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IDENTIFICATION OF INDICATOR SPECIES OF ZOOPLANKTON ORGANISMS BY COI GENE FRAGMENT FOR ESTIMATION OF ECOLOGICAL STATE OF A WATER BODY

Ludmila Leonidovna Frolova*, Arthur Maratovich Husainov
Kazan Federal University, Department of Genetics, 18 Kremlyovskaya St., 420008 Kazan, Republic of Tatarstan, Russian Federation
Email: Lucie.Frolova@gmail.com

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Abstract

Nowadays various methods are used for estimation of ecological state of water bodies; the method of bioindication is one of them. Identification of indicator species of aquatic organisms via the visual method with the aid of a microscope may be performed with a high degree of certainty only by highly experienced zoologists. This method requires deep knowledge of morphological description of every organism, which includes approximately from 15 to 25 characteristics, thus the method greatly depends on subjectiveness of an investigator. At the same time the known methods of molecular genetics are used for determination of the taxonomy of organisms but have not been still used in ecological investigations of water bodies. The method of DNA barcoding which was used by us for identification of indicator species of zooplankton in freshwater bodies in the city of Kazan is one of such molecular methods. The experiment resulted in sequence analysis of the four base sequences of COI gene fragments which were added to the GenBank international database under the following unique numbers: Scapholeberismucronata - HQ336794 (658 bp), Moinamicrura - HQ336797 (658 bp), Mesocyclopsleuckarti - HQ336795 (658 bp), Brachionuscalyciflorus - HQ336793 (660 bp). The estimation of three lakes in Kazan city is based on identification of indicator species of zooplankton by COI gene.

Key words: DNA barcoding, zooplankton, COI gene, ecological state of water bodies

Introduction

Monitoring of the state of water bodies is of urgent necessity due to high human impact on biosphere especially in the cities where large number of water bodies is located. At present ecological state of water bodies is estimated based on the assessment of saprobity of organisms by the ratio of indicator species in a water sample [1]. As is known one water sample contains variety of indicator species indicative of ecological state of a water body. Zooplankton is one

of the most widely studied subjects of research of freshwater bodies by the method of bioindication [2]. Traditionally identification of organism species in a water sample is done by highly experienced specialists-zoologists by the visual method with use of a microscope and guides [3]. At the same time the modern methods of molecular-genetics analysis allow for instrumental identification of organisms to an accuracy of specie. Thus for example DNA barcoding method is used for such purposes, this is an up-to-date taxonomical method utilizing short genetic markers in DNA of organisms in order to determine their affiliation with a definite specie [4]. The method is based on nucleotide sequence in DNA-barcode, which is specific for only one species, for example for animals this sequence is represented by a variable CO1 gene fragment with the length of 600-700 base pairs [5, 6]. Identification of species by barcode has special importance if traditionally used methods are not helpful. For example if there are undistinguishable by appearance sibling species or vice versa species is characterized by sexual dimorphism [7, 8, 9, 10]. It is also important that the selected DNA sections will remain the same for the species at any developmental stage, i.e. from germ cells or semen to adult mature organisms [11]. Therefore DNA-barcode may be used for identification of any water organism inclusive of zooplankton even if a tiny fragment of any tissue is present.

Numerous DNA-barcodes distributed by organism species are accumulated in GenBank, the international database of nucleotide sequences [12]. The GenBank database at NCBI website contains CO1 nucleotide sequences for only 247 indicator species of zooplankton living in freshwater bodies from 1226 species included in the list of Sladechek V. [1,12]. These sequences are represented by 29 world countries, mainly from Europe and America. The larger data amount is represented by Great Britain and Germany, i.e. 15% of total amount; the USA, Canada and Mexico presented 10%, Russia 4% or 14 records. It is obvious that the scientist across the world make their first steps in genetic study of zooplankton organisms inclusive of indicator ones. In Russia the scientists from Moscow, Saint Petersburg and Irkutsk perform pilot research in this sphere. There has not been any similar research works in Kazan. Therefore this study is aimed at identification of indicator species of zooplankton organisms of freshwater bodies in Kazan city by COI gene fragment for estimation of ecological state of a water body by the method of bioindication.

Materials and Methods

Sampling

The samples of zooplankton were taken from the lakes of Kazan city namely Verkhny Kaban, Sredny Kaban, Nizhny Kaban. Sampling and samples processing was performed according to the standard hydrobiological methods [13,14]. Species composition of zooplankton was identified by means of identification guides[15,16].

Separation and PCR-amplification of COI gene fragment

Mitochondrial DNA was isolated from zooplankton Scapholeberis mucronata, Keratella cochlearis (Sredny Kaban Lake), Brachionus calyciflorus, Moinamicrura (Nizhny Kaban Lake), Mesocyclops leacrarta (Verkhny Kaban Lake). PCR-amplification of COI gene was made with the aid of Taq-polymerase and universal primers (Table 1) supplied by Syntol company (Russia) with use of MJ Mini (BioRad) device under the conditions as recommended by the manufacturer.

Table 1: Universal primers for PCR-amplification of COI gene fragment.

Primer description	Primer sequence
LCO1490 COIproject	5'-ggtcaacaaatcataaagatattgg-3'
HC02198 COIproject	5'-taaacttcagggtgaccaaaaaatca-3'

PCR mode: DNA denaturation was performed at the temperature of 95ºC within 1 minute, and then in cycle: the temperature of 95ºC was set for the period of 30 sec, annealing of oligonucleotides was performed within 30 sec at the temperature of 72ºC (time on the basis of 1000 nucleotides per minute), total number of cycles made 35, after which final synthesis was performed at the temperature of 72ºC over the period of 5 min.

PCR reaction was analyzed with use of 1% agarose gel.

Isolation of DNA from agarose gel

DNA was isolated from agarose gel by means of BS353-50reps EZ-10 Spin Columns DNA Gel Extraction Kit according to the methods offered by the manufacturer (https://store.biobasic.com).

Ligation procedure

DNA ligation was performed at the temperature of 4°C within 18 hours in 5 mcl of the reaction mixture containing 1 mcl of ligase buffer and 0.5 mcl T4 ligase per 3.5 µg of DNA.

Inoculation of overnight culture E.coli

8 ml of LB growing medium was diluted with 8 mcl of antibiotic (kanamycin). A bacteria colony was partitioned by means of annealed loop and mixed in a test tube with the growing medium. The culture was incubated within 14-16 hours at the temperature of 37°C with use of a shaking apparatus.

Preparation of competent cells and transformation

For transformation fresh overnight culture was taken (incubated within 4 hours at the temperature of 37°C), then followed incubation on ice over the period of 10 minutes, the mixture was divided into two plastic test tubes and

centrifuged within 10 minutes at the rate of 6000 rpm at the temperature of 4°C for sedimentation of the culture. The sediment was diluted with the cooled solution of $CaCl_2$+$MgCl_2$ and gently stirred until the culture dissolution. The resulting solution was centrifuged within 10 minutes at the rate of 6000 rpm and the temperature of 4°C. The sediment was diluted with 1 ml of 0.1M $CaCl_2$ and thoroughly resuspended. 100 mcl of the resuspended solution was diluted with 5 mcl of ligation mixture and incubated on ice within 30 minutes. After that the cells were subjected to heat shock, i.e. were incubated at the temperature of 42°C within 90 sec and placed back on ice. The solution was diluted with 800 mcl of LS-LB growing medium and incubated with use of a shaker within 1 hour at the temperature of 37°C. The culture was disseminated onto Petri plates with ampicillin and with addition of 10 mcl of 1M IPTG and 40 mcl of X-GAL.

PCR procedure with use of colonies

PCR-amplification of COI gene fragment taken from colonies was performed with the aid of Taq-polymerase and universal primers (Table 1).

PCR mode: DNA denaturation was carried out at the temperature of 95ºC within 1 minute, the temperature of 95ºC was set for the period of 30 sec, annealing of oligonucleotides was performed within 30 sec at the temperature of 72ºC (time on the basis of 1000 nucleotides per minute), total number of cycles made 35, after which final synthesis was performed at the temperature of 72ºC over the period of 5 min. PCR result was analyzed with use of 1.5% agarose gel.

Isolation of plasmid DNA from agarose gel

Plasmid DNA was isolated from agarose gel by means of Roche High Pure Plasmid Isolation Kit according to the methods described by the manufacturer, i.e. binding buffer was cooled before isolation. Bacterial cells were sedimented from 0.5 – 4.0 ml of Colixl1 blue culture and supernatant fluid was extracted. The resulting solution was dissolved with 250 mcl of Suspension Buffer+Rnase and resuspended with the aid of a vibration mixer. After that 250 mcl of lysis buffer was added. The eppendorf was gently turned over 3-6 times (mixing with use of a vibration mixer and shaking is not allowed since DNA may break up), the solution was incubated within 5 minutes (5 minutes maximum) at the temperature of 15°-20°C. After that the solution was diluted with 350 mcl of the cooled binding buffer.

The eppendorf was turned over 3-6 times and the solution was incubated on ice over the period of 5 minutes. After that the solution was centrifuged within 10 minutes at the highest speed rate. Thereafter the high pure filter tube was

inserted into the collection tube and the total amount of supernatant was displaced to the filter tube which was then centrifuged within 1 minute at the highest speed rate. After centrifuging the filter tube was removed from the collection tube, the collection tube was emptied and the filter tube was inserted back. Then the solution in the filter tube was diluted with 700 mcl of wash buffer II for washing and centrifuged within 30-60 sec at the highest speed rate, the contents of the collection tube was drained off. The tube was centrifuged within 1 minute at the maximum speed rate. The filter tube was inserted into a clean eppendorf (1.5 ml) and the solution in the tube was diluted with 100 mcl of elution buffer and 200 mcl of distilled water (pH from 8.0 to 8.5) and centrifuged within 1 minute at the maximum speed rate.

DNA restriction and PCR procedure

DNA restriction was made with the aid of restriction enzymes BamHI and EcoRI (Sibenzyme, Russia) within 30 minutes at the temperature of 37°C under the conditions recommended by the manufacturer. The content of 10 mcl of reaction mixture: buffer for restriction enzyme – 1.0 mcl, plasmid DNA– 8 μg, enzyme – 10 units. PCR amplification was performed according to the above description.

Sedimentation of mixture fragments

DNA sedimentation was carried out at the temperature of -24°C over the period of 12 h in 70 mcl of reaction mixture containing 65 mcl of 96% ethanol, 2 mcl of 3M NaCl and 125mM EDTA per 20 mcl of DNA.

After that the solution was centrifuged within 15 minutes at the maximum speed rate. The supernatant fluid was carefully removed and the sediment was diluted with 100 mcl of 70% ethanol. The solution was centrifuged within 5 minutes at the maximum speed rate and the supernatant fluid was carefully removed. The eppendorf tubes were dried in a temperature-controlled cabinet at the temperature of 37°C. The solution was diluted with 20 mcl of Hi-Di formamide and mixed in the vibration mixer. Then the solution was incubated in SeqPrep mode at the PCR amplifier (95°C within 2 minutes, storage at the temperature of 4°C). The samples were then sent for sequence analysis.

Results And Discussions

Molecular analysis

The investigators used the universal primers (see Table 1) for isolation of the fragments of mitochondrial genes CO1 from Scapholebecis mucronata, Brachionus calyciflorus, Keratella cochlearis, Moina micrura, Mesocyclops leacrarta organisms in the course of polymerase chain reaction (PCR). Electrophoregram of the DNA fragments isolated from the aquatic organisms as a result of PCR is shown on Figure 1.

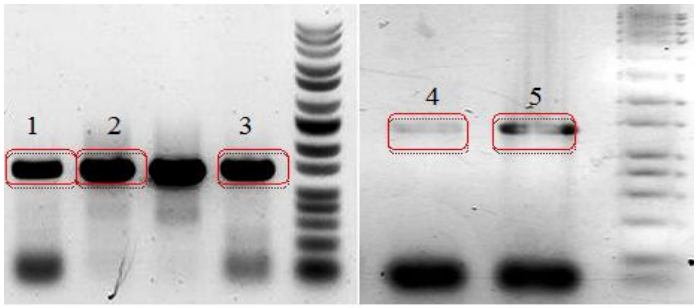

Fig.1. Electrophoregram of DNA fragments isolated from water organisms: 1- Keratella cochlearis, 2 - Brachionus calyciflorus, 3 - Scapholebecis mucronata, 4 -Moina micrura, 5 - Mesocyclops leacrarta

Ligation of the fragment was made with the help of PTZ57R/T vector. Preparation of the competent cells and transformation with use of PTZ57R/T vector was followed by checking the result of transformations by the colony PCR method Only white colonies were taken into consideration since blue colonies failed to transform.

Transformation was successful. The colonies of clones corresponding to the DNA fragments shown on Figure 2 were used for the further work.

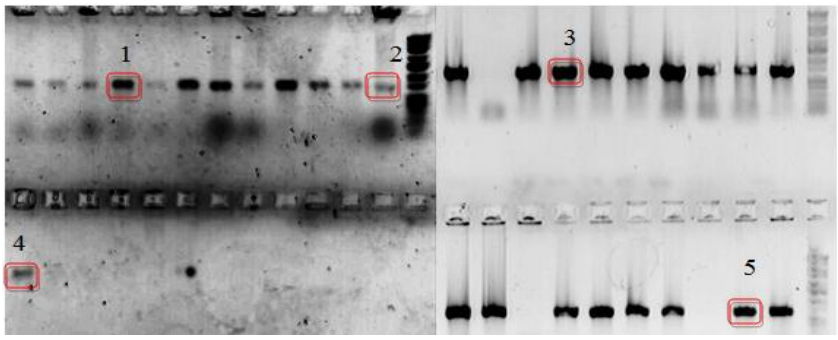

Fig.2. Electrophoregram of DNA fragments isolated in the course of colonies PCR 1- Brachionus calyciflorus, 2 - Scapholebecis mucronata, 3 - Mesocyclops leacrarta, 4 - Keratella cochlearis, 5 -Moinamicrura

Then the bacterial cultures were reinoculated by the pour plate method. After colonies growth plasmid DNA was isolated by Roche High Pure Plasmid Isolation Kit, after which restriction was performed. Restriction was analyzed by the electrophoretic technique with use of agarose gel. (Fig.3).

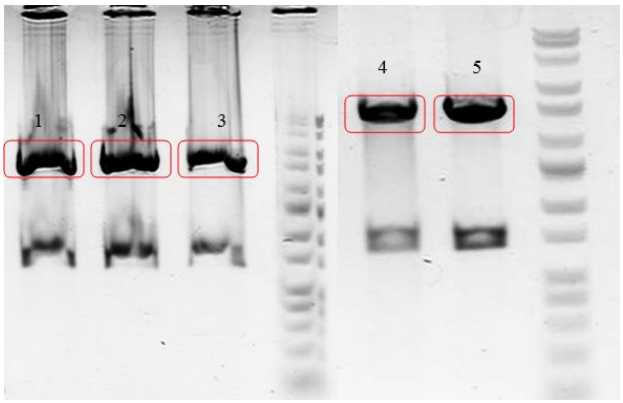

Fig.3. Electrophoregram of DNA fragments after restriction:

1- Brachionus calyciflorus, 2 - Scapholebecis mucronata, 3 - Mesocyclops leacrarta, 4 - Keratella cochlearis, 5 - Moinamicrura

In the consequence of the experiment there were sequenced five base sequences of the fragments of CO1 gene, four of which were entered into the GenBank international database under the unique numbers, i.e. Scapholeberismucronata - HQ336794 (658 bp),Moinamicrura - HQ336797 (658 bp), Mesocyclops leuckarti - HQ336795 (658 bp), Brachionus calyciflorus - HQ336793 (660bp).

Bioinformation analysis

Comparative analysis of the four experimentally obtained base sequences of COI of zooplankton organisms with base sequences of COI genes from GenBank database showed that:

COI base sequence of Scapholeberismucronata (HQ336794, 658 bp) demonstrated 88% homology (580bp/658bp) with the base sequences of COI genes of Scapholeberissp. (HM377195, JN233968);

COI base sequence of Moinamicrura (HQ336797, 658 bp) demonstrated 99% homology (657bp/658bp) with the base sequences of COI genes of Moinasp. (KC617394, KC617393, KC61739);

COI base sequence of Mesocyclopsleuckarti (HQ336795, 658 bp) demonstrated 97% homology (636bp/658bp) with the base sequence of COI gene of Mesocyclopsleuckarti (KF357729);

COI base sequence of Brachionuscalyciflorus (HQ336793, 660 bp) demonstrated 99% homology (658bp/660bp) with the base sequences of COI genes of Brachionuscalyciflorus (KC495195, KC489694, GU232579, GU232577) [17].

Environmental analysis

The following studied zooplankton species belong to indicator ones and are used for estimation of ecologic state of water bodies: Mesocyclopsleuckarti (HQ336795, 658 bp) is an indicator of an oligosaprobic zone (o, indicative weight 1.25), was drawn out of Verkhny Kaban Lake which is characterized by ecologists as an oligosaprobic water body. Scapholeberismucronata (HQ336794, 658 bp) is an indicator of a beta- mesosaprobic zone (b, indicative weight 2.0), was drawn out of Sredny Kaban Lake which is characterized as a beta-mesosaprobic water body according to the studies of ecologists. Moinamicrura (HQ336797, 658 bp) is an indicator of a beta-mesosaprobic zone (b, indicative weight 2.20), was drawn out of Nizhny Kaban Lake which is described by ecologists as a polysaprobic water body. Brachionuscalyciflorus (HQ336793, 660 bp) is an indicator of a beta-alpha- mesosaprobic zone (b-a, indicative weight 2.50), was drawn out of Nizhny Kaban Lake which is characterized by ecologists as a polysaprobic water body [18].

Findings

Following the results of the study there were isolated the fragments of COI mitochondrial genes of zooplankton organisms of the Kaban Lakes of Kazan city. The base sequences were recorded in the GenBank database under the unique numbers i.e. Scapholeberismucronata - HQ336794 (658 bp), Moinamicrura - HQ336797 (658 bp), Mesocyclopsleuckarti - HQ336795 (658 bp), Brachionuscalyciflorus - HQ336793 (660 bp). According to the experiment outcomes it was demonstrated that the pair of universal primers LCO1490 COI project and HC02198 COI project is suitable for isolation of fragment of COI mitochondrial gene of zooplankton organisms. The proportion of homology of the experimentally obtained CO1 sequences of zooplankton organisms as compared to the base sequences of COI gene of zooplankton organisms in the GenBank database made 88% – 99%.

On the basis of identification of the indicator species of zooplankton by COI gene along with other indicator species it is possible to assess ecological state of the three lakes of Kazan city, for example: Mesocyclopsleuckarti (o, indicative weight 1.25) is an indicator of an oligosaprobic zone, Scapholeberismucronata (b, indicative weight 2.0) is an indicator of a beta-mesosaprobic zone, Moinamicrura (b, indicative weight 2.20) is an indicator of a beta-mesosaprobic zone, Brachionuscalyciflorus (b-a, indicative weight 2.50) is an indicator of a beta-alpha-mesosaprobic zone. Considerable amount of the stated organisms in a test sample is indicative of oligosaprobic characteristic of Verkhny Kaban water body, beta-mesosaprobic characteristic of Sredny Kaban water body, and polysaprobic characteristic of Nizhny Kaban which is in complete accord with the assessments of ecologists. Therefore the method of identification of zooplankton organisms by COI marker genes may be used for assessment of ecologic state of water bodies.

Conclusion

Identification of the indicator species of zooplankton organisms by means of COI marker genes offers an advantages of replacement of the visual subjective method by the instrumental objective method for evaluation of ecological state of water bodies. Such new modern approach will allow for reliable and timely assessment of ecological state of a water body.

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