CRISPR/Cas9-Mediated Genome Editing and Mutagenesis of EcChi4 in Exopalaemon carinicauda

Tianshu Gui,*† Jiquan Zhang,*†‡ Fengge Song,*† Yuying Sun,*§ Shijun Xie,*† Kuijie Yu,* and Jianhai Xiang*‡
*Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China, †University of Chinese Academy of Sciences, Beijing 100049, China, ‡Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266000, China, and §College of Marine Life and Fisheries, Huaihai Institute of Technology, Lianyungang 222005, China

ORCID ID: 0000-0001-5063-339X (J.Z.)

ABSTRACT The development of the type II clustered regularly interspaced short palindromic repeats (CRISPR) system has resulted in the revolution of genetic engineering, and this technology has been applied in the genome editing of various species. However, there are no reports about target-specific genome editing in shrimp. In this research, we developed a microinjection method for the ridgetail white prawn Exopalaemon carinicauda and successfully applied CRISPR/Cas9 technology to the genome editing of E. carinicauda. Through coinjection of mRNA of Cas9 nuclease and gRNA specialized for E. carinicauda chitinase 4 (EcChi4), shrimps with indel mutations were obtained. Further analysis showed that the mutations could be transmitted to the next generation. This is the first time that site-specific genome editing has been successfully demonstrated in a decapod, and will further contribute to the study of functional genomics in decapods.

KEYWORDS microinjection Exopalaemon carinicauda CRISPR/Cas9 chitinase

Shrimp are widespread all over the world and play a very important role in mariculture. It is a key goal in genetic analysis to identify which genes contribute to specific biological phenotypes and diseases. Although plenty of functional genes of shrimp had been identified and their functions had forecasted through bioinformatics methods, such research in shrimp remains at the gene (real-time polymerase chain reaction [PCR], in situ hybridization, and RNAi, etc.) and protein levels (recombinant expression, Western blot analysis, immunohistochemistry, protein–protein interactions, and protein–DNA interactions, etc.).

In the last few decades, researchers had done a lot of work on the integration of exogenous genes into the shrimp’s genome, including spermatophore-microinjection (Tsai et al. 1997; Li and Tsai 2000), electroporation (Powers et al. 1995; Tseng et al. 2000), and gene gun bombardment (Gendreau et al. 1995). However, compared with microinjection, these methods have a plenty of disadvantages, such as, laborious work, low efficiency of integration, and strong randomness of integration. More importantly, it has not been possible to determine where the exogenous gene will locate. Therefore, more efficient genome editing methods, which are site-specific, are essential for further research on the functional genes of shrimp.

Fortunately, recent advances on the clustered regularly interspaced short palindromic repeats (CRISPR) system have made site-specific genome editing quite easy. Since the CRISPR/Cas9 system was first introduced, with the aim of producing site-specific genetic changes in mammalian cells (Cho et al. 2013; Cong et al. 2013; Jinek et al. 2013; Mali et al. 2013), it has mediated site-specific genome editing in more than 20 different animals, including Drosophila melanogaster (Bassett et al. 2013), zebrafish (Chang et al. 2013), Bombyx mori (Wang et al. 2013), and others (Harrison et al. 2014). In crustaceans, the CRISPR/Cas9 system also has been used to introduce mutations in Daphnia magna (Nakanishi et al. 2014) and Parhyale hawaiensis (Martin et al. 2016).

The exoskeleton of arthropods, made up of chitin and sclerotized proteins, is a rigid scaffold. In order to grow and develop, arthropods have developed a mechanism of molting to replace their old exoskeleton (Merzendorfer and Zimoch 2003). During the molting process, the chitinases play a dominant role in degrading the old cuticles (Elyakova 1972; Funke and Spindler 1987; Buchholz 1989; Watanabe and Kono 1997; Ote et al. 2005; Rocha et al. 2012). A wide range of
chitinase research has been conducted in insects to investigate their structure, function, and regulation (Merzendorfer and Zimoch 2003; Arakane and Muthukrishnan 2010). Some studies of the chitinases in crustations also exist. However, most research has focused on the cloning and characteristic expression patterns of chitinase genes. Recently, RNAi of a chitinase gene was conducted on the salmon louse *Lepeophtheirus salmonis* (Eichner et al. 2014, 2015). Knock-down of *LsChi2* by RNAi induced strong downregulation of *LsChi2* expression in the larval stages as a result and evinced changes in the body dimensions, locomotive behavior, and ability to infect fish of salmon lice (Eichner et al. 2015).

In our previous research, we purified and characterized two kinds of chitinase (EcChi1 and EcChi2) from the hepatopancreas of *Exopalaemon carinicauda* (Wang et al. 2015), which is one of the most important commercial shrimp in China (Li et al. 2012). According to the

![Figure 1](https://example.com/figure1.png)

**Figure 1** Structure of the EcChi4 gene. (A) Nucleotide sequence of EcChi4 gene. Nucleotides are numbered with reference to the translation initiation site (+1). Nucleotides and amino acids are numbered on both sides of the sequence. Uppercase letters indicate exon sequences and lowercase letters represent intron sequences. The gRNA (guide RNA) site is double underlined and the PAM (protospacer adjacent motif) site is indicated by rectangles. The primers used to amplify the CRISPR target site of EcChi4 are single underlined. (B) Schematic representation of the genomic structures of EcChi4. The numbers represent the length of the exon or intron.

chitinase research has been conducted in insects to investigate their structure, function, and regulation (Merzendorfer and Zimoch 2003; Arakane and Muthukrishnan 2010). Some studies of the chitinases in crustations also exist. However, most research has focused on the cloning and characteristic expression patterns of chitinase genes. Recently, RNAi of a chitinase gene was conducted on the salmon louse *Lepeophtheirus salmonis* (Eichner et al. 2014, 2015). Knock-down of *LsChi2* by RNAi induced strong downregulation of *LsChi2* expression in the larval stages and resulted in evident changes in the body dimensions, locomotive behavior, and ability to infect fish of salmon lice (Eichner et al. 2015).

In our previous research, we purified and characterized two kinds of chitinase (EcChi1 and EcChi2) from the hepatopancreas of *Exopalaemon carinicauda* (Wang et al. 2015), which is one of the most important commercial shrimp in China (Li et al. 2012). According to the
transcriptome data of \textit{E. carinicauda}, the full-length cDNA sequences of \textit{EcChi1} and \textit{EcChi2} were obtained (Wang \textit{et al.} 2015).

In this research, we obtained a new kind of chitinase from \textit{E. carinicauda} and named it as \textit{EcChi4}. In order to clarify the function of \textit{EcChi4}, we tried to knock-out \textit{EcChi4} by CRISPR/Cas9 technology.

**MATERIALS AND METHODS**

**Rearing and hatching of \textit{E. carinicauda}**

The ridgetail white prawn \textit{E. carinicauda} have been cultured in our laboratory for 5 yr. Mature gravid females and mature male shrimps were selected randomly and cultivated in a 180 liter tank containing 100 liter seawater. After spawning, the one-cell stage embryos were collected from the abdomen and transferred to 10 ml sterilized seawater in a petri dish. Before being subjected to microinjection, the one-cell stage embryos were stored at 4°C to keep them from developing.

After injection, embryos were put in petri dishes containing sterilized seawater and cultured on a shaker at 100 rpm at room temperature. After 15 d, the shrimp were hatched and the Mysis larvae of \textit{E. carinicauda} were fed with anemia larvae. When the Mysis larvae grew into juvenile prawns, they were fed with bait.

**Preparation of EGFP mRNA**

The EGFP sequence was inserted in the multiple cloning site (MCS) between EcoRI and NotI of the plasmid pIZT/V5-His (Invitrogen) to construct the recombinant plasmid, and renamed pIZ-EGFP; then, the DNA fragment SP6-EGFP-pA for EGFP mRNA synthesis was amplified from the constructed plasmid pIZ-EGFP. The primers were as follows: SP6-EGFP-F (forward): 5’-GCATTTAGGTGACACTATAGAAACAGGCCACCATGTTGAGCAAGGGCGAGGA-3’; SP6-EGFP-R (reverse): 5’-CGCGCTTGAAAGGAGTGTGTA-3’.

The DNA fragment SP6-EGFP-pA was used as the template for \textit{in vitro} transcription, and the capped EGFP mRNA was synthesized using a SP6 mMESSAGE mMACHINE Kit (Ambion). The synthesized EGFP mRNA was then purified and extracted though phenol–chloroform and stored in aliquots at −80°C.

**Designation and preparation of gRNA**

The gRNA target site of the chitinase gene \textit{EcChi4} was identified by online tool ZiFiT (http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx) (Sander \textit{et al.} 2010).

\textit{In vitro} transcription was performed with the Thermo Scientific TranscriptAid T7 High Yield Transcription Kit. The synthesized gRNA was purified by phenol–chloroform extraction and stored in aliquots at −80°C.

**Preparation of Cas9 mRNA**

The pCMV-Cas9 vector (Sigma-Aldrich) was linearized by XbaI (Takara, Japan) and purified by ethanol precipitation. The purified linearized fragment was used as a template for \textit{in vitro} transcription with a mMESSAGE mMACHINE T7 Ultra Kit (Ambion) to synthesize the capped and poly(A) tailed mRNA of Cas9. The synthesized Cas9 mRNA was purified and extracted though phenol–chloroform, and stored at −80°C.

**Microinjection of \textit{E. carinicauda} embryos**

Microinjection was carried out using a Warner PLI-100A Pico-Injector microinjector (Warner Instruments) with standardized Femtotip II sterile microcapillaries (Eppendorf, Germany). Embryos were separated...
using a dissecting needle. After separation, the embryos were injected under a dissecting microscope using a MN-152 micromanipulator (Narishige). All injected mixtures were prepared in water containing 0.05% of the inert dye phenol red. The injection volume was approximately 0.5 nl.

The EGFP mRNA was injected into one-cell stage embryos of *E. carinicauda* at a concentration of 250 ng/µl; Cas9 mRNA at a concentration of 200 ng/µl and gRNA at concentration of 100 ng/µl were coinjected into one-cell stage embryos of *E. carinicauda*.

**Extraction and amplification of genomic DNA**

The Genomic DNA of Mysis larvae or juvenile prawns was extracted and the target fragment was amplified using a MightyAmp Genotyping Kit (Takara) according to the manufacturer’s instructions.

The genomic region flanking the CRISPR target site for EcChi4 was amplified and the product was purified using a Gel Extraction Kit (OMEGA) following the manufacturer’s protocol. The primers used to amplify the target fragment containing the CRISPR target site are as follows: EcChi4-10F (forward): 5’-TAGAGTTAAATTACATCAGCCTGA-3’; EcChi4-10R (reverse): 5’-GTTGTTATCCTACCTGAT TGAGAT-3’.

**Mutation detection by T7 Endonuclease I (T7EI) assay and Sanger sequencing**

The PCR products were subjected to a reannealing process to enable heteroduplex formation: 95°C for 10 min, 95–85°C ramping at 2°C/sec, 85–25°C at 0.3°C/sec, and holding at 25°C for 1 min (Guschin et al. 2010). After reannealing, the hybrid product was incubated with T7 Endonuclease I (NEB) for 20 min and analyzed on a 3% agarose gel. The amplified PCR products were isolated and cloned into the pMD19-T Simple Vector (Takara), which was used for Sanger sequencing.

**Data availability**

All mentioned tagged lines are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

**RESULTS**

**Structure and characterization of EcChi4**

Based on the transcriptomic data of *E. carinicauda*, the full-length nucleotide and deduced amino acid sequences of EcChi4 were obtained (Figure 1A). The open reading frame (ORF) of EcChi4 encoded 389 amino acids, with a predicted molecular weight of ~24,843,35 Da and a theoretical isoelectric point (pI) of ~4.93. The deduced amino acid sequence of EcChi4 contains a signal peptide at position 1–17 and a glycosyl hydrolase family 18 (Glyco_18) domain at position 18–366.

Using the primers (EcChi4-InF and EcChi4-InR), a specific fragment of 3390 bp was amplified from the genomic DNA and then sequenced. The EcChi4 cDNA sequence showed 100% identity to the corresponding sequence obtained from the genome. Comparison of the genomic
DNA sequence with the cDNA sequence of EcChi4 revealed that there are seven exons and six introns in EcChi4. The six introns divide the ORF into seven parts. The sequences at the exon–intron boundaries conformed to the typical eukaryotic splice sites, including an invariant GT at the intron 5′ boundary and an invariant AG at its 3′ boundary (Figure 1A). The organization of the gene is illustrated in Figure 1B.

Expression of exogenous EGFP mRNA in shrimp embryos

The EGFP mRNA was in vitro synthesized using the SP6 polymerase. First, the DNA fragment of Sp6-EGFP-pA was cloned from the constructed plasmid pIZ-EGFP, by the primers SP6-EGFP-F/R. The forward primer, SP-EGFP-F, included an upstream spacer (GC), a SP6 promoter (5′-ATTAGGTGACACTATAGAA-3′), a downstream spacer (5′-ACAG-3′), a Kozak sequence (5′-GCCACC-3′), and a gene-specific sequence for EGFP (5′-ATGGTGAGCAAGGGC-3′). The Kozak sequence can enhance the translation initiation of the mRNA (Kozak 1989). The OpIE2 polyadenylation sequence can regulate the transcription termination of mRNA (Theilmann and Stewart 1992). Then, the DNA fragment of Sp6-EGFP-pA was used as the template for RNA synthesis. Through the use of the mMESSAGE mMACHINE Kit (Ambion), the synthesized EGFP mRNA gained a 7-methyl guanosine cap structure [m7G(5′)-ppp(5′)G] at the 5′ end.

To test whether the exogenous mRNA could be expressed in the shrimp embryos, the synthesized EGFP mRNA was imported into one-cell stage embryos of E. carinicauda through microinjection (as described in the Materials and Methods). After 20 hr, the embryos were monitored for EGFP-induced fluorescence under a fluorescence microscope. Compared with embryos of the control group, which were not injected with EGFP mRNA, the mRNA-injected embryos exhibited abundant specific green fluorescence and the fluorescence was visible in almost all the injected embryos (Figure 2). This result indicated that the exogenous mRNA could be expressed in shrimp embryos.

### Table 1 Mutation frequencies induced by microinjection of Cas9 mRNA and gRNA

| RNA Concentration | Injected Embryos | Hatched Zoea Stage | Survival Postlarvae | Mutant Postlarvae | Survival Rate | Mutant Rate  |
|-------------------|------------------|--------------------|---------------------|------------------|---------------|--------------|
| 100 ng/μl (gRNA-EcChi4) | 200 ng/μl (pCMV-Cas9) | 247 | 88 | 35 | 18 | 14.17% | 7.29% |

gRNA, guide RNA.
CRISPR/Cas9-induced mutagenesis in *E. carinicauda* is highly efficient and that the survival rate of the injected embryos is quite high.

After a further 50 d, the injected embryos developed to adult *E. carinicauda* and their weight and length were statistically analyzed (Table 2). As the data show, the average weight and length of the mutant shrimp are slightly higher those of the control shrimp. However, there is no significant difference between them when these data are assessed using single factor analysis of variance. In addition, no significant morphological change in the mutants was observed. Thus, we speculated that the mutations on the target site of *EcChi4* do not have significant influence on the development and growth of *E. carinicauda*.

### Table 2 Weight and length of mutant and control shrimp

| Number | Mutants | Controls |
|--------|---------|----------|
|        | Weight (g) Length (cm) | Weight (g) Length (cm) |
| 1      | 0.17 2.2 | 0.34 3.0 |
| 2      | 0.28 2.8 | 0.26 2.4 |
| 3      | 0.24 2.7 | 0.22 2.6 |
| 4      | 0.08 1.8 | 0.10 2.1 |
| 5      | 0.11 2.0 | 0.09 2.0 |
| 6      | 0.28 2.7 | 0.31 2.9 |
| 7      | 0.30 2.9 | 0.11 1.9 |
| 8      | 0.18 2.5 | 0.20 2.5 |
| 9      | 0.23 2.6 | 0.25 2.7 |
| 10     | 0.38 3.0 | 0.29 2.8 |
| 11     | 0.63 3.4 | 0.11 2.0 |
| 12     | 0.10 1.8 | 0.12 2.0 |
| 13     | 0.21 2.5 |
| 14     | 0.30 3.0 |
| 15     | 0.21 2.5 |
| Means  | 0.25 2.5 | 0.21 2.5 |

Transmission of mutations to subsequent generations

In order to investigate whether the mutations could be transmitted to the next generation, four mutant shrimps were crossed with four wild-type shrimps to produce the filial generations (G1). Through Sanger sequencing, the genotypes of parents and subsequent generations were mapped. As the result show, the wild-type parent was homozygous, the mutant parent was heterozygous, and half of the filial generations were heterozygous (Figure 6). Further identification showed the mutation of the heterozygous progeny from the same family, with one wild-type allele and one allele harboring 5 bp deletions (as described in the Materials and Methods) (Figure 7). Those heterozygous progeny (G1) from the same family were then crossed to produce the filial generations (G2). Of 30 sequenced G2 postlarvae, 8 were wild-type, 16 were heterozygous, and 6 were homozygous mutants. The ratio of three genotypes was 1:2:1, indicating Mendelian inheritance of the mutant. The results indicate that CRISPR/Cas9-mediated mutagenesis can be generated within the germline and that the mutations can be transmitted to the offspring in *E. carinicauda*. There is no significant morphological change among wild-type, heterozygous, and homozygous mutants of G2 postlarvae at the *EcChi4* locus. In addition, the 5 bp deletions in exon 4 of *EcChi4* cause a shift in the reading frame of *EcChi4*. Therefore, we conclude that the mutation of *EcChi4* does not influence to the development and growth of *E. carinicauda*.

### DISCUSSION

In this research, we showed four major findings: (1) that the microinjection method was successfully applied to the embryos of *E. carinicauda*; (2) exogenous mRNA was successfully expressed in shrimp embryos; (3) the CRISPR/Cas9 system efficiently generated double-strand breaks (~50%) to induce a wide range of indels at the *EcChi4* locus in the genome of *E. carinicauda*; and (4) that heritable genome editing can be conducted in *E. carinicauda* via the CRISPR/Cas9 system.

At present, RNAi remains instrumental and universal in decapod research (Sagi et al. 2013). However, it involves the injection of adult decapods. The construction of the microinjection system in shrimp breaks down the barrier to the research of functional genes in decapods. Using the microinjection system, a wide range of RNAi knock-down studies on embryos of decapods can be conducted and will accelerate the basic research of decapod animals.

More importantly, this system can be used as a genome editing tool for other decapods. Up to now, genome editing has only been...
Conducted in several crustaceans. In the amphipod crustacean Parhyale hawaiiensis, the transposable element Minos was used to produce transgenic lines (Pavlopoulos and Averof 2005). In the cladoceran crustaceans D. magna and D. pulex, TALEN and CRISPR/Cas9 were used to mediate targeted mutagenesis (Hiruta et al. 2014; Nakanishi et al. 2014; Naitou et al. 2015). However, this study represents the first time that gene editing has been realized on a decapod crustacean. Using the CRISPR/Cas9 system, 18 EcChi4 locus mutants of the ridgetail white prawn were established. By crossing the mutant shrimp with the wild-type shrimp, we also got the mutant offspring. Since whole genome data for E. carinicauda is not currently available, the evaluation of the off-target effects cannot be conducted. Nonetheless, no morphological change in the 18 mutants and their offspring was observed.

Through the CRISPR/Cas9 system, we have knocked-out a specific gene in shrimp for the first time. This represents a great advance for the research of functional genes in shrimp. It will also provide a knock-in technology approach that can integrate exogenous genes into the genome of E. carinicauda. Thus, the CRISPR/Cas9 system is an efficient tool for the genome editing of shrimp that can be used in both scientific research and breeding improvement in aquaculture. In addition, the ridgetail white prawn E. carinicauda may be used as a novel model organism for decapod crustaceans to reveal the function of genes relevant to their development, growth, metabolism, and reproduction in vivo.

ACKNOWLEDGMENTS

The project was supported by The National Natural Science Foundation of China (Nos. 31172449, 41306165, 41376165), The National High Technology Research and Development Program of China (Nos. 31172449, 41306165, 41376165), The Scientific and Technological Innovation Project financially supported by the Qingdao National Laboratory for Marine Science and Technology (No. 2015ASJ02). The authors declare no competing interests.

LITERATURE CITED

Arakane, Y., and S. Muthukrishnan, 2010 Insect chitinase and chitinase-like proteins. Cell. Mol. Life Sci. 67: 201–216.

Bassett, A. R., C. Tibbit, C. P. Ponting, and J. L. Liu, 2013 Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. Cell Rep. 4: 220–228.

Buchholz, F., 1989 Molt cycle and seasonal activities of chitinolytic enzymes in the integument and digestive-tract of the antarctic krill, Euphausia superba. Polar Biol. 9: 311–317.

Chang, N. N., C. H. Sun, L. Gao, D. Zhu, X. F. Xu et al., 2013 Genome editing with RNA-guided Cas9 nuclease in Zebrafish embryos. Cell Res. 23: 465–472.

Cho, S. W., S. Kim, J. M. Kim, and J. S. Kim, 2013 Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31: 230–232.

Cong, L., F. A. Ran, D. Cox, S. L. Lin, R. Barretto et al., 2013 Multiplex genome engineering using CRISPR/Cas systems. Science 339: 819–823.

Eichner, C., F. Nilsen, S. Grotmol, and S. Dalvin, 2014 A method for stable gene knock-down by RNA interference in larvae of the salmon louse (Lepeophtheirus salmonis). Exp. Parasitol. 140: 44–51.

Eichner, C., E. Harasimczuk, F. Nilsen, S. Grotmol, and S. Dalvin, 2015 Molecular characterisation and functional analysis of LsChi2, a chitinase found in the salmon louse (Lepeophtheirus salmonis, Kroyer 1838). Exp. Parasitol. 151: 39–48.

Eylakova, L., 1972 Distribution of cellulases and chitinases in marine invertebrates. Comp. Biochem. Physiol. B 43: 67–70.

Funke, B., and K. Spindler, 1987 Developmental changes of chitinolytic enzymes and ecdysteroid levels during the early development of the brine shrimp Artemia. pp. 67–78 in Artemia Research and Its Applications. Universa Press, Wetteren, Belgium.

Gendreau, S., V. Lardans, J. P. Cadoret, and E. Mialhe, 1995 Transient expression of a luciferase reporter gene after ballistic introduction into Artemia franciscana (Crustacea) embryos. Aquaculture 133: 199–205.

Guschin, D., A. Waite, G. Katibah, J. Miller, M. Holmes et al., 2010 A rapid and general assay for monitoring endogenous gene modification. pp. 247–256 in Engineered Zinc Finger Proteins, edited by Mackay, J. P., and D. J. Segal, Humana Press, Totowa, NJ.

Harrison, M. M., B. V. Jenkins, K. M. O’Connor-Giles, and J. Wildonger, 2014 A CRISPR view of development. Genes Dev. 28: 1859–1872.

Hiruta, C., Y. Ogino, T. Sakuma, K. Toyota, S. Miyagawa et al., 2014 Targeted gene disruption by use of transcription activator-like effector nuclease (TALEN) in the water flea Daphnia pulex. BMC Biotechnol. 14: 8.

Jinek, M., A. East, A. Cheng, S. Lin, E. B. Ma et al., 2013 RNA-programmed genome editing in human cells. eLife 2: 9.

Kozak, M., 1989 The scanning model for translation: an update. J. Cell Biol. 108: 229–241.

Li, J., J. Han, P. Chen, Z. Chang, Y. He et al., 2012 Cloning of a heat shock protein 90 (HSP90) gene and expression analysis in the ridgetail white prawn Exopalaemon carinicauda. Fish Shellfish Immunol. 32: 1191–1197.

Li, S. S., and H. J. Tsai, 2000 Transfer of foreign gene to giant freshwater prawn (Macrobrachium rosenbergii) by spermatophore-microinjection. Mol. Reprod. Dev. 56: 149–154.

Mali, P., L. H. Yang, K. M. Esvelt, J. Aach, M. Guell et al., 2013 RNA-guided human genome engineering via Cas9. Science 339: 823–826.

Martin, A., J. M. Serano, E. Jarvis, H. S. Bruce, J. Wang et al., 2016 CRISPR/Cas9 mutagenesis reveals versatile roles of Hox genes in crustacean limb specification and evolution. Curr. Biol. 26: 14–26.

Figure 7 EcChi4 fragments were amplified and cloned into the pMD19-T simple vector from the filial generations for sequencing. The brown rectangle is the PAM sequence. The black arrowhead is the cleavage site. The yellow rectangle is the 20 bp genomic target site. Deletion is represented by a dashed line. PAM, protospacer adjacent motif; WT, wild-type.
Merzendorfer, H., and L. Zimoch, 2003 Chitin metabolism in insects: structure, function and regulation of chitin syntheses and chitinases. J. Exp. Biol. 206: 4393–4412.

Naitou, A., Y. Kato, T. Nakanishi, T. Matsuura, and H. Watanabe, 2015 Heterodimeric TALENs induce targeted heritable mutations in the crustacean Daphnia magna. Biol. Open 4: 364–369.

Nakanishi, T., Y. Kato, T. Matsuura, and H. Watanabe, 2014 CRISPR/Cas-mediated targeted mutagenesis in Daphnia magna. PLoS One 9: 7.

Ote, M., K. Mita, H. Kavasaki, T. Daimon, M. Kobayashi et al., 2005 Identification of molting fluid carboxypeptidase A (MF-CPA) in Bombyx mori. Comp. Biochem. Phys. B 141: 314–322.

Pavloupolos, A., and M. Averof, 2005 Establishing genetic transformation for comparative developmental studies in the crustacean Parhyale ha-waiensis. Proc. Natl. Acad. Sci. USA 102: 7888–7893.

Powers, D. A., V. L. Kirby, T. Cole, and L. Hereford, 1995 Electroporation as an effective means of introducing DNA into abalone (Haliotis rufescens) embryos. Mol. Mar. Biol. Biotechnol. 4: 369–376.

Rocha, J., F. L. Garcia-Carreno, A. Muhlia-Almazan, A. B. Peregrino-Uriarte, G. Yepiz-Plascencia et al., 2012 Cuticular chitin synthase and chitinase mRNA of whiteleg shrimp Litopenaeus vannamei during the molting cycle. Aquaculture 330: 111–115.

Sagi, A., R. Manor, and T. Ventura, 2013 Gene silencing in crustaceans: from basic research to biotechnologies. Genes (Basel) 4: 620–645.

Sander, J. D., M. L. Maeder, D. Reyon, D. F. Voytas, J. K. Joung et al., 2010 ZIFIT (Zinc Finger Targeter): an updated zinc finger engineering tool. Nucleic Acids Res. 38: W462–W468.

Theilmann, D. A., and S. Stewart, 1992 Molecular analysis of the trans-activating IE-2 gene of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus. Virology 187: 84–96.

Tsai, H. J., C. H. Lai, and H. S. Yang, 1997 Sperm as a carrier to introduce an exogenous DNA fragment into the oocyte of Japanese abalone (Haliotis diversicolor supertexta). Transgenic Res. 6: 85–95.

Tseng, F. S., H. J. Tsai, I. C. Liao, and Y. L. Song, 2000 Introducing foreign DNA into tiger shrimp (Penaeus monodon) by electroporation. Theriogenology 54: 1421–1432.

Wang, J., J. Q. Zhang, F. G. Song, T. S. Gui, and J. H. Xiang, 2015 Purification and characterization of chitinases from ridgetail white prawn Exopalaemon carinicauda. Molecules 20: 1955–1967.

Wang, Y., Q. Z. Li, J. Xu, B. S. Zeng, L. Ling et al., 2013 The CRISPR/Cas system mediates efficient genome engineering in Bombyx mori. Cell Res. 23: 1414–1416.

Watanabe, T., and M. Kono, 1997 Isolation of a cDNA encoding a chitinase family protein from cuticular tissues of the Kuruma prawn Penaeus japonicus. Zoolog. Sci. 14: 65–68.

Communicating editor: W. S. Davidson