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(54) **CONSTRUCTION METHOD FOR GENE EDITING SYSTEM OF APOSTICHOPUS JAPONICUS**

(57) The present invention belongs to the field of aquatic genetic breeding, and particularly relates to a construction method for a gene editing system of *Apostichopus japonicus.* Sperms and ova of *Apostichopus japonicus* are acquired, the ova are immobilized and arranged into a single cell row and fertilized, a prepared mixed solution containing the sgRNA of target genes of *Apostichopus japonicus* is injected into the fertilized ova of *Apostichopus japonicus* arranged into a single cell row, the injected dose is 8%-12% of the volume of a *Apostichopus japonicus* embryo, and embryos after microinjection are incubated at 23 °C, so as to obtain a gene editing system of *Apostichopus japonicus*. With the construction method of the present invention, embryos with expected phenotype can be obtained after development to a target stage, and the success rate is more than 90%. The application of the present invention can significantly enhance the breeding efficiency, realize site-directed and accurate alteration of DNA sequences of *Apostichopus japonicus*, obtain a new variety of *Apostichopus japonicus* with target traits, and improve the product quality and economic benefits of *Apostichopus japonicus*.

Dit octrooi is verleend ongeacht het bijgevoegde resultaat van het onderzoek naar de stand van de techniek en schriftelijke opinie. Het octrooischrift komt overeen met de oorspronkelijk ingediende stukken.

CONSTRUCTION METHOD FOR GENE EDITING SYSTEM OF APOSTICHOPUS JAPONICUS

Technical Field

5 The present invention belongs to the field of aquatic genetic breeding, and particularly relates to a construction method for a gene editing system of *Apostichopus japonicus*.

Background

10 Gene editing is a new genetic engineering technology that can modify specific target genes in the genome of an organism accurately. In the breeding process, the gene editing technology can realize the modification and alteration of genetic loci of target traits in varieties, so as to accelerate the improvement of varieties. The gene editing technology is still in the initial development stage in animal breeding, but has 15 a broad prospect in genetic breeding with genome selection as the core, and can achieve efficient and accurate improvement of target traits of target species. Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 are three different gene editing tools. The CRISPR/Cas system is essentially an 20 RNA-guided nuclease. Unlike ZFN and TALEN nucleases which recognize target sequences through protein-DNA interaction, the CRISPR/Cas nuclease recognizes target sequences through RNA and DNA base pairing. The CRISPR/Cas9 provides a low-cost, efficient and easy-to-use gene editing system. Compared with TALENS, CRISPR adopts small proteins that are easy to deliver, so gene editing is faster; and 25 compared with ZFN, CRISPR can target a specific locus without pairs of proteins, so CRISPR is more convenient to use. CRISPR/Cas has the ability of promoting multiple genomic modifications at one time, and is much easier to design than the other two DNA nucleases.

Compared with other marine economic animals, *Apostichopus japonicus* will 30 take a longer time to reach sexual maturity, which is usually more than 3 years. Therefore, the breeding cycle of a new variety is at least 12-13 years, and the breeding is limited by the factors such as long breeding cycle and low breeding efficiency. In addition, as the key quantitative traits such as length, weight, number of

parapodia, length of parapodia and color are difficult to be determined quantitatively and qualitatively, the analysis of basic quantitative genetic characteristics is not clear. Therefore, the traditional breeding technologies represented by hybrid breeding and selective breeding can no longer meet the current technical requirements on
5 timeliness and reliability of breeding good varieties of *Apostichopus japonicus*. As a result, it is an urgent need to explore molecular markers with breeding value, carry out the optimal design and breeding of new color varieties (species) of *Apostichopus japonicus* with excellent target traits, establish a gene editing and breeding technology for *Apostichopus japonicus*, and realize an accurate breeding system of
10 new color varieties of *Apostichopus japonicus*, thus to achieve the healthy and sustainable development of the *Apostichopus japonicus* industry.

With the improvement and development of the CRISPR technology, gene editing has become more and more widely used in aquatic animals. Aquatic products are the third largest source of animal protein in the world, and aquatic animals provide an
15 economical and high-quality animal protein. Therefore, the application of the gene editing technology in aquatic animals enables us to obtain more fishery resources with high quality, and contributes to the healthy and sustainable development of fisheries. At present, CRISPR/Cas9 has been successfully applied to fish such as zebra fish, tilapia, Atlantic salmon, carp, grass carp and tonguefish and to shellfish
20 such as *Crepidula fornicata*, *Lymnaea stagnalis*, cuttlefish and *Crassostrea gigas*. However, for *Echinodermata*, CRISPR/Cas9 has only been successfully applied to sea urchins. *Apostichopus japonicus* is an important representative species of *Echinodermata*, but no gene editing system has been established yet, and the research on microinjection of *Apostichopus japonicus* is urgently needed.
25 Taking zebra fish with relatively mature application of gene editing as an example, the preliminary preparation process of microinjection is generally as follows: on the day before injection, adult zebra fish are placed in breeding tanks with a female to male ratio of 1:1 or 2:1, and each breeding tank is separated by a partition board. On the morning the day of injection, the partition boards are removed, and the
30 female and male zebra fish begin to mate. Generally, the parent fish will oviposit and make ova fertilized after about more than 10 minutes. Generally, the partition boards of two tanks are removed at the first time, and then the partition boards of the other tanks are removed in due time according to the progress of the experiment. To save

the time for cleaning embryos, an inner tank together with parent fish can be transferred to another clean outer tank before a partition board is removed. After parent fish oviposit, ova on the bottom of the outer tank are collected by a filter screen with a bore diameter of 180 μm and washed with aquaculture water for 2-3 times. Fertilized ova shall be collected within 20 minutes as far as possible (i.e., for the parent fish in one tank, the time interval between two collections of fertilized ova shall not exceed 20 minutes, so as to ensure the consistency of development stages). After the fertilized ova are sorted and cleaned, the collected ova are placed in a plate petri dish through a pipette, foreign matters in the dish and water around embryos are removed, and the embryos are treated by dry injection.

Due to species specificity, the method for acquiring sperms and ova of zebra fish and the like is not suitable for *Apostichopus japonicus*, and even the method for acquiring sperms and ova of a closest relative of *Apostichopus japonicus*, sea urchins, through injection of KCL is also not suitable for *Apostichopus japonicus*. In addition, zebra fish and the like are treated by dry injection, but *Apostichopus japonicus* cannot be treated by dry injection, as *Apostichopus japonicus* will die in large numbers during dry injection. Therefore, it is extremely urgent to establish a special gene editing system suitable for *Apostichopus japonicus*.

Summary

In order to realize the construction of an accurate breeding system of a new variety of *Apostichopus japonicus* body color and solve the difficulties of the site-directed precision technology in the genetic breeding of *Apostichopus japonicus*, the present invention aims to provide a construction method for a gene editing system of *Apostichopus japonicus*, so as to obtain a new variety of *Apostichopus japonicus* with excellent target traits, thus providing support for the healthy development of the *Apostichopus japonicus* industry.

To achieve the above purpose, the present invention adopts the following technical solution:

A construction method for a gene editing system of *Apostichopus japonicus*, comprising the steps of acquisition of sperms and ova, immobilization for microinjection, sgRNA design of target genes, preparation of an injection system, and microinjection, wherein sperms and ova of *Apostichopus japonicus* are acquired, the

ova are immobilized and arranged into a single cell row and fertilized, a prepared microinjection mixed solution containing sgRNA of target genes of *Apostichopus japonicus* is injected into the fertilized ova of *Apostichopus japonicus* arranged into a single cell row, the injected dose is 8%-12% of the volume of a *Apostichopus*
5 *japonicus* embryo, and embryos after microinjection are incubated at 23℃, so as to obtain a gene editing system of *Apostichopus japonicus*.

The obtained ova of *Apostichopus japonicus* are immobilized in a petri dish containing a protamine sulfate solution and arranged in a single cell row, and then semen is added to the petri dish to fertilize the ova; and the immobilized fertilized
10 ova of *Apostichopus japonicus* are injected with a microinjection mixed solution containing the sgRNA of target genes of *Apostichopus japonicus* under a microscope.

The immobilization is realized by drawing a line on the back surface of the bottom of the petri dish for microinjection, rinsing the inner part of the petri dish with a 0.1%-1% protamine sulfate solution, and then using a sucking mouth part to orderly
15 arrange the ova in the position with the line on the surface of an injection dish coated with protamine sulfate.

The rinsing is realized by applying a 0.1%-1% protamine sulfate solution to the petri dish, draining, and bleaching with deionized water immediately, or drying, and bleaching with deionized water, for use.

20 The acquisition of ova of *Apostichopus japonicus* is realized by promoting maturation of *Apostichopus japonicus* by using a neuropeptide NGIWY-NH2 to obtain ova.

NGIWY-NH2 (10 μM) is injected into *Apostichopus japonicus* body cavities, the injected dose is about 0.1% (v/w) of *Apostichopus japonicus*, *Apostichopus*
25 *japonicus* discharges ova 15-20 minutes after shaking, and the ova of *Apostichopus japonicus* are collected.

The mixed solution containing the sgRNA of target genes of *Apostichopus japonicus* comprises 500 ng of Cas9 albumen, 300 ng of the sgRNA of target genes, 0.2 μg of 20% glycerine, 0.2 μl of FITC and 0.16 μl of RNase-free water per
30 microliter.

The concentration ratio of Cas9 albumen to sgRNA in the microinjection mixed solution containing the sgRNA of target genes of *Apostichopus japonicus* is 30:1 to 1:1.

The sgRNA of *Apostichopus japonicus* for gene editing related to growth and development is acquired by designing gRNA sequences according to CRISPRscan (http://www.crisprscan.org/?page=sequence) and selecting gRNAs with high scores according to CRISPRscan score, and when the microinjection mixed solution is prepared, the sequences can be used individually or in a mixed manner or in a combined manner.

Further, the steps of constructing a gene editing system of *Apostichopus japonicus* are as follows:

1) Collecting and acquiring breeding *Apostichopus japonicus* in Laizhou sea area of Yantai in Shandong province in May. Sperms of *Apostichopus japonicus* are obtained by dissection, and ova of *Apostichopus japonicus* are obtained by promoting maturation by injecting a neuropeptide NGIWY-NH2. NGIWY-NH2 (10 μM) is injected into *Apostichopus japonicus* body cavities, and the injected dose is about 0.1% (v/w) of *Apostichopus japonicus*. After the polypeptide is injected, *Apostichopus japonicus* begins to shake, and discharges sperms or ova after about 15 minutes.

2) Rinsing a petri dish for microinjection with a 0.1%-1% protamine sulfate solution, drawing a 1/3 line from the edge on the back surface of the bottom of the petri dish, and scratching behind this line. Applying protamine sulfate to the petri dish, draining, and bleaching with deionized water immediately, or drying, and bleaching with deionized water, and then storing in a dust-free container for later use. Using a sucking mouth part to orderly arrange the ova on the surface of an injection dish coated with protamine sulfate. Arranging the ova in a row greatly facilitates rapid and successful microinjection. At the beginning of microinjection, injecting 1 ul of diluted semen into the petri dish with a pipettor to finish fertilization.

The 0.1%-1% protamine sulfate solution is prepared as follows: adding 0.4-0.5 g of protamine sulfate to 40-400 ml of distilled water, and preparing a 0.1%-1% protamine sulfate solution in a 50 ml bacteria-free taper pipe until the protamine sulfate is dissolved completely (> 1 h). The solution can be used for at least three months if stored at 4℃. Some protamine sulfate will precipitate after being stored at 4℃, and the solution shall be heated to room temperature for the protamine sulfate to be dissolved completely in use every time.

3) Designing gRNA for gene editing of *Apostichopus japonicus* by using

CRISPRscan (http://www.crisprscan.org/?page=sequence). A target locus contains two guanine nucleotides at the 5' end for initial gRNA transcription using T7 RNA polymerase, while the 3' end is adjacent to an NGG motif (PAM) in Endo16 Module A. For preliminary evaluation, gRNAs with high CRISPRscan scores are considered
5 first. CRISPRscan provides a guide sequence with a T7 sequence and a tail sequence. Ordering selected gRNA sequences provided by CRISPRscan and 80-nucleotide tail primer sequences from Eton, and annealing and extending gRNA and tail primers through PCR by using Phusion Master mix (Phusion High-Fidelity PCR Master Mix with HF Buffer). Then purifying gRNA by a QIAquick PCR purification kit.
10 Carrying out in vitro transcription by using a MEGAshortscript™ T7 Transcription Kit, and carrying out purification through alcohol precipitation.

4) Preparing a microinjection mixture containing Cas9 albumen, sgRNA, 20% glycerine, FITC and RNase-free water, with the final volume of 5 ul. The concentration range of the Cas9 albumen is 250 ng/ul-750 ng/ul. The concentration
15 range of sgRNA is 100 ng/ul-400 ng/ul. Before microinjection, placing the mixture on ice.

5) Injecting the solution into the fertilized ova of *Apostichopus japonicus* immobilized in the petri dish. The diameter of the injected solution is about 1/3-1/4 of an embryo (less than 25% of the volume of an embryo). Experimental controls shall
20 include Cas9 protein injection alone, without sgRNA, to evaluate the effects of exogenous protein injection and expression. Incubating injected embryos and control embryos at 23℃. After the embryos reach the desired stage, extracting genomic DNA for genotyping, and extracting RNA for gene expression level assessment and imaging.

25 6) If sgRNA/Cas9 injected embryos have no detectable phenotype, increasing the injection concentration of sgRNA/Cas9. However, a high dose of Cas9 protein is toxic to *Apostichopus japonicus* embryos. On the other hand, if the injected embryos exhibit a severe nonspecific phenotype, reducing the injection concentration. The proportion of the Cas9 albumen amount to sgRNA can be adjusted (from 30:1 to 1:1).
30 Through tests, the gene editing efficiency of *Apostichopus japonicus* can reach more than 90%.

The injection site in step 1) is the abdomen of breeding *Apostichopus japonicus*.

The petri dish for microinjection in step 2) is rinsed with 0.1%-1% protamine

sulfate, the protamine sulfate will produce a solution with positive charges, and the negatively charged surface of an embryo will adhere to the solution. Immobilizing embryos in this manner is conductive to rapid injection without affecting development.

5 The present invention has the advantages and positive effects that:

1. The present invention can efficiently and stably acquire ova of *Apostichopus japonicus* in the breeding season for microinjection; and the petri dish rinsed with protamine sulfate can be used to immobilize ova of *Apostichopus japonicus* without affecting fertilization and development of the ova.

10 2. The microinjection system and injection method for gene editing of *Apostichopus japonicus* explored in the present invention can avoid the damage of *Apostichopus japonicus* embryos, and the editing efficiency can reach more than 90%.

3. The application of the present invention can significantly enhance the

15 breeding efficiency, realize site-directed and accurate alteration of DNA sequences of *Apostichopus japonicus*, obtain a new variety of *Apostichopus japonicus* with target traits, and improve the product quality and economic benefits of *Apostichopus japonicus*.

20 **Description of Drawing**

Fig. 1 is a schematic diagram of ova immobilized for microinjection provided in an embodiment of the present invention;

Fig. 2 is a schematic diagram of arranging ova with a sucking mouth part provided in an embodiment of the present invention.

25

Detailed Description

Detailed description of the present invention is further illustrated below in combination with examples. It shall be noted that the detailed description described herein is only used to illustrate and explain the present invention, not limited to the

30 present invention.

Breeding *Apostichopus japonicus* used for the following experiments is obtained from Laizhou sea area of Yantai in Shandong province.

Embodiment 1

In the embodiment, a gene editing system of *Apostichopus japonicus* is constructed for the key gene ALX1 in the skeleton of *Apostichopus japonicus* embryos based on the CRISPR/Cas9 technology:

5 1) Design of gRNA sequences for ALX1 gene of *Apostichopus japonicus* and synthesis of sgRNA:

Designing gRNA sequences of the ALX1 gene of *Apostichopus japonicus* by using CRISPRscan (http://www.crisprscan.org/?page=sequence). Entering the DNA sequences of the ALX1 gene of *Apostichopus japonicus* in the "Submit sequence"
10 page of the CRISPRscan website, selecting "Sea urchin - Strongylocentrotus purpuratus", "No mismatch", "Cas9-NGG" and "In vitro T7 promoter", and then clicking the "Get sgRNAs" button to obtain a list of gRNA sequences.

A target locus contains two guanine nucleotides at the 5' end for initial gRNA transcription using T7 RNA polymerase, while the 3' end is adjacent to an NGG motif
15 (PAM) in ALX1. According to CRISPRscan score (Moreno-Mateos et al., 2015) provided in the CRISPRscan website and blast alignment of the gRNA sequences with the *Apostichopus japonicus* genome, four gRNA sequences with high CRISPRscan scores and fewer identical sequences after alignment are selected for subsequent experiments (see Table 1). Ordering target gRNA sequences provided by
20 CRISPRscan and tail primer sequences (GTTTTAGAGCTAGAA), and annealing and extending gRNA and tail primers through PCR by using Phusion Master mix (Phusion High-Fidelity PCR Master Mix with HF Buffer). Then purifying gRNA by a QIAquick PCR purification kit. Carrying out in vitro transcription by using a MEGAshortscript™ T7 Transcription Kit, and carrying out purification through
25 alcohol precipitation to obtain sgRNA.

Table 1 gRNA Sequences of Alx1 Gene of *Apostichopus japonicus* Designed in This Experiment

Name	Sequence (5' to 3')
gRNA1	GGGGCTTATCGAGGTGGCGT
gRNA2	GGGTTGGCGCCGCCCGGCTC
gRNA3	GGGTTCGACTCTCGCCGCAT
gRNA4	GGAGGCGGCTAACTCGTGTA

2) Preparation of microinjection mixed solution:

Preparing a microinjection mixture containing Cas9 albumen (purchased from

GenScript), sgRNA, glycerine with the mass concentration of 20%, FITC fluorescent dye (purchased from Molecular Probes) and RNase-free water, with the final volume of 5 ul. Before preparation, the concentration of the Cas9 albumen is 2.5 μ g/ul, and the concentration of sgRNA is 1.25 μ g/ul. Before microinjection, placing the
5 mixture on ice of 4℃.

5 ul of microinjection mixture in experiment group 1 comprises 1 ul of Cas9 albumen, 1 ul of 20% glycerine, 1 ul of dye (FITC), 1.2 ul of sgRNA (0.3 ul of sgRNA1, 0.3 ul of sgRNA2, 0.3 ul of sgRNA3 and 0.3 ul of sgRNA4), and 0.8 ul of RNase-free water. Finally, the injection concentration of the Cas9 albumen in the
10 experiment group 1 is 500 ng/ul, and the concentration of sgRNA is 300 ng/ul.

5 ul of microinjection mixture in experiment group 2 comprises 1.5 ul of Cas9 albumen, 0.8 ul of 20% glycerine, 1 ul of dye (FITC), 1.5 ul of sgRNA (0.375 ul of sgRNA1, 0.375 ul of sgRNA2, 0.375 ul of sgRNA3 and 0.375 ul of sgRNA4), and 0.2 ul of RNase-free water. Finally, the injection concentration of the Cas9 albumen
15 in the experiment group 2 is 750 ng/ul, and the concentration of sgRNA is 375 ng/ul.

5 ul of microinjection mixture in experiment group 3 comprises 0.5 ul of Cas9 albumen, 1 ul of 20% glycerine, 1 ul of dye (FITC), 1.0 ul of sgRNA (0.25 ul of sgRNA1, 0.25 ul of sgRNA2, 0.25 ul of sgRNA3 and 0.25 ul of sgRNA4), and 1.5 ul of RNase-free water. Finally, the injection concentration of the Cas9 albumen in the
20 experiment group 3 is 250 ng/ul, and the concentration of sgRNA is 250 ng/ul.

5 ul of microinjection mixture in experiment group 4 is the same as that in the experiment group 1, except the injected dose is different, which is used to test the influence of the injected dose on the gene editing effects of *Apostichopus japonicus*.

5 ul of microinjection mixture in a control group comprises 1 ul of Cas9
25 albumen, 1 ul of 20% glycerine, 1 ul of dye (FITC), and 2 ul of RNase-free water. Finally, the injection concentration of the Cas9 albumen in the control group is 500 ng/ul, and the concentration of sgRNA is 0.

3) Acquisition of sperms and ova of *Apostichopus japonicus*:

Obtaining sperms by dissecting *Apostichopus japonicus* from the above region;
30 and obtaining ova of *Apostichopus japonicus* by promoting maturation by injecting a neuropeptide NGIWY-NH2, specifically as follows: NGIWY-NH2 (10 μM) is injected into *Apostichopus japonicus* body cavities, and the injected dose is about 0.1% (v/w) of *Apostichopus japonicus*. After the polypeptide is injected,

Apostichopus japonicus begins to shake, and discharges sperms or ova after about 15 minutes.

4) Immobilization and fertilization of ova of *Apostichopus japonicus*:

Immobilizing the ova obtained above in a petri dish for microinjection, specifically as follows: adding 0.4 g of protamine sulfate to 40 ml of distilled water, and preparing a 1% protamine sulfate solution in a 50 ml bacteria-free taper pipe until the protamine sulfate is dissolved completely (> 1 h) for use. Drawing a line parallel to the diameter of the petri dish in the 1/3 position of the area of the petri dish on the back surface of the petri dish, then adding a 1% protamine sulfate solution to the petri dish to rinse the petri dish, draining, and bleaching with deionized water immediately, or drying, and bleaching with deionized water, for use; and storing in a dust-free container for later use. Finally, using a sucking mouth part to orderly arrange and immobilize the ova in the position with the marked line of the petri dish coated with protamine sulfate (see Fig. 1 and Fig. 2). At the beginning of microinjection, injecting 1 ul of diluted semen into the petri dish with a pipettor to finish fertilization.

5) Microinjection:

Placing the immobilized embryos under a stereomicroscope, focusing the embryos with a low power objective, gently lowering a needle tip, pushing the injection needle tip into the center of the field of view, and adjusting the position of the injection needle through fine adjustment of a micro operation system until the needle tip can be seen clearly. Further adjusting the focal distance of the microscope and the positions of the injection needle and embryos to achieve the best clarity of the embryos and the injection needle tip. Pushing a control level, and carefully inserting the needle to make the injection needle tip enter the embryos. Pushing a foot switch to inject a sample into the embryos. The injected dose of the experiment groups 1-3 and the control group is 10% of the volume of a *Apostichopus japonicus* embryo, generally 1 nL. The injected dose of the experiment group 4 is 15% of the volume of a *Apostichopus japonicus* embryo, generally 1.5 nL. Each of the four experimental groups and one control group is injected with 500 *Apostichopus japonicus* embryos. After injection, the success of the injection is judged according to the fluorescence in the embryos under a fluorescence microscope. According to the microscopy results, 488 embryos are successfully injected in the experiment group 1, 478 embryos are successfully injected in the experiment group 2, 490 embryos are successfully

injected in the experiment group 3, 450 embryos are successfully injected in the experiment group 4, and 489 embryos are successfully injected in the control group.

6) Embryo culture after injection:

Incubating the injected embryos in a 23℃ incubator until the stage of developing into auricularia larva.

7) Gene editing success rate

For normally developing *Apostichopus japonicus* embryos, each larva has only one solid skeleton at the stage of auricular larva, and the position of the skeleton is fixed, only on the right side of the back. However, at the early stage of the development of doliolaria, the number and positions of skeletons of the embryos begin to change, and some embryos begin to have two skeletal elements in different positions.

For the injected embryos in the experiment group 1, 450 living embryos develop to the stage of auricular larva, among which 439 embryos have skeletons disappeared, and the gene editing success rate is 97.5%; for the injected embryos in the experiment group 2, 419 living embryos develop to the stage of auricular larva, among which 365 embryos have skeletons disappeared, and the gene editing success rate is 87.1%; for the injected embryos in the experiment group 3, 446 living embryos develop to the stage of auricular larva, among which 387 embryos have skeletons disappeared, and the gene editing success rate is 86.7%; for the injected embryos in the experiment group 4, only 42 living embryos develop to the stage of auricular larva, the other injected embryos become malformed or die, 35 of 42 normal embryos have skeletons disappeared, and the gene editing success rate is 83.3%; and for the injected embryos in the control group, 459 living embryos develop to the stage of auricular larva, among which no embryo has skeletons disappeared, and the gene editing success rate is 0. The experiment proves that the gene editing system of *Apostichopus japonicus* is successfully constructed.

It can be seen from the above embodiment that the present invention constructs a gene editing breeding system of *Apostichopus japonicus* based on the CRISPR/Cas9 technology, solving (1) the problem that ova and sperms of *Apostichopus japonicus* for gene editing cannot be acquired in time; and meanwhile, the fixation and efficient microinjection of ova of *Apostichopus japonicus* of the present invention can be used to complete the injection of a large number of embryos in a short time, significantly

improving the experimental efficiency, so as to improve the gene editing efficiency according to the reagent ratio and the injected dose of the optimized microinjection system of *Apostichopus japonicus*. The present invention can realize site-directed and accurate genetic transformation of *Apostichopus japonicus*, be applied to different genes according to the target traits for breeding of *Apostichopus japonicus*, and realize the editing of a plurality of target genes and multiple target traits by constructing a gene editing system, so as to obtain an excellent variety of *Apostichopus japonicus* with target traits, thus providing a new way and method for breeding of *Apostichopus japonicus*.

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Conclusies

1. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus,* welke stappen omvat voor verkrijgen van sperma en eicellen, immobilisatie van eicellen voor micro-injectie, ontwerpen van sgRNA voor
5 doelgenen, voorbereiding van een injectiesysteem, en microinjectie, waarbij het sperma en de eicellen van *Apostichopus japonicus* verworven worden, de eicellen geïmmobiliseerd worden en in een enkele celrij geordend en bevrucht worden, een bereide gemengde oplossing bevattende sgRNA voor de doelgenen van *Apostichopus japonicus* geïnjecteerd wordt in de bevruchte eicellen van de *Apostichopus japonicus*
10 die in een enkele celrij geordend zijn, de geïnjecteerde dosis 8%-12% van het volume bedraagt van een *Apostichopus japonicus* embryo, en embryo's na microinjectie geïncubeerd worden bij 23°C, zodat een genbewerkingssysteem wordt verkregen voor *Apostichopus japonicus.*

15 2. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus* volgens conclusie 1, waarbij de verkregen eicellen van *Apostichopus japonicus* geïmmobiliseerd worden in een petrischaal welke een protaminesulfaatoplossing bevat en de eicellen geordend worden in een enkele celrij, en vervolgens het sperma aan de petrischaal wordt toegevoegd om de eicellen te
20 bevruchten; en de geïmmobiliseerde bevruchte eicellen van *Apostichopus japonicus* onder een microscoop geïnjecteerd worden met een gemengde microinjectie-oplossing welke de sgRNA voor de doelgenen van *Apostichopus japonicus* bevat.

25 3. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus* volgens conclusie 2, waarbij de immobilisatie gerealiseerd wordt door een lijn te trekken op de achterzijde van de bodem van de petrischaal welke gebruikt wordt voor de microinjectie, het binnenste gedeelte van de petrischaal te spoelen met een 0.1%-1% protaminesulfaatoplossing, en vervolgens een zuigmonddeel te

gebruiken om de eicellen ordelijk te rangschikken in positie met de lijn op het oppervlak van een injectieschaal bedekt met protaminesulfaat.

4. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus*
5 *japonicus* volgens conclusie 2, waarbij het spoelen wordt gerealiseerd door een 0.1%-1% protaminesulfaatoplossing op de petrischaal aan te brengen, de oplossing af te voeren, de petrischaal onmiddellijk te bleken met gedeïoniseerd water, of de petrischaal voor gebruik te drogen en te bleken met gedeïoniseerd water.

10 5. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus* volgens conclusie 1 of 2, waarbij het verkrijgen van de *Apostichopus japonicus* eicellen wordt gerealiseerd door de maturatie van *Apostichopus japonicus* te bevorderen door een neuropeptide NGIWY-NH2 te gebruiken.

15 6. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus* volgens conclusie 5, waarbij het NGIWY-NH2 (10 μM) geïnjecteerd wordt in lichaamsholten van *Apostichopus japonicus*, de geïnjecteerde dosis ongeveer 10%-15% van het volume van *Apostichopus japonicus* bedraagt, de *Apostichopus japonicus* 15-20 minuten na schudden eicellen loost, en de eicellen van *Apostichopus*
20 *japonicus* worden verzameld.

7. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus* volgens conclusie 1 of 2, waarbij de gemengde oplossing welke het sgRNA voor de doelgenen van *Apostichopus japonicus* bevat 500 ng Cas9 eiwit, 300 ng van
25 de sgRNA voor de doelgenen, 0.2 μg 20% glycerine, 0.2 μl FITC en 0.16 μl RNase-vrij water per microliter omvat.

8. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus japonicus* volgens conclusie 7, waarbij de concentratieverhouding tussen het Cas9

eiwit en sgRNA in de gemengde microinjectie-oplossing welke de doelgenen voor *Apostichopus japonicus* bevat 30:1 tot 1:1 is.

9. Werkwijze voor het construeren van een genbewerkingssysteem voor *Apostichopus*
5 *japonicus* volgens conclusie 7, waarbij het sgRNA voor de doelgenen van *Apostichopus japonicus* wordt verkregen door gRNA-sequenties te ontwerpen volgens CRISPRscan (http://www.crisprscan.org/?page=sequence) en gRNA's met hoge scores te selecteren volgens CRISPRscan-score, en de sequenties afzonderlijk, gezamenlijk of in combinaties gebruikt kunnen worden bij het bereiden van de
10 gemengde microinjectie-oplossing.

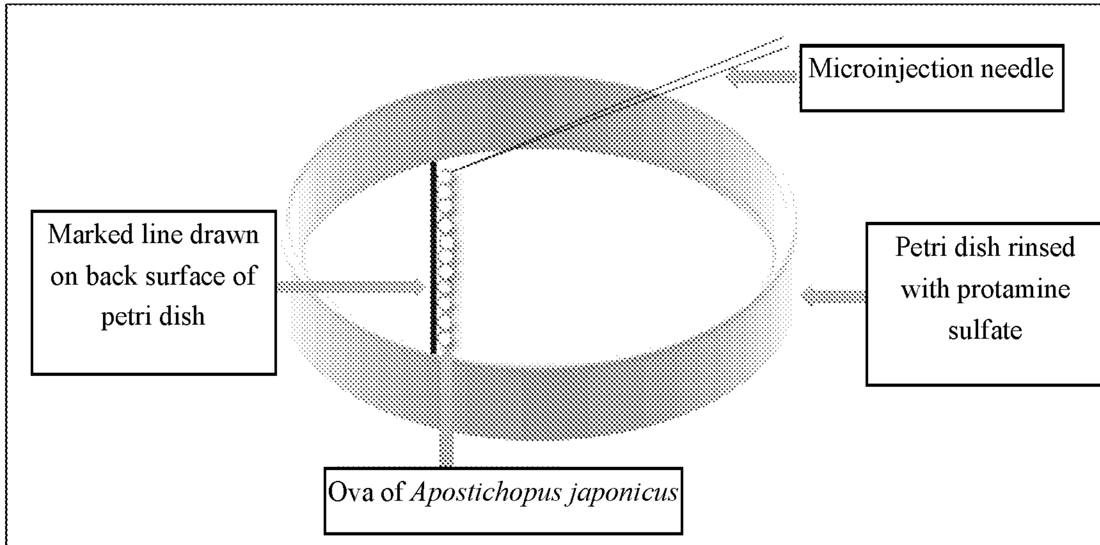

FIG. 1

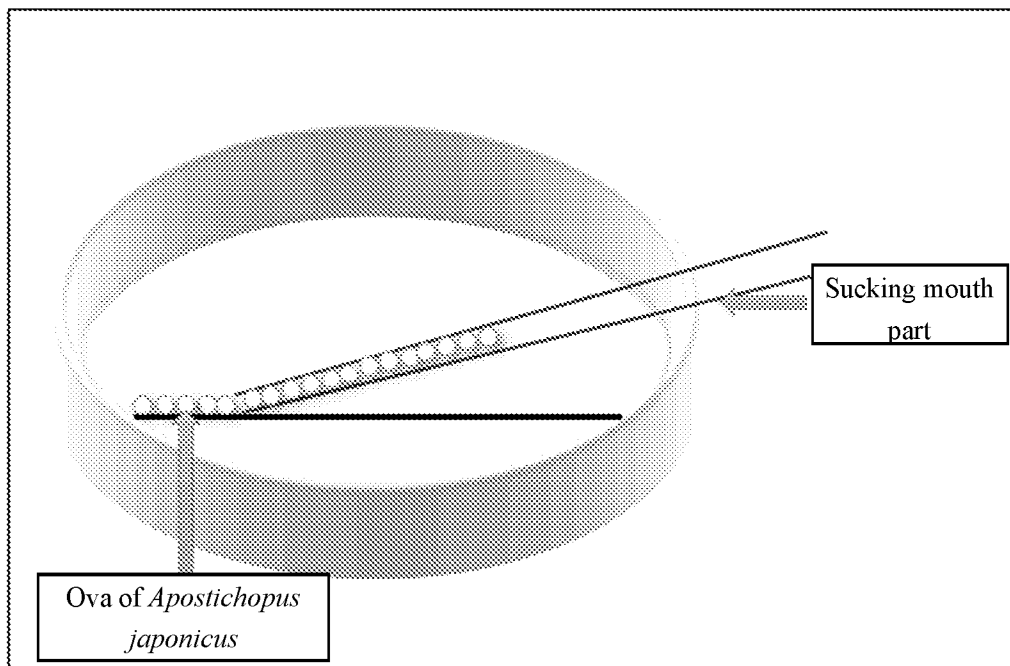

FIG. 2

Rijksdienst voor Ondernemend Nederland

OCTROOIAANVRAAG NR.:

NO 142425
NL 2033411

ONDERZOEKSRAPPORT

BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK

RELEVANTE LITERATUUR

Categorie[1]	Literatuur met, voor zover nodig, aanduiding van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s) nr:	Classificatie(IPC)
Y	LIN CHE-YI ET AL: "Genome editing in sea urchin embryos by using a CRISPR/Cas9 system", DEVELOPMENTAL BIOLOGY, ELSEVIER, AMSTERDAM, NL, deel 409, nr. 2, 26 november 2015 (2015-11-26), bladzijden 420-428, XP029388419, ISSN: 0012-1606, DOI: 10.1016/J.YDBIO.2015.11.018 * bladzijde 421 * -----	1-8	INV. A01K67/033 C07K14/435 C12N15/87 C12N15/113
Y	PIEPLOW ALICE ET AL: "CRISPR-Cas9 editing of non-coding genomic loci as a means of controlling gene expression in the sea urchin", DEVELOPMENTAL BIOLOGY, deel 472, april 2021 (2021-04), bladzijden 85-97, XP093033628, AMSTERDAM, NL ISSN: 0012-1606, DOI: 10.1016/j.ydbio.2021.01.003 * bladzijde 86; figuur 1 * ----- -/--	1-9	

Onderzochte gebieden van de techniek
A01K
C07K
C12N

Indien gewijzigde conclusies zijn ingediend, heeft dit rapport betrekking op de conclusies ingediend op:

Plaats van onderzoek:	Datum waarop het onderzoek werd voltooid:	Bevoegd ambtenaar:
München	22 maart 2023	Deleu, Laurent

[1] NDERLINCATEGORIE VAN DE VERMELDE LITERATUUR

X: de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur
Y: de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht
A: niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft
O: niet-schriftelijke stand van de techniek
P: tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur
T: na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding
E: eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven
D: in de octrooiaanvrage vermeld
L: om andere redenen vermelde literatuur
&: lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

2

EOB FORM 02.83 (P0414B)

RELEVANTE LITERATUUR		
Categorie[1]	Literatuur met, voor zover nodig, aanduiding van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s) nr:
Y	Foltz Kathy R. ET AL: "Chapter 3: Echinoderm Eggs and Embryos: Procurement and Culture" In: "Development of Sea Urchins, Ascidians, and Other Invertebrate Deuterostomes: Experimental Approaches", 2004, Elsevier, XP093033631, ISSN: 0091-679X ISBN: 978-0-12-480278-0 deel 74, bladzijden 39-74, DOI: 10.1016/S0091-679X(04)74003-0, Gevonden op het Internet: URL:http://dx.doi.org/10.1016/S0091-679X(04)74003-0> * bladzijde 64 - bladzijde 67 * -----	1-9
Y	HUO DA ET AL: "The regulation mechanism of lncRNAs and mRNAs in sea cucumbers under global climate changes: Defense against thermal and hypoxic stresses", SCIENCE OF THE TOTAL ENVIRONMENT, deel 709, maart 2020 (2020-03), bladzijde 136045, XP093033700, AMSTERDAM, NL ISSN: 0048-9697, DOI: 10.1016/j.scitotenv.2019.136045 * bladzijde 13 * -----	1-9
Y	CHEATLE JARVELA ALYS M. ET AL: "A Method for Microinjection of Patiria minata Zygotes", JOURNAL OF VISUALIZED EXPERIMENTS, nr. 91, 2014, bladzijden 5191310-3791, XP093033632, DOI: 10.3791/51913 Gevonden op het Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4828045/pdf/jove-91-51913.pdf> * figuren 2-3 * ----- -/--	1-3

[1] CATEGORIE VAN DE VERMELDE LITERATUUR

X: de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur
Y: de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht
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E: eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven
D: in de octrooiaanvrage vermeld
L: om andere redenen vermelde literatuur
&: lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

EOB FORM 02.83 (P0414C)

RELEVANTE LITERATUUR

Categorie[1]	Literatuur met, voor zover nodig, aanduiding van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s) nr:
Y	DMOCHOWSKI IVAN J. ET AL: "Quantitative imaging of cis-regulatory reporters in living embryos", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, deel 99, nr. 20, oktober 2002 (2002-10), bladzijden 12895-12900, XP093033818, ISSN: 0027-8424, DOI: 10.1073/pnas.202483199 * figuur 1 * -----	1-9
Y	STEPICHEVA NADEZDA A. ET AL: "High Throughput Microinjections of Sea Urchin Zygotes", JOURNAL OF VISUALIZED EXPERIMENTS, nr. 83, 21 januari 2014 (2014-01-21), bladzijden 5084110-3791, XP093033670, DOI: 10.3791/50841 Gevonden op het Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4089436/pdf/jove-83-50841.pdf> * bladzijde 2 - bladzijde 7 * -----	2,3
Y	KATO S ET AL: "Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, Apostichopus japonicus", DEVELOPMENTAL BIOLOGY, ELSEVIER, AMSTERDAM, NL, deel 326, nr. 1, februari 2009 (2009-02), bladzijden 169-176, XP025995054, ISSN: 0012-1606, DOI: 10.1016/J.YDBIO.2008.11.003 [gevonden op 2008-11-19] * samenvatting * ----- -/--	5,6

[1] CATEGORIE VAN DE VERMELDE LITERATUUR

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2

EOB FORM 02.83 (P0414C)

RELEVANTE LITERATUUR

Categorie[1]	Literatuur met, voor zover nodig, aanduiding van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s) nr:
Y	Yaguchi Junko ET AL: "Microinjection methods for sea urchin eggs and blastomeres" In: "Development of Sea Urchins, Ascidians, and Other Invertebrate Deuterostomes: Experimental Approaches", 1 januari 2019 (2019-01-01), Elsevier, XP093033710, ISSN: 0091-679X ISBN: 978-0-12-480278-0 deel 150, bladzijden 173-188, DOI: 10.1016/bs.mcb.2018.09.013, Gevonden op het Internet: URL:http://dx.doi.org/10.1016/bs.mcb.2018.09.013> * bladzijde 180 - bladzijde 187 * -----	1-9
A	ELASWAD AHMED ET AL: "Effects of CRISPR/Cas9 dosage on TICAM1 and RBL gene mutation rate, embryonic development, hatchability and fry survival in channel catfish", SCIENTIFIC REPORTS, deel 8, nr. 1, 7 november 2018 (2018-11-07), XP093033634, DOI: 10.1038/s41598-018-34738-4 Gevonden op het Internet: URL:https://www.nature.com/articles/s41598-018-34738-4> * bladzijde 5 - bladzijde 14 * -----	6-9

[1] CATEGORIE VAN DE VERMELDE LITERATUUR

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SCHRIFTELIJKE OPINIE

DOSSIER NUMMER	INDIENINGSDATUM	VOORRANGSDATUM	AANVRAAGNUMMER
NO142425	28.10.2022	01.12.2021	NL2033411
CLASSIFICATIE INV. A01K67/033 C07K14/435 C12N15/87 C12N15/113			
AANVRAGER INSTITUTE OF OCEANOLOGY, CHINESE ACADEMY OF SCIENCES			

Deze schriftelijke opinie bevat een toelichting op de volgende onderdelen:

- ☒ Onderdeel I Basis van de schriftelijke opinie
- ☐ Onderdeel II Voorrang
- ☐ Onderdeel III Vaststelling nieuwheid, inventiviteit en industriële toepasbaarheid niet mogelijk
- ☐ Onderdeel IV De aanvraag heeft betrekking op meer dan één uitvinding
- ☒ Onderdeel V Gemotiveerde verklaring ten aanzien van nieuwheid, inventiviteit en industriële toepasbaarheid
- ☐ Onderdeel VI Andere geciteerde documenten
- ☐ Onderdeel VII Overige gebreken
- ☒ Onderdeel VIII Overige opmerkingen

	DE BEVOEGDE AMBTENAAR
	Deleu, Laurent

Aanvraag nr.:

SCHRIFTELIJKE OPINIE

NL2033411

Onderdeel I   Basis van de Schriftelijke Opinie

1. Deze schriftelijke opinie is opgesteld op basis van de meest recente conclusies ingediend voor aanvang van het onderzoek.

2. Deze motivering is opgesteld, met betrekking tot **nucleotide- en/of aminozuursequenties** die genoemd worden in de aanvraag, op basis van een sequentielijst die:

   a. ☒ is opgenomen in de aanvraag zoals deze oorspronkelijk is ingediend

   b. ☐ aangeleverd is na de indieningsdatum ten behoeve van het onderzoek

   ☐ en vergezeld ging van een verklaring dat de sequentielijst niet meer informatie bevat dan de aanvraag zoals deze oorspronkelijk is ingediend.

3. ☐ Deze motivering is opgesteld, met betrekking tot nucleotide- en/of aminozuursequenties die genoemd worden in de aanvraag, voor zover een zinvolle motivering gevormd kon worden zonder een sequentielijst die voldeed aan WIPO standaard ST.26.

4. Overige opmerkingen:

Onderdeel V   Gemotiveerde verklaring ten aanzien van nieuwheid, inventiviteit en industriële toepasbaarheid

1. Verklaring

Nieuwheid	Ja:	Conclusies	1-9
	Nee:	Conclusies	
Inventiviteit	Ja:	Conclusies	
	Nee:	Conclusies	1-9
Industriële toepasbaarheid	Ja:	Conclusies	1-9
	Nee:	Conclusies	

2. Citaties en toelichting:

   Zie aparte bladzijde

Onderdeel VIII   Overige opmerkingen

De volgende opmerkingen met betrekking tot de duidelijkheid van de conclusies, beschrijving, en figuren, of met betrekking tot de vraag of de conclusies nawerkbaar zijn, worden gemaakt:

Zie aparte bladzijde

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 Cited documents

Reference is made to the following documents:

D1 LIN CHE-YI ET AL: "Genome editing in sea urchin embryos by using a CRISPR/Cas9 system",
DEVELOPMENTAL BIOLOGY, ELSEVIER, AMSTERDAM, NL,
deel 409, nr. 2, 26 november 2015 (2015-11-26), bladzijden 420-428, XP029388419,
ISSN: 0012-1606, DOI: 10.1016/J.YDBIO.2015.11.018

D2 PIEPLOW ALICE ET AL: "CRISPR-Cas9 editing of non-coding genomic loci as a means of controlling gene expression in the sea urchin",
DEVELOPMENTAL BIOLOGY,
deel 472, 1 april 2021 (2021-04-01), bladzijden 85-97, XP093033628,
AMSTERDAM, NL
ISSN: 0012-1606, DOI: 10.1016/j.ydbio.2021.01.003

D3 Foltz Kathy R. ET AL: "Chapter 3: Echinoderm Eggs and Embryos: Procurement and Culture"
In: "Development of Sea Urchins, Ascidians, and Other Invertebrate Deuterostomes: Experimental Approaches", 1 januari 2004 (2004-01-01), Elsevier, XP093033631,
ISSN: 0091-679X
ISBN: 978-0-12-480278-0
deel 74, bladzijden 39-74, DOI: 10.1016/S0091-679X(04)74003-0,
Gevonden op het Internet:
URL:http://dx.doi.org/10.1016/S0091-679X(04)74003-0

D4 HUO DA ET AL: "The regulation mechanism of lncRNAs and mRNAs in sea cucumbers under global climate changes: Defense against thermal and hypoxic stresses",
SCIENCE OF THE TOTAL ENVIRONMENT,
deel 709, 1 maart 2020 (2020-03-01), bladzijde 136045,

XP093033700,
AMSTERDAM, NL
ISSN: 0048-9697, DOI: 10.1016/j.scitotenv.2019.136045

D5 CHEATLE JARVELA ALYS M. ET AL: "A Method for Microinjection of Patiria minata Zygotes",
JOURNAL OF VISUALIZED EXPERIMENTS,
nr. 91, 1 januari 2014 (2014-01-01), bladzijden 5191310-3791,
XP093033632,
DOI: 10.3791/51913
Gevonden op het Internet:
URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4828045/pdf/jove-91-51913.pdf

D6 DMOCHOWSKI IVAN J. ET AL: "Quantitative imaging of cis-regulatory reporters in living embryos",
PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,
deel 99, nr. 20, 1 oktober 2002 (2002-10-01), bladzijden 12895-12900, XP093033818,
ISSN: 0027-8424, DOI: 10.1073/pnas.202483199

D7 STEPICHEVA NADEZDA A. ET AL: "High Throughput Microinjections of Sea Urchin Zygotes",
JOURNAL OF VISUALIZED EXPERIMENTS,
nr. 83, 21 januari 2014 (2014-01-21), bladzijden 5084110-3791,
XP093033670,
DOI: 10.3791/50841
Gevonden op het Internet:
URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4089436/pdf/jove-83-50841.pdf

D8 KATO S ET AL: "Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, Apostichopus japonicus",
DEVELOPMENTAL BIOLOGY, ELSEVIER, AMSTERDAM, NL,
deel 326, nr. 1, 1 februari 2009 (2009-02-01), bladzijden 169-176,
XP025995054,
ISSN: 0012-1606, DOI: 10.1016/J.YDBIO.2008.11.003
[gevonden op 2008-11-19]

2 Novelty

D1 and D2 disclose a method for gene editing in the Echinodermata sea urchin using CRISPR/Cas9 injected in an *in vitro* fertilized embryo (see D1, page 421; D2, figure 1).

D3 discloses methods for the *in vitro* fertilization of gametes of sea cucumber (see D3, pages 64-67).

None of the available prior art documents discloses a method for gene editing *Apostichopus japonicus* using a guide RNA.

Thus claims 1-9 appear to be new.

3 **Inventive step**

D1 or D2 can be regraded as the closest prior art. The problem underlying the claims may be regarded as the provision of methods for genetically editing *Apostichopus japonicus.* It is clear from the general knowledge in the art and D4 in particular, that the skilled person would have the motivation to gene edit sea cucumber using CRISPR (see D4, page 13).

The skilled person would apply the common technologies taught in D1 or D2 to microinject embryos that have been obtained by the technique of D3. The arrangement of eggs in a single row is obvious to facilitate high-throughput processing (see D5, figure 3D; D6, figure 1).

Therefore claim 1 does not involve the exercise of inventive skills.

Claims 2-3 are directed to the use of protamine sulfate to immobilise the eggs and facilitate their microinjection. This is commonly done in the art and does not involve the exercise of inventive skills (see D7, pages 2-7).

The use of neuropeptide for maturation of *Apostichopus japonicus* is commonly known in the art (see D8, Abstract). Therefore claims 5 and 6 do not involve the exercise of inventive skills.

Claims 7 and 8 are directed to common technologies that the skilled person would consider when designing and injecting sgRNA (see D2, page 86). Therefore said claims do not involve the exercise of inventive skills.

Re Item VIII

Certain observations on the application

1 Claim 1 comprises the step of fertilizing the eggs. Said step is an essentially biological process excluded from patentability.

2 The term "gene processing system" in claim 1 is vague and renders the subject-matter for which protection is sought unclear.

3 Claim 9 refers to a webpage. However, the content of the webpage may be modified over time and become inaccessible. Therefore, the subject-matter for which protection is sought is unclear.