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RESEARCH ARTICLE

Microinjection-based CRISPR/Cas9 mutagenesis in the decapoda crustaceans *Neocaridina heteropoda* and *Eriocheir sinensis*

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ABSTRACT

CRISPR/Cas9 technology has been applied to many arthropods. However, application of this technology to crustaceans remains limited because of the unique characteristics of embryos. Our group has developed a microinjection system to introduce the CRISPR/Cas9 system into *Neocaridina heteropoda* embryos (one-cell stage). Using the developed method, we mutated the target gene *Nh-scarlet* (*N. heteropoda* scarlet), which functions in eye development and pigmentation. The results showed that both eye color and shape were altered in individuals in which *Nh-scarlet* was knocked out. Furthermore, this system was also successfully applied to another decapod crustacean, *Eriocheir sinensis*. DNA sequencing revealed that the zoeae with red eyes had an edited version of *Es-scarlet*. This study provides a stable microinjection method for freshwater crustaceans, and will contribute to functional genomics studies in various decapods.

KEY WORDS: Genome modification, Gene editing, Functional genomics, *Scarlet*, Embryo, Freshwater shrimp, Crab

INTRODUCTION

To explore gene function, various genome modification tools have been developed such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Kanchiswamy et al., 2016; Liu et al., 2012; Segal and Meckler, 2013). Although ZFNs and TALENs were the most popular genome modification tools in previous decades, off-target effects may lead to cellular toxicity, and complex experimental procedures have prompted the development of a new generation of gene-editing tools, including the clustered regularly interspaced short palindromic repeats and its associated endonuclease 9 (CRISPR/Cas9) system (Jinek et al., 2012). CRISPR/Cas9 has been used for gene editing in plants and animals for several years (Hsu et al., 2014; Chen et al., 2020; Tsuchimatsu et al., 2020). The system consists of the endonuclease Cas9 and the guide RNA (sgRNA) guiding Cas9 to the target site, where it cleaves the target sequence to induce DNA double strand breaks in the target genome (Hsu et al., 2014). CRISPR/Cas9 technology has been applied successfully to many arthropods (Bassett et al., 2013; Hu et al., 2019; Wang et al., 2016; Wei et al., 2014; Zhang et al., 2016).

Multiple methods can be used to deliver the CRISPR/Cas9 system into arthropod embryos, such as microinjection (Gratz et al., 2014; Yu et al., 2013), recombinant virus transfection (Dong et al., 2016; Martínez-Solís et al., 2019) and receptor-mediated ovary transduction of cargo (Chaverra-Rodriguez et al., 2018, 2020). Compared with other methods, microinjection has several advantages (Yan and Yu, 2008): (1) only a small volume of nucleic acid or protein is required, which is less stressful to the embryos and decreases the likelihood of cell death compared with viral infection or liposome-mediated transfection; (2) recombinant plasmid or protein is delivered into embryos directly; hence, a small sample can have a potent effect; and (3) it does not require large quantities of specific reagents to penetrate the cell membrane; hence, it can be applied to the embryos of model and non-model animals. For these reasons, microinjection is the preferred method for delivering CRISPR/Cas9 into arthropod embryos. However, currently, application of CRISPR/Cas9 to crustaceans is limited to *Exopalaemon carinicauda* (Gui et al., 2016), *Parhyale hawaiensis* (Nakanishi et al., 2014) and *Daphnia magna* (Kumagai et al., 2017). It is not easy to deliver the CRISPR/Cas9 system into embryos of crustaceans by microinjection, especially shrimp embryos, because bacterial and fungal infection can kill embryos during culturing *in vitro* (Gil-Turnes et al., 1989; Porntrai and Damrongphol, 2008). Moreover, peroxidase and dual oxidases promote egg membrane hardening within a very short time after oviposition (Dias et al., 2013; Toyota et al., 2016), increasing the difficulty of needle insertion. Finally, egg components may leak during microinjection because of the difference in osmotic pressure between the inside and outside of the egg (Elendt and Bias, 1990; Toyota et al., 2016). In addition, most shrimps have a large body size and long growth cycle, which makes them unsuitable for culturing in the lab (Li et al., 2020b). Accordingly, we chose *Neocaridina heteropoda*, which has a short growth cycle (~2 months from larva to sextual maturity), numerous embryos (20–50 per female adult), and a frequent reproduction cycle (~1 month at 26°C) (Tropea et al., 2015), as our model organism for gene editing.

Until now, many attempts have been made in several species to solve problems in the microinjection process. To overcome microbial infection, researchers usually use antibiotics for *in vitro* culturing of crustacean embryos. For example, malachite green and formalin can inhibit microbe infection (Bao et al., 1999; Liao and Zhao, 2001). However, both have been banned because they may damage the environment, and many researchers prefer to refresh the water more frequently when culturing embryos *in vitro* (Porntrai and Damrongphol, 2008; Zhang et al., 2021, 2018). To dissolve the hard egg membrane, some studies suggest keeping embryos on ice after oviposition because the low temperature can inhibit enzyme-

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catalyzed reactions (Hiruta et al., 2013; Kato et al., 2011). However, for some shrimps, such as *N. heteropoda*, a violent temperature change may damage the embryo. Because the ice incubation method is unsuitable for all embryos, many researchers prefer to collect the fertilized eggs as early as possible and inject before the membrane becomes hard, which can decrease the difficulties of microinjection (Bassett et al., 2013; Koutroumpa et al., 2016; Perry et al., 2016; Tanaka et al., 2016; Wang et al., 2020).

As for the adjustment of osmotic pressure, in general, high concentration medium or buffer can regulate the osmotic pressure of embryos. For example, M4 medium with 80 mmol l^{-1} sucrose can inhibit embryo leakage during embryo microinjection in *D. magna* (Elendt and Bias, 1990; Hiruta et al., 2013; Kato et al., 2011). However, for some freshwater crustaceans, treating their embryos with high osmotic pressure medium may damage them. Some studies reported that the osmotic pressure of embryos can also be regulated by the culture environment. For example, placing embryos of *Vanessa cardui* in a dry environment can decrease the water content (Zhang and Reed, 2016). Wet conditions not only keep embryos moist but also regulate osmotic pressure. For example, placing embryos of pea aphid on wet filter paper can assist hydration and regulate osmotic pressure (Le Trionnaire et al., 2019).

Concerning the edited genes, those targeted by the CRISPR/Cas9 technology system should be involved in some obvious phenotype to screen individual mutants; moreover, the mutations should not adversely impact animal lifespan or health (Sun et al., 2017). In *Drosophila*, White, Brown and Scarlet are eye pigment transporters belonging to the ATP-binding cassette (ABC) transporter subfamily harboring an AAA (ATPases associated with a variety of cellular activities) domain and several transmembrane domains (TMDs) (Schmitz et al., 2001). Mutation of the *scarlet* gene may lead to a transparent eye in *D. magna* (Ismail et al., 2018), while in *Tribolium castaneum*, mutation of the *scarlet* gene induces a white eye color (Grubbs et al., 2015). Thus, mutation of the *scarlet* gene would be expected to induce similar phenotype changes in *N. heteropoda* and *Eriocheir sinensis*.

In the present study, we constructed a CRISPR/Cas9 delivery system in *N. heteropoda* embryos by microinjection. The *N. heteropoda scarlet* (*Nh-scarlet*) gene was selected as the target gene to verify the gene-editing effect. This system was also tested in *E. sinensis*. The results showed that the CRISPR/Cas9 system was successfully delivered into *N. heteropoda* and *E. sinensis* embryos, and the *Nh-scarlet* gene was mutated as intended, with mutated individuals displaying transparent or light-colored eyes in *N. heteropoda* and red-colored eyes in *E. sinensis*.

MATERIALS AND METHODS

Neocaridina heteropoda and *Eriocheir sinensis* rearing, hatching and embryo staining with DAPI

Neocaridina heteropoda (Liang 2002) and *Eriocheir sinensis* (H. Milne-Edwards 1853) were purchased from a local aquatic market and cultured in our lab over 6 months. The tank was sterilized using potassium permanganate ($KMnO_4$), and freshwater was aerated for 24 h before being added to the tank.

Neocaridina heteropoda embryos were collected at 1, 2, 3, 4 and 5 h post-spawning, and then irrigated with freshwater and fixed with 4% paraformaldehyde overnight. Fixed embryos were stained with DAPI (10 µg ml^{-1}; SparkJade) for 30 min, and then washed with freshwater, and the number of nuclei was observed under a fluorescence microscope to confirm the best collection time to ensure embryo survival.

Following this initial step (see Results), one-cell stage embryos were collected at 4 h post-spawning from the abdomen of shrimps and irrigated 3 times with sterilized freshwater. Before microinjection, embryos were kept at room temperature to make sure that they could develop normally.

Microinjection of tracers and embryo survival

After wetting a filter paper with sterilized freshwater, one-cell stage *Neocaridina heteropoda* embryos were placed on the paper and kept moist. Dextran (1000 ng $µl^{-1}$, MW 10,000; Invitrogen) and Phenol Red (0.05%; BBI) were used as tracers to confirm that the injection solution had been injected successfully into embryos. After microinjection, the injected embryos were cultured on the paper and irrigated 3 times with sterilized freshwater every day to avoid microbial infection.

The mortality of injected embryos was used to evaluate which tracer was more suitable for *N. heteropoda* embryos.

Nh-scarlet and *Es-scarlet* cloning and analysis

Primers ScaF, ScaR, EscaF and EscaR were designed by Primer premier 5.0 software according to the cDNA sequence of *Nh-scarlet* (GenBank accession no. OK398107) and the genomic sequence of *Es-scarlet* (GenBank assembly accession: GCA_003336515.1). Then, the partial genomic sequences of the *Nh-scarlet* and *Es-scarlet* genes were amplified using all these primers (Table S1).

Genomic DNA was extracted from *N. heteropoda* and *E. sinensis* embryos using a TIANamp Marine Animals DNA kit (TianGen). Primers ScaF, ScaR, EscaF and EscaR were used to amplify the partial genomic sequence of *Nh-scarlet* (GenBank accession no. MZ670769) and *Es-scarlet* (GenBank accession no. OK398106) using PrimeSTAR HS DNA Polymerase (TaKaRa) according to the manufacturer's instructions. All sequences analyzed in this study were sequenced by GeneWiz (Beijing).

Domain structure and signal peptide analyses were performed by SMART (http://smart.embl-heidelberg.de) and Signal IP (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). Multiple sequence alignment and neighbor-joining phylogenic construction were performed by MEGA 7.0 (MEGA) with 2000 bootstrap replicates. The amino acid sequence of the Scarlet protein was analyzed by DNAMAN (Lynnon Biosoft; https://www.lynnon.com/dnaman.html).

Measurement of *Nh-scarlet* expression at different embryo development stages

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To determine the expression level of *Nh-scarlet* at different embryo development stages, samples of embryos at the one-cell stage, morula, blastula, gastrula, nauplius, flea larva and eyes of juvenile and adult shrimps were collected for total RNA extraction.

Total RNA was reverse-transcribed into cDNA by a PrimeScript RT reagent kit (TaKaRa). To analyze the expression profiles of the *Nh-scarlet* gene in different embryo development stages, real-time PCR assays were performed on a LightCycler 480 System (Roche). Results were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization against the 18S ribosomal RNA gene (GenBank accession no. HQ534061.1). Primers qScaF/qScaR and 18sF/18sR were used to amplify *Nh-scarlet* and *18S*, respectively. The amplification procedure involved one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s.

Data were analyzed using SPSS 16.0 (IBM) with the one-way analysis of variance (ANOVA) method.

Journal of Experimental Biology (2022) 225, jeb243702. doi:10.1242/jeb.243702

Preparation of sgRNA and Cas9 protein for *in vitro* digestion

Three sgRNAs for the *scarlet* gene were designed by CRISPOR (http://crispor.tefor.net). The partial genomic sequences of *Nh-scarlet* and *Es-scarlet* were used as templates. Genome information for *Penaeus vannamei* (white shrimp, NCBI Assembly GCF_003789085.1) served as a reference genome. Primers Nsg-Scarlet1, Nsg-Scarlet2, Nsg-Scarlet3, Esg-Scarlet1 and T7 sg-re (Table S1) were used to synthesize the template for sgRNAs with Phusion High-Fidelity DNA Polymerase (NEB). Nsg-Scarlet1, 2 and 3 targeted the sites at 163 bp, 209 bp and 1093 bp of the genome fragment, respectively. Templates were purified using phenol (pH 7.9, BBI) and chloroform extraction. The purified DNA templates of sgRNAs were transcribed into sgRNAs using a MEGA shortscript kit (Invitrogen). The sgRNAs were purified by phenol (pH 4.8, BBI) and chloroform extraction. Cas9 protein was purchased from PNA Bio. Both the Cas9 protein and sgRNAs were stored at −80°C.

To evaluate the digestion efficiency, sgRNAs were tested by *in vitro* digestion. Partial *Nh-scarlet* DNA was amplified by PCR, and Cas9 protein (NEB) was used to perform the *in vitro* digestion. According to the manufacturer's instructions, 30 ng DNA was digested using 300 ng sgRNA and 1 µl Cas9 (1 µmol l^{-1}) for 15 min at 37°C. The results of *in vitro* digestion were assessed by 1% agarose gel electrophoresis.

Microinjection of *N. heteropoda* and *E. sinensis* embryos

Microinjection was carried out using a Femto Jet 4i microinjector (Eppendorf) with standardized Femtotip II sterile microcapillaries (Eppendorf). On the basis of work done (Li et al., 2017; Nakayama et al., 2014) in other labs and our pre-experiments, Cas9 protein (PNA Bio) and sgRNAs were diluted with sterilized Milli-Q water to a final concentration of 300 ng μl^{-1} and 200 ng μl^{-1}, respectively. Cas9 and sgRNAs were incubated at 25°C for 10 min before microinjection. The injection volume was ∼0.8 nl for each *N. heteropoda* embryo and ∼0.2 nl for each *E. sinensis* embryo.

Embryos were placed on wet filter paper to perform microinjection, and injected embryos were cultured on the paper and gently irrigated 3 times every day with sterilized freshwater. To achieve a significant mutant phenotype, all three sgRNAs were injected into *N. heteropoda* embryos.

Mutation genotype and phenotype verification

To test whether the editing system would work, embryos were divided into three groups and injected with Nsg-Scarlet1, 2 or 3. Then they were cultured to the gastrula stage for initial detection. Meanwhile, some embryos were injected with all three sgRNAs together and cultured until the flea larva stage. Then, their eye color was observed to confirm whether the mutated *Nh-scarlet* gene induced an abnormal eye in *N. heteropoda*. The crab embryos were cultured to the zoea stage and screened for abnormal eye color. Embryos were killed for genomic DNA extraction. Primers ScaF, ScaR, EscaF and EscaR (Table S1) were used to amplify the mutated *Nh-scarlet* gene and *Es-scarlet* gene, using PrimeSTAR HS DNA Polymerase (TaKaRa) according to the manufacturer's instructions, and PCR products were sequenced to see whether introduction of the mutation was successful. PCR products were appended with poly-A tails using an A-Tailing kit (TaKaRa); A-tailed *Nh-scarlet* was cloned into the pMD-18T vector (TaKaRa) for sequencing; and sequences were analyzed by BioEdit software (Hall, 1999).

RESULTS

Determining the optimal time to collect embryos for injection

If *N. heteropoda* embryos were collected immediately after spawning, manipulation may have damaged them. Thus, at 1, 2, 3, 4 and 5 h post-spawning, embryos were collected to evaluate which time was best for manipulation. The results showed that at 4 h post-spawning, manipulation had the least impact on embryos, and the egg membrane was suitable for microinjection. Additionally, this injection time ensured that normal fertilization was unaffected: nearly all eggs taken from adult females were able to become fertilized. The results of DAPI staining also suggested that collecting embryos at 4 h post-spawning did not negatively impact development. Additionally, microinjection at 4 h post-spawning can be performed before the first cell division (Fig. 1). Thus, all embryos used for microinjection in this study were collected at 4 h post-spawning.

Microinjection

Dextran and Phenol Red were tested as tracers for microinjection of *N. heteropoda* embryos. Green fluorescence could be detected under a fluorescence microscope after microinjection of dextran (Fig. 2A). At 4 h post-injection, green fluorescence could still be detected, and embryos continued dividing (Fig. 2B). However, the survival rate of embryos injected with dextran was only about 10% when counted at the cell division stage (Table 1). By contrast, when Phenol Red was used as the tracer for microinjection, the survival rate of embryos (∼50%) in the same period was better than that in the dextran-injected group (Table 1). Although mortality gradually increased in both groups as embryo development continued, the survival rate of the Phenol Red group remained higher than that of the dextran group. When using a wet filter paper in the microinjection process (see Materials and Methods), although the embryos might leak slightly, they were able to survive the damage induced by injection (Fig. S1A). Moreover, because the injection sites on the embryos were exposed to the air, the leaked components might have solidified and closed the pore induced by the injection, halting the leakage (Fig. S1B).

Genomic sequence and expression profile of *Nh-scarlet*

Nh-scarlet complete cDNA sequence was acquired from transcriptome data obtained in our previous study, containing the 5′ and 3′ untranslated region (UTR). Through sequence analysis and PCR verification, the open reading frame (ORF) of *Nh-scarlet* was delimited to 2013 bp in length and encoded a putative protein of 671 amino acids with a predicted molecular weight of 75.62 kDa (Fig. S2). The results of signal peptide and TMD analysis indicated that Nh-Scarlet contained several TMDs (Fig. S3A) but no signal peptide (Fig. S3B). Analysis of the SMART results indicated that Nh-Scarlet had an AAA domain and five TMDs (Fig. S3C). Phylogenetic analysis of Nh-Scarlet revealed a close genetic relationship with its homolog in *Penaeus monodon*, suggesting that the gene from *N. heteropoda* did indeed encode *scarlet* (Fig. S3D).

The partial genomic sequence of *Nh-scarlet* is 1177 bp in length and contains two exons (236 bp and 88 bp) and an intron of 853 bp (Fig. S3E).

The results of real-time PCR indicated that in early development stages of *N. heteropoda* embryos (single-cell, morula, blastula and gastrula stages), the expression level of *Nh-scarlet* was too low to be detected (Fig. 3). However, *Nh-scarlet* expression was detected in the nauplius stage, and levels peaked in the flea larva stage (Fig. 3). The expression level of *Nh-scarlet* in the flea larva stage was significantly higher than that in the nauplius, juvenile shrimp and

Journal of Experimental Biology (2022) 225, jeb243702. doi:10.1242/jeb.243702

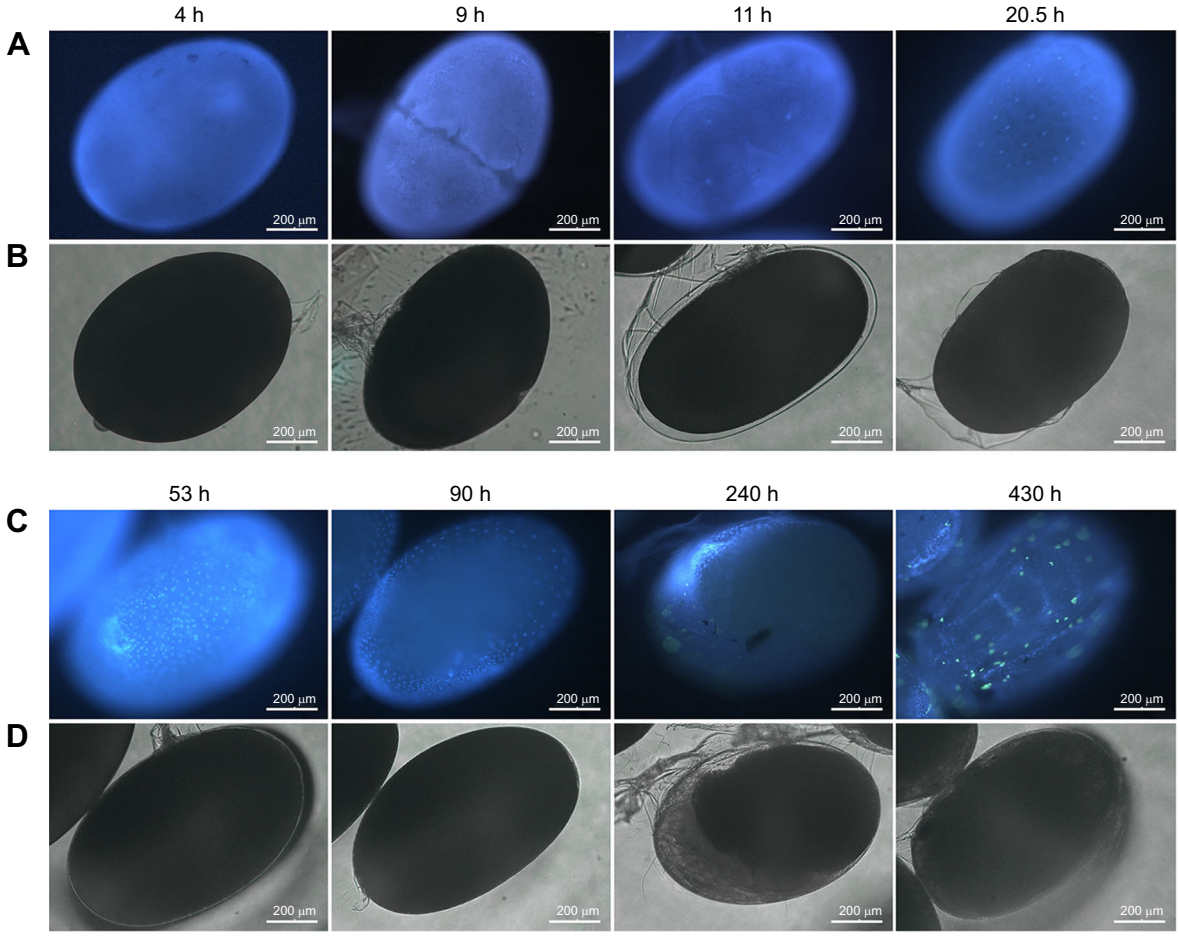

Fig. 1. DAPI staining of *Neocaridina heteropoda* embryos at different developmental stages from 4 h post-spawning. (A) DAPI staining of (from left to right) the single-cell, two-cell, four-cell and morula stages. (B) Observation of the single-cell, two-cell, four-cell and morula stages under bright field illumination. (C) DAPI staining of (from left to right) the blastocyst, gastrula, nauplius and flea larva stages. (D) Observation of the blastocyst, gastrula, nauplius and flea larva stages under bright field illumination. Scale bars: 200 μm.

adult shrimp stages (P<0.01). *Nh-scarlet* expression levels among the nauplius, juvenile shrimp and adult shrimp stages were not significantly different (P>0.05; Fig. 3).

Mutation of *Nh-scarlet*

Three sgRNAs were used to edit the *Nh-scarlet* gene; sg-Scarlet1 was designed to digest the sense strand, and sg-Scarlet2 and

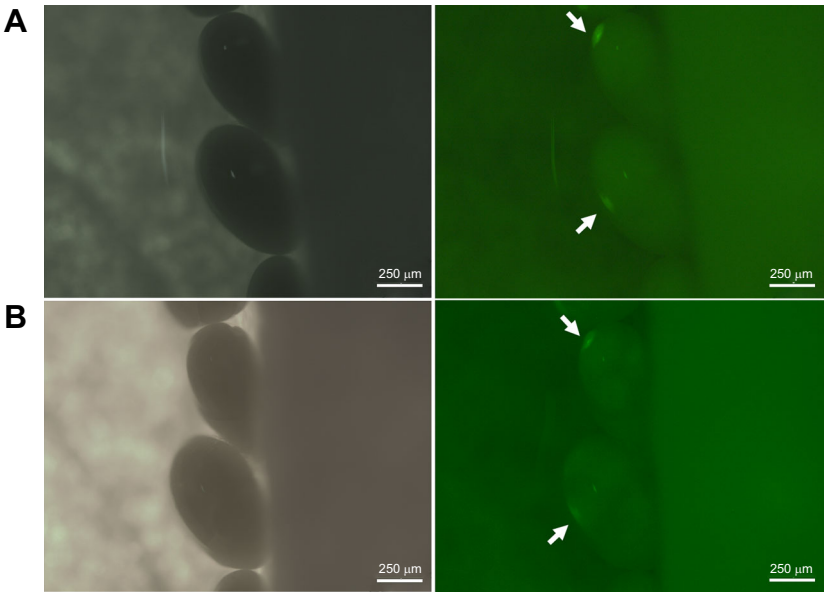

Fig. 2. *Neocaridina heteropoda* embryo development after microinjection with dextran. (A) Observations were made immediately after microinjection. Green fluorescence indicates that dextran was successfully injected into the embryos. (B) Observation at 4 h after microinjection. The results confirm embryo survival after microinjection. Scale bars: 250 μm.

Journal of Experimental Biology (2022) 225, jeb243702. doi:10.1242/jeb.243702

Table 1. Survival rate of *Neocaridina heteropoda* embryos using different tracers

Tracer	Survival rate (%)		
	Cell division	Blastula	Flea larva
Dextran	10.15±3.99	4.86±2.58	2.04±3.53
Phenol Red	59.46±18.22	39.75±10.67	23.57±9.71

sg-Scarlet3 were designed to digest the antisense strand (Fig. 4A). Delivery of all three sgRNAs into embryos induced a large fragment deletion. All three sgRNAs were pre-evaluated for their digestion effect *in vitro*. The larger digestion fragments were predicted to be ∼1 kb, ∼960 bp and ∼1 kb corresponding to Nsg-sgRNA 1, 2 and 3, respectively. The results of agarose gel electrophoresis indicated that sg-Scarlet1 and sg-Scarlet2 could induce target DNA breaks (Fig. 4B, lanes 2 and 3). However, sg-Scarlet3 had no digestion effect *in vitro* (Fig. 4B, lane 4). To ensure that *Nh-scarlet* was mutated, all three sgRNAs were injected into the embryo to generate a mutant phenotype.

Following injection of these three sgRNAs, embryos were cultured to the gastrula stage, genomic DNA was extracted, PCR amplification of *scarlet* was carried out with primers ScaF and ScaR (Table S1) and the fragments were sequenced. The sequencing results revealed a double peak at the target sites of sg-Scarlet1 and sg-Scarlet2 (Fig. 4C), indicating that there were mutant genotypes among these clones. Then, the mutant (MT) *Nh-scarlet* PCR fragments were cloned into the pMD-18T vector for sequencing. The results confirmed that sg-Scarlet1 induced two types of mutation: a 156 bp fragment deletion and some base substitutions (Fig. 4D; sgRNA1), whereas sg-Scarlet2 induced a 3-base deletion and a 2-base substitution (Fig. 4D; sgRNA2).

Embryos injected with all three sgRNAs were cultured until they reached the flea larva stage for screening of *Nh-scarlet* mutated phenotype. Embryos whose eyes were different from those of WT embryos were collected for DNA sequencing. Comparison between WT and MT embryos mostly showed less pigment deposition in MT embryo eyes (Fig. 5B,C). Moreover, in some MT embryos, the eye had disappeared and a translucent orbit was visible (Fig. 5D). Sequencing results showed that multiple fragments of *Nh-scarlet* had been deleted in MT embryos. Individual sequencing results revealed a 10-base insertion at the sg-Scarlet2 targeting site, and some base substitutions at the sg-Scarlet1 and sg-Scarlet3 targeting sites (Fig. 6A); in some individuals, sg-Scarlet1 and sg-Scarlet2 targeting sites had been deleted from *Nh-scarlet*. Moreover, sg-Scarlet3 induced two types of mutation: base deletion and fragment deletion (Fig. 6B). Other sequences showed that sg-Scarlet1 and sg-Scarlet2 targets had also been deleted, and the sg-Scarlet3 targeting site displayed several base deletions and replacements (Fig. 6C). A summary of the mutations is given in Table 2.

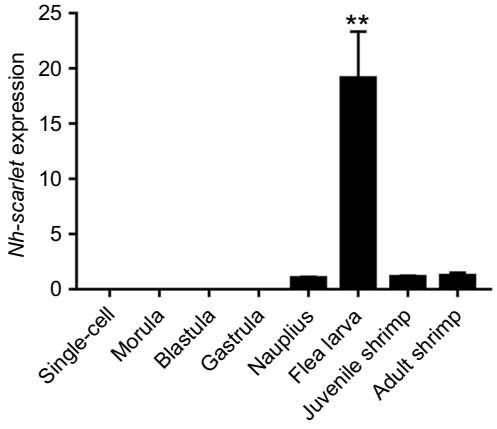

Fig. 3. Expression levels of *Nh-scarlet* in different *N. heteropoda* embryo developmental stages. Expression level of *Nh-scarlet* in the single-cell, morula, blastula, gastrula, nauplius and flea larva stages, and in the eyes of juvenile and adult shrimps (flea larva versus nauplius, P=0.0018; flea larva versus juvenile shrimp, P=0.0018; flea larva versus adult shrimp, P=0.0019; nauplius versus juvenile shrimp, P=0.3858; nauplius versus adult shrimp, P=0.3059; juvenile shrimp versus adult shrimp, P=0.5769; one-way ANOVA).

Mutation of *Es-scarlet*

We assessed whether our microinjection system could successfully deliver the CRISPR/Cas9 complex into an economically important freshwater crustacean species, *E. sinensis*, to target the *Es-scarlet* gene. After determining the partial genomic sequence of *scarlet*, a sgRNA was designed and tested *in vitro* (data not shown). Es-sgRNA1 (Fig. 7A) was injected together with Cas9 protein into the embryos. After *in vitro* hatching, about 10% of the injected embryos survived and were screened for eye phenotype (Table 2). Compared with normal crab zoeae (Fig. 7B; Movie 1), about 50% of the zoea survivors showed red eyes and red pigment aggregation in the form of granules (Fig. 7C,D; Movie 2). Extraction and sequencing of genomic DNA from one zoea revealed a 694 bp deletion at the 5′ end of Es-sgRNA1 (Fig. 7E).

DISCUSSION

For most arthropods, collecting embryos for microinjection as early as possible after oviposition is an effective method to avoid egg membrane hardening (Bassett et al., 2013; Itokawa et al., 2016; Khan et al., 2017; Markert et al., 2016; Tanaka et al., 2016). However, if embryos of *N. heteropoda* are collected too early after oviposition, manipulation may damage them and prevent normal development. Moreover, embryos may need time to form an intact egg membrane that is strong enough to resist the impact of the environment. For example, embryos of *P. hawaiensis* should be collected for microinjection at 1 h post-spawning to ensure that eggs have a hard outer chorion (Rehm et al., 2009). Our results showed that collecting *N. heteropoda* embryos at 4 h post-spawning results in eggs with a harder outer chorion that protects them against damage.

To avoid embryo component leakage induced by the difference in osmotic pressure inside and outside the embryo, researchers usually try to modify the osmotic pressure to slightly dehydrate the embryos. High concentration medium or reagent is used to modify the osmotic pressure. For example, to modify the osmotic pressure of *D. magna* and *Daphnia pulex* embryos, M4 medium with 80 and 60 mmol l^{-1} sucrose has been used (Hiruta et al., 2013; Kato et al., 2011). However, *N. heteropoda* and their embryos should be cultured in freshwater; highly concentrated medium or reagents may lead to embryo death (Viau et al., 2016). Thus, it was necessary to establish a method that altered osmotic pressure without using a reagent. To avoid embryo component leakage, the embryos of *V. cardui* are placed in a desiccant chamber to dehydrate them before microinjection (Zhang and Reed, 2016). However, dry conditions are detrimental to the survival of *N. heteropoda* embryos (Sonakowska et al., 2015). For microinjection of pea aphids, embryos are placed on a wet filer paper to keep them hydrated (Le Trionnaire et al., 2019). By combining the methods for *V. cardui* and pea aphid, and considering the characteristics of *N. heteropoda* embryos, we developed injection conditions in which the upper side of the embryos was exposed to air and the lower side was immersed in water.

Journal of Experimental Biology (2022) 225, jeb243702. doi:10.1242/jeb.243702

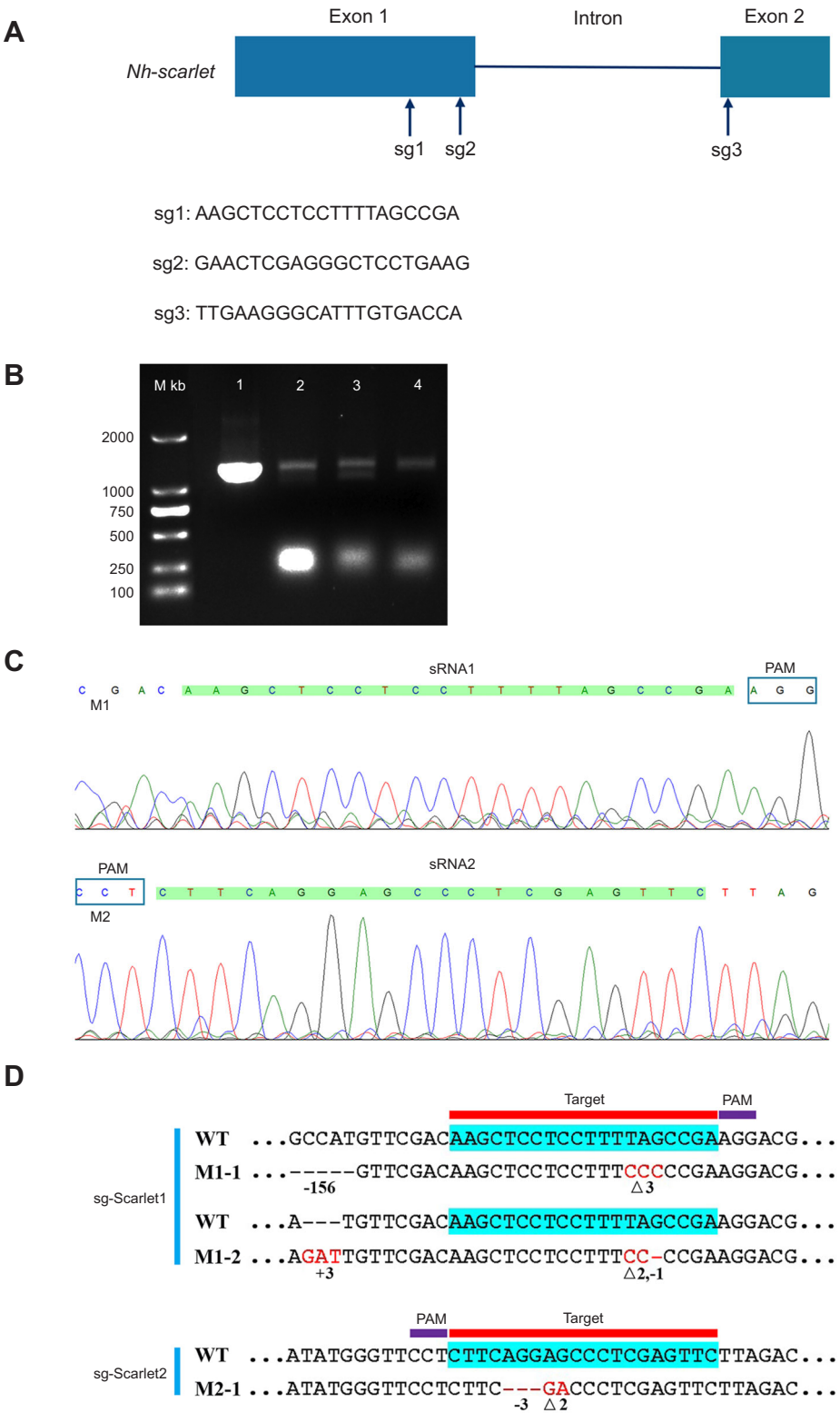

Fig. 4. Mutation of *Nh-scarlet* in *N. heteropoda* embryos and sequencing of mutant (MT) *Nh-scarlet*. (A) Target sites of sg-Scarlet1, sg-Scarlet2 and sg-Scarlet3 in *Nh-scarlet*. (B) Agarose gel electrophoresis analysis of sgRNA digestion effects *in vitro*. M, DL-2000 DNA markers; 1, wild-type (WT) *Nh-scarlet*; 2, digestion products of sg-Scarlet1; 3, digestion products of sg-Scarlet2; 4, digestion products of sg-Scarlet3. (C) Following injection with the three sgRNAs and *in vitro* culturing, embryos were collected and *Nh-scarlet* PCR products were sequenced. There were double peaks around the target sites of sg-Scarlet1 and sg-Scarlet2 on the sequencing map but no double peaks in the sg-Scarlet3 group. (D) *Nh-scarlet* fragments were amplified and cloned into the pMD18-T vector for sequencing. The Nsg-Scarlet1 and Nsg-Scarlet2 groups showed mutations at the target sites. No mutation was detected in Nsg-Scarlet3 group. PAM, protospacer adjacent motif.

Although a previous study reported that dextran is safe for cells and embryos (Li et al., 2015a), it induced higher mortality than Phenol Red when used as a tracer in our study (Table 1), and it did not spread in embryos (Fig. 2). This may be because dextran is water soluble and cannot dissolve in yolk, the main components of which are lipids (Bai et al., 2011; Luby-Phelps, 1988; Yang et al., 2015). Moreover, dextran may be blocked outside the cell membrane during cell remodeling, and this may explain why it is insoluble in *N. heteropoda* embryos (Ludtke et al., 2002). Many studies have used 0.05–0.5% Phenol Red as a tracer with Cas9 protein and sgRNAs (Gui et al., 2016; Martin et al., 2016; Rehm et al., 2009). The survival rate of embryos injected with a Phenol Red tracer was better than that of embryos injected with dextran in the present study (Table 1). Thus, Phenol Red was used as a tracer in subsequent

Journal of Experimental Biology (2022) 225, jeb243702. doi:10.1242/jeb.243702

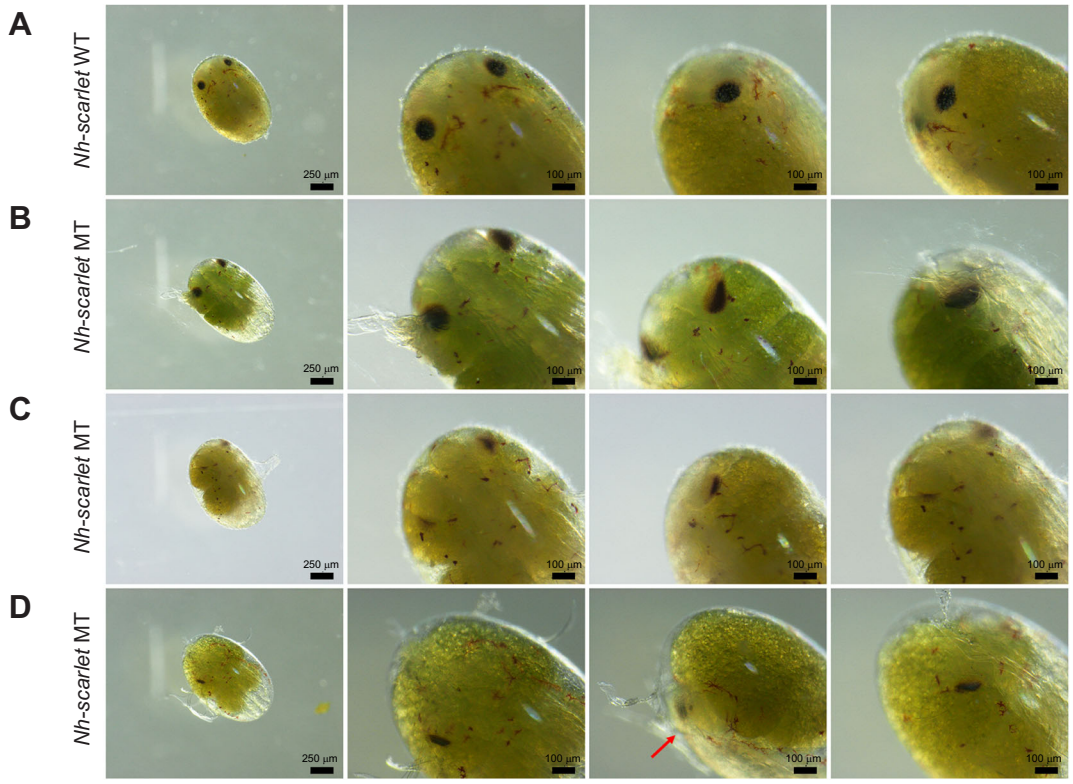

Fig. 5. *Neocaridina heteropoda* eye color following *Nh-scarlet* mutation. (A) WT embryos with normal eye color and shape. (B–D) MT embryos injected with all three sgRNAs and Cas9 protein showed altered eye color compared with that of WT embryos (A); in some, the eyes had almost disappeared (C); and in some, one eye had disappeared (D; the red arrow indicates the translucent orbit). Scale bars: 100 μm.

experiments. The reason why dextran induced higher mortality in *N. heteropoda* embryos requires further study. Studies on *P. hawaiensis* have shown that embryos can survive even if some leakage of embryo components occurs after microinjection (Martin et al., 2016; Rehm et al., 2009). Thus, in the present study, embryos showing slight leakage were not removed from the filter paper, and some survived (Fig. S1).

Scarlet forms a complex with White to transport a tryptophan-derived precursor, 3-hydroxykynurenine, from the cytosol to pigment granules, resulting in the generation of a brown-colored ommochrome pigment, whereas the White and Brown heterodimer transports a guanine-derived precursor that leads to the production of the bright red pigment drosopterin (Ewart and Howells, 1998). Thus, *white* appears to be more important in eye color pigment

Fig. 6. Sequencing results for *N. heteropoda* embryos displaying altered eye color and shape. The blue shading indicates the target sequence, and the pink shading indicates the PAM sequence. Δ represents the total number of replaced bases;+represents the total number of inserted bases;–represents the total number of deleted bases. (A) For embryos in Fig. 5B, the sequencing results indicate that mutation occurred only in the sg-Scarlet2 targeting site. (B) For embryos in Fig. 5C, the sequencing results indicate that mutation occurred in the targeting sites of all three sgRNAs. (C) In embryos in Fig. 5D, the sequencing results indicate that mutation occurred in the targeting sites of all three sgRNAs in the *Nh-scarlet* genome.

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Table 2. Summary of gene-edited mutations

Species	Phenotype	Gene mutation	No. of mutants	No. survived	% Mutants
Neocaridina heteropoda	Altered eye shape	Insertion/replacement/deletion	2	28	7
	Eye disappeared	Insertion/replacement/deletion	1	28	3
Eriocheir sinensis	Altered eye color	Deletion	8	17	47

transport pathways. However, there are multiple white orthologs in *D. magna*, and mutated *scarlet* also induced a change in eye color (Ismail et al., 2018), consistent with the results of *N. heteropoda* transcriptome analysis. It is time consuming to identify which *white* gene is involved in eye color determination in *N. heteropoda*; hence, we selected *Nh-scarlet* as the target gene to generate the mutant phenotype.

Before use, sgRNAs should be tested for digestion activity *in vitro* (Hum and Loots, 2016). In this study, sg-Scarlet1 and sg-Scarlet2 performed well for target gene digestion. However, digestion by sg-Scarlet3 could not be detected by agarose gel electrophoresis (Fig. 4B). Studies indicate that if the digestion efficiency of sgRNAs is too low, the results of *in vitro* digestion may be difficult to observe by agarose gel electrophoresis (She et al., 2018). Thus, we suggest two reasons for the digestion results for sg-Scarlet3: sg-Scarlet3 may have no digestion activity toward the target sequence, or the digestion efficiency of sg-Scarlet3 may be too low to be detected by agarose gel electrophoresis. Considering that gene-editing efficiency may be affected by DNA structure and to make sure that *Nh-scarlet* had been completely mutated, the mutated phenotype was assessed after injecting all three sgRNAs into *N. heteropoda* embryos simultaneously.

Some embryos that received an injection of all three sgRNAs displayed a different eye phenotype with reduced pigment. Others displayed altered eye shape, and eyes were completely absent in some cases (Fig. 5). In *D. magna*, mutation of *scarlet* impacted the pigment transportation process, inducing eye transparency (Ismail et al., 2018). In *Nilaparvata lugens* lacking *scarlet*, a mosaic eye was observed, reflecting partial loss of pigment (Jiang and Lin, 2018). Our results are generally consistent with those of previous studies. The sequencing results proved that in embryos showing slight pigment loss, *Nh-scarlet* had a 10-base insertion (Figs 5B and 6A). In embryos showing severe eye abnormalities, *Nh-scarlet* gene showed large fragment deletion at sg-Scarlet1 and sg-Scarlet2 targeting sites, as well as insertion and replacement at the sg-Scarlet3 targeting site (Fig. 6B,C). These results also indicate that sg-Scarlet3 works *in vivo*.

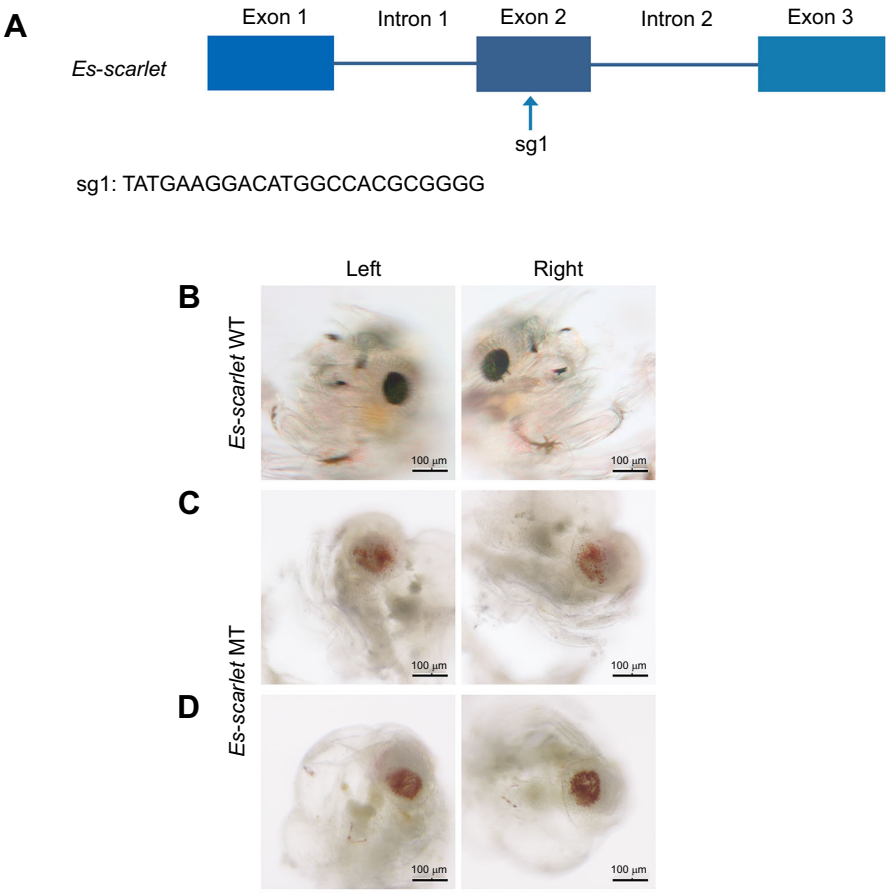

Fig. 7. *Eriocheir sinensis* eye color and sequencing verification following *Es-scarlet* mutation. (A) Target sites of sg-Scarlet1 in *Es-scarlet*. (B) WT embryos with normal eye color and shape. (C,D) Embryos injected with sg-Scarlet1 and Cas9 protein showed red eyes. Scale bars: 100 μm. (E) Sequencing results of one red-eye zoea. The blue shading indicates the target sequence, and the pink shading indicates the PAM sequence.–represents the total number of deleted bases.

Some embryos contained two types of mutated *Nh-scarlet* (Fig. 6C). This may have occurred as a result of cell division because gene editing by CRISPR/Cas9 is only highly effective at certain stages in the cell cycle, such as G1, S and G2 (Lin et al., 2014). If Cas9 protein and sgRNAs are delivered into embryos at other cell cycle stages, then they may separate randomly into the two daughter cells (Yan and Yu, 2008; Elaswad et al., 2018), leading to different edited gene types.

In summary, we delivered CRISPR/Cas9 into *N. heteropoda* and *E. sinensis* embryos by microinjection and successfully mutated *Nh-scarlet* and *Es-scarlet*. However, the gene-editing efficiency was only 10% for *N. heteropoda* (three embryos with mutant phenotypes out of 28 injected and surviving embryos). Injection of *E. sinensis* embryos was able to achieve 47% gene-editing efficiency but mortality of *in vitro* hatching was still high. This technical system needs to be further improved. Moreover, because there is no genome information available for *N. heteropoda*, the off-target effects and digestion accuracy need to be investigated in future work.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.L.; Methodology: R.L., Q.M., J.Q.; Software: J.Y.; Validation: J.Q.; Formal analysis: Q.M., J.H.; Investigation: L.H.; Resources: R.L., J.H., J.S.; Data curation: R.L., L.H.; Writing - original draft: R.L., Q.M.; Writing - review & editing: R.L., J.S.; Visualization: J.S.; Supervision: R.L., J.S.; Project administration: J.S.; Funding acquisition: Y.Z., J.S.

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