange and blue boxes, respectively. Red letters and dashes indicate the identified mutations. The size of deletions and insertions are shown to the right of each mutated sequence (Δ; deletions, +; insertions). Numbers next to insertions/deletions indicate the numbers of mutated clones identified from all analyzed clones from each sample. aa, predicted protein size in amino

acids; brackets, appearance position of the stop codon. **(B)** Subcloned sequences observed in F_1 progenies of *Dll*_T3-injected F_0 founders (#1, 2). Binding sites, insertions/deletions, number of mutated clones and predicted protein size indicated as in **(A)**.

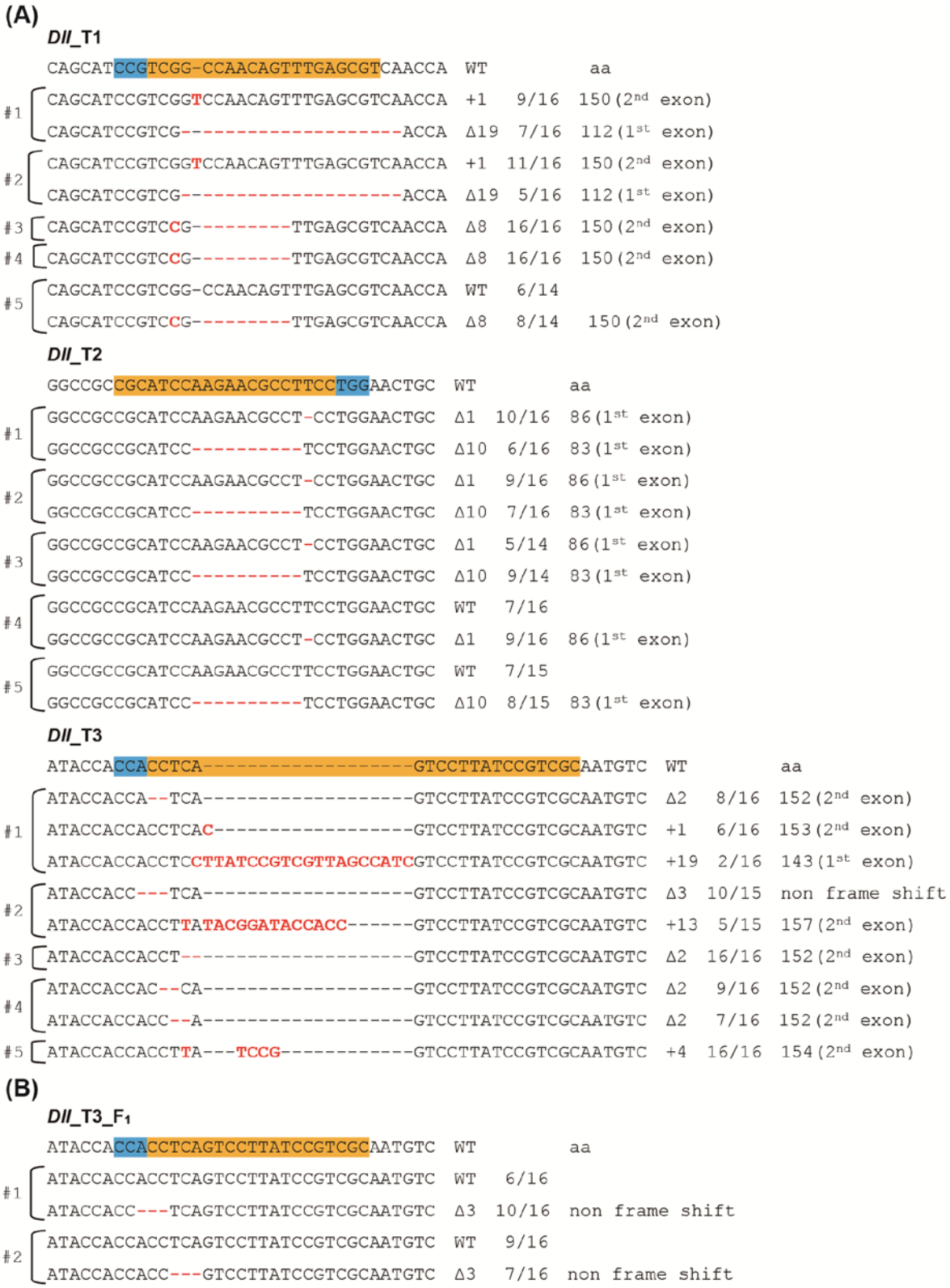

Fig. 4. Major *Dll*_CRISPR/Cas9 phenotypes.

The left and right columns show representative phenotypes of uninjected controls and

individuals injected with *Dll*_T3 Cas9 RNPs, respectively. **(A, B)** Lateral view of first instar juvenile. **(C, D)** Lateral view of the second antenna. **(E, F)** Second thoracic limb (T2). *Dr*, dorsal ramus; *En*, endopodite; *Ep*, epipodite; *Ex*, exopodite; *Gn*, gnathobase; *Pe*, peduncle; *Vr*, ventral ramus. Scale bars = 200 μm in **A**, **B**; 100 μm in **C**, **D**, **E**, and **F**.

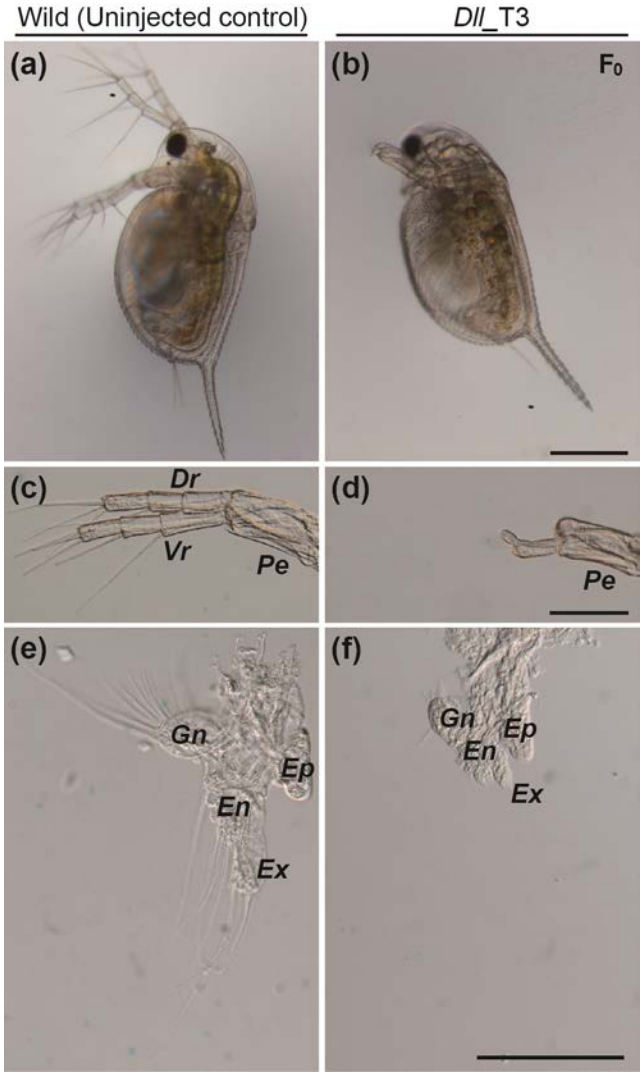

Table 1. Summary of CRISPR/Cas9 results.

CRISPR/Cas9	sgRNAs concentration (ng/μl)	Cas9 protein concentration (ng/μl)	Injected embryos	Juveniles[*1]	Viability (%)	Shortened 2nd antennae[*2]
***Dll*_T1**	500	600	61	13	21.3	5
***Dll*_T2**	500	600	123	30	24.4	10
***Dll*_T3**	500	600	173	35	20.2	15

	250	600	119	35	29.4	4
	125	600	70	34	48.6	1
	0	600	89	60	67.4	0
	500	300	99	43	43.4	3
	500	150	120	69	57.5	1
	500	0	156	121	77.6	0
Uninjected	-	-	165	132	80.0	0

[*1]Total number of injected embryos survived by the first instar juvenile stage (including both normal and abnormal phenotypes).

[*2]Number of juveniles whose second antennae were shortened.

Figure S1. Phenotypes of *Dll*_T3 Cas9 RNPs-injected juveniles.

The left and right columns show representative phenotypes of uninjected controls and individuals injected with *Dll*_T3 Cas9 RNPs, respectively. **(a, b)** First thoracic limb (T1). The exopodite and endopodite were shortened by *Dll*_T3 Cas9 RNPs. **(c, d)** Third and fourth thoracic limbs (T3/4), having the same morphology. The exopodite was shrunken in *Dll*_T3-injected juveniles. **(e, f)** Fifth thoracic limb (T5). The exopodite was shortened by *Dll*_T3 Cas9 RNPs. **(g, h)** Lateral view of the rostrum and head; arrowhead indicates an ocellus. The ocellus was lost in *Dll*_T3-injected juveniles. **(i, j)** Lateral view of abdomen; the arrow and arrowhead show an abdominal claw and abdominal seta, respectively. The abdominal claw was shrunken and abdominal seta was lost in *Dll*_T3-injected juveniles. *En*, endopodite; *Ep*, epipodite; *Ex*, exopodite; *Fc*, filter comb. Scale bars = 50 μm.

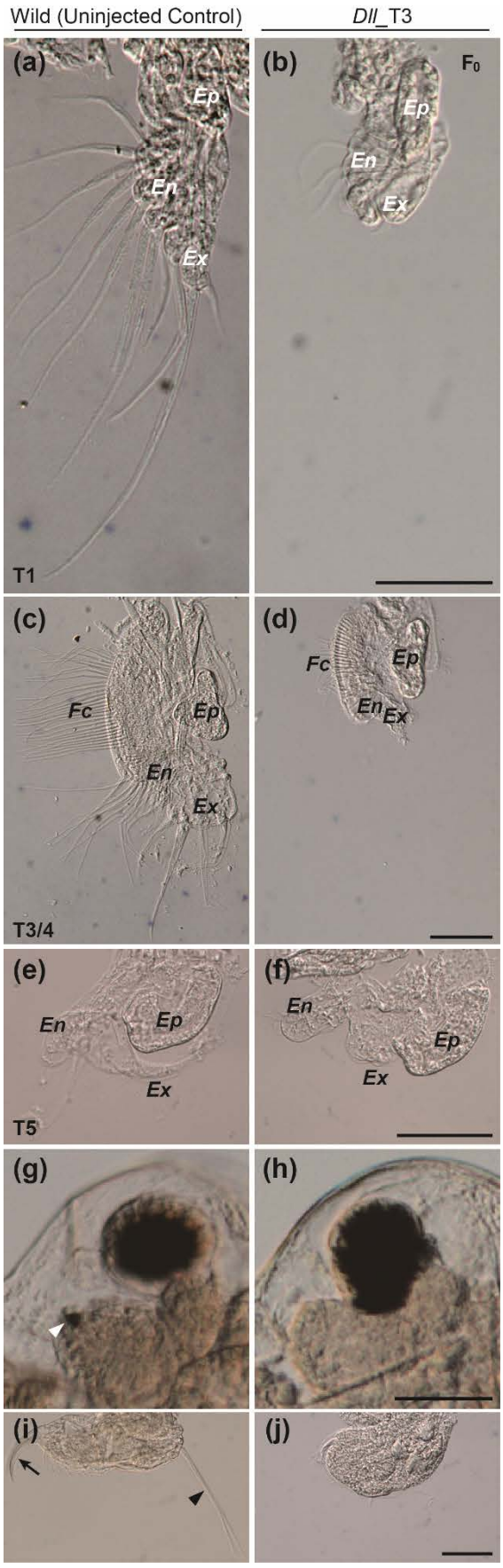
Wild (Uninjected Control)
Dll_T3
(a)
Ep
En
Ex
T1
(b)
F0
Ep
En
Ex
(c)
Fc
Ep
En
Ex
T3/4
(d)
Fc
Ep
En
Ex
(e)
En
Ep
Ex
T5
(f)
En
Ep
Ex
(g)
(h)
(i)
(j)

Table S1. The list of primer sequences.

Application	Fwd (5’ to 3’)	Rev (5’ to 3’)
sgRNA DNA template *Dll*_T1	TAATACGACTCACTATAGACGC TCAAACTGTTGG	TTCTAGCTCTAAAACTCGGCCAA CAGTTTGAGCG
sgRNA DNA template *Dll*_T2	TAATACGACTCACTATAGCGCAT CCAAGAACGCC	TTCTAGCTCTAAAACGGAAGGCG TTCTTGGATGC
sgRNA DNA template *Dll*_T3	TAATACGACTCACTATAGGCGA CGGATAAGGACT	TTCTAGCTCTAAAACCCTCAGTC CTTATCCGTCG
T7EI assay for *Dll*_T1	AAGAACGCCTTCCTGGAACT	TCTCTGGGAGGTGATGGACA
T7EI assay for *Dll*_T2	ATGTCGTCGACGACGCC	CATCTGGCCGTAAGGGTTG
T7EI assay for *Dll*_T3	CCTTACGGCCAGATGCAG	TCTCTGGGAGGTGATGGACA
Mutation analysis	CGACCAGCAACAACAACAACA	TCTCTGGGAGGTGATGGACA