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THE EXPERIENCE IN APPLICATION OF MOLECULAR GENETIC METHODS FOR INSECTS SPECIES IDENTIFICATION – PREPARING OF SAMPLES TO DNA SEQUENCING

Abstract

The article is devoted to the experience of samples preparing for molecular genetic species identification of insects in the framework of some international projects carried out in Kostanay State Pedagogical University named after U. Sultangazin. Individuals of different Coleoptera families collected in territory of the Kostanay region were an object of this research.

DNA-barcoding methods were used for invertebrates of Kostanay region for the first time.

Key words: *DNA-barcoding, gel-electrophoresis, insects, molecular genetic methods, PCR machine.*

1 Introduction

Morphological and anatomic features of collected organisms and collectable control samples of leading research center collections are used for species identification of biological object generally. Certainly, traditional methods of identification of species diversity on morphological features are highly demanded. On the other hand, there are cases where it is necessary to define the species, not having a whole organism, but only a part of an object. In large objects (mammals, birds, fish), in some cases it is possible to determine the species using their derivatives (feathers, wool, fish scales, etc.). For insects, species determination using derivatives is often difficult, and the results are unreliable. In addition, intrapopulation polymorphism and geographical variability are often observed in insects [1-3]. With the development of new technologies in recent years, research in the field of the systematics of organisms has been radically changed based on the use of molecular genetic methods.

This paper describes the experience of using molecular genetic methods for determining the species affiliation of some invertebrates in the Kostanay region. The objects of research are presented both by the collections of the SRC PEB (Science-Research Center for Ecology and Biology of the Kostanay State Pedagogical University named after Umirzak Sultangazin), and the materials collected during the implementation of the initiative topic «Assessment of the impact of land use and climate change on soil invertebrate communities (macrofauna)» registered in JSC SCSSTE

(No. 0119РКИ0195). This initiative topic was developed in 2018 in the framework of the international scientific project «Innovations in the development of methods of sustainable agricultural management in the changing climate in the dry steppes of Kazakhstan and South-West Siberia (ReKKS)», which is part of the ICBF-Financing measure «CLIENT II – international partnerships for sustainable innovation».

Taking part in the science research project «Training the new generation of entomologists in DNA-based molecular and genomic methods – international network (EntoMol)» (project №CPEA-2015/10069, Curator of the Project Ass. Professor V. Gusarov) we got the possibility to identify some representatives of invertebrate animal groups in the DNA laboratory of the Museum of Natural History (Oslo, Norway). The research work in the laboratory was carried out during the scientific internship (02/01/2019 – 02/15/2019) of the Professor, Doctor of Biological Sciences T.M. Bragina (Co-ordinator of the project in Kazakhstan, Head of the Science-Research Center), with project participants – M.A. Bobrenko, Master of Biology, and M.M. Rulyova, Senior Lecturer, Master of Biology.

In this study, we tested DNA-barcoding to re-define the species diversity of some groups of invertebrates in the Kostanay region and prepared samples for sequencing.

There are several molecular genetic approaches in this field, to which a number of works are devoted [4, 5]. This article describes the application of the molecular identification method, which allows determining the organism`s belonging in a particular taxon called DNA barcoding via short genetic markers in DNA. By such a DNA barcode a living creature can be identified even by a tiny fragment of any tissue, practically without damaging the body. The idea of barcoding arose back in 2003. Barcoding, as applied to the identification of species belonging to zoological objects, was covered in the work of the Canadian zoologist Paul Hebert [6], where he writes: «We establish that the mitochondrial genecytochromecoxidase I (COI) can serve as the core of a global bioidentification system for animals».

2 Materials and methods

The samples and specimens were collected during some field expeditions in the Kostanay region as part of ongoing projects in the Kostanay, Mendykara and Naurzum districts in 2017-2018. Soil invertebrates (macrofauna) were collected by methods of soil-zoological samples [7]. Some of the specimens were collected manually, night gatherings were carried out using the light traps. All specimens were labelled and placed in test tubes with 90–99% ethanol. After a couple of hours, the alcohol was replaced, and the samples were placed in the freezer. For control, several previously collected specimens stored in alcohol for several years were taken.

Work with samples in the laboratory consisted of several stages:

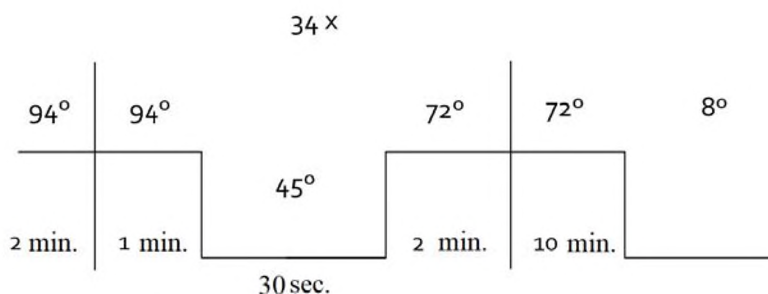
1. Morphological determination of specimens. Insects were determined before molecular genetic analysis. An insect body sample was then taken for further analysis and placed in a numbered tube. The rest, fixed in 100% ethanol, was deposited in the museum's laboratory.
2. Extraction part 1:
   2.1 Check Buffer ALT. Clear at 55^0 C if precipitated.
   2.2 Set incubator to 55^0 C.
   2.3 Vacuum dry samples. Remove excess alcohol first, and dry samples in the vacuum centrifuge for 5'.
   2.4 Crush samples. Add a lead ball to each tube containing a sample. Set machine to 20 Hz for 1'. Centrifuge down remains at 8000 rpm for 1' if necessary.
   2.5 Add 180 μl ATL.
   2.6 Add 20 μl proteinase K.
   2.7 Short vortex.
   2.8 Incubate at 55^0 C for 24 hours.
3. Extraction part 2.
   3.1 Check buffer AL. Clear at 55^0 C if precipitated.
   3.2 Set incubator to 70^0 C.

3.3 Vortex samples 15».
3.4 Add 200 µl buffer AL, vortex immediately and thoroughly.
3.5 Incubate at 70^0 C for 10'.
3.6 Add 200 µl ethanol, vortex thoroughly.
3.7 Transfer to the labeled mini spin column. Centrifuge 8000 rpm, 1'. Move the mini spin column to new collection tube.
3.8 Add 500 µl buffer AW1. Centrifuge 8000 rpm, 1'. Move the mini spin column to new collection tube.
3.9 Add 500 µl buffer AW2. Centrifuge 14000 rpm, 3'. Move the mini spin column to labelled Eppendorf.
3.10Add 100/200 µl buffer AE. Incubate 1' at room temperature. Centrifuge 8000 rpm 1'. Keep flow through (contains the DNA).
3.11Repeat step 2.10.

During the two stages of extraction, the mitochondrial DNA of each sample was isolated for further analysis.

4. PCR, Taq Polymerase:
4.1 Prepare PCR machine (Picture 1) according to the scheme (Picture 2).

Picture 1 – PCR machine



Picture 2 – The scheme of PCR machine

Under the given conditions in the course of PCR machine work, the amplification of DNA occurs. To do this, before placing the samples in a PCR machine, add master mix 1 and 2.
4.2 Prepare Master Mix 1 in an Eppendorf tube. Example for 16 samples:

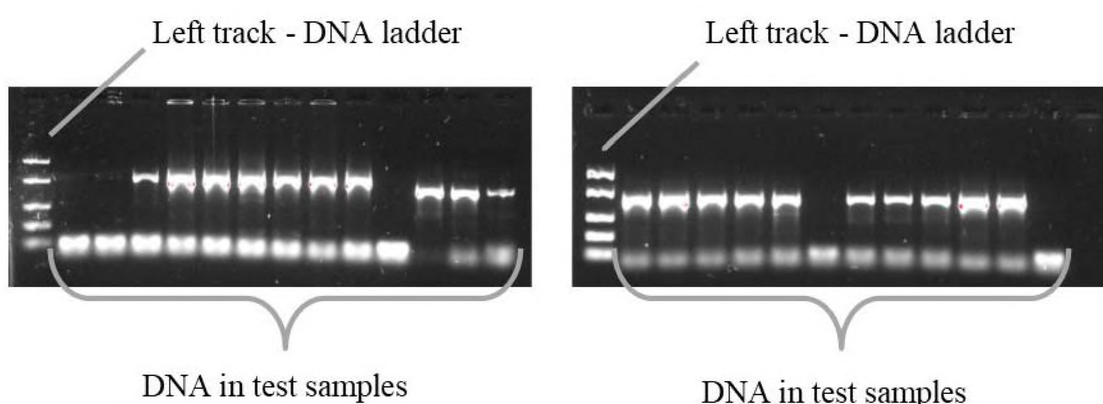
dsH$_2$O – 6.8 * 16 = 108.8 μl
BSA – 1.2 * 16 = 19.2 μl
MgCl$_2$ – 2.5 * 16 = 40 μl
dNTPs – 1 * 16 = 16 μl
Primer 1 – 1.25 * 16 = 20 μl
Primer 2 – 1.25 * 16 = 20 μl
Total – 14 * 16 = 224 μl

4.3 Prepare Master Mix 2 in an Eppendorf tube. Wait with the Polymerase. Example for 16 samples:

dsH$_2$O – 5.3 * 16 = 84.8 μl
Taq buffer – 2.5 * 16 = 40 μl
Polymerase – 0.2 * 16 = 3.2 μl
Total – 8 * 16 = 128 μl

4.4 Add Master Mix 1 to wells, 14 μl per well.
4.5 Add DNA to wells, 3 μl per well.
4.6 Add Polymerase to Master Mix 2, vortex for 4–5 seconds and spin down the mix. Keep on ice!
4.7 Add master Mix 2 to wells, 8 μl per well.
4.8 Spin down strips and place them in PCR machine.
4.9 PCR takes 2–3 hours.

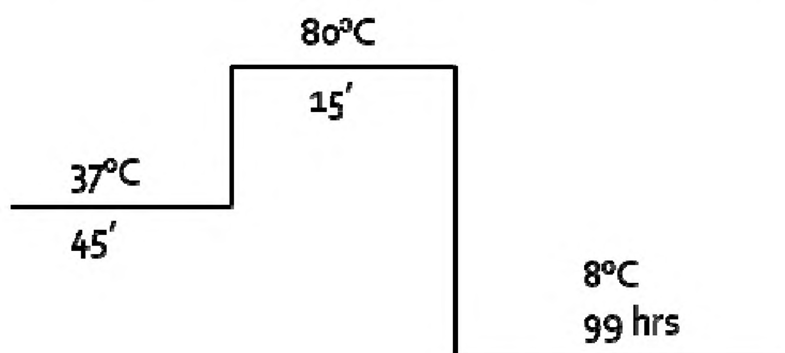
5. Gel electrophoresis:
5.1 Prepare a gel. Add agarose to produce a 1% gel (1 g of SeaKem LE agarose per 100ml of 0.5 X TBE buffer). Dissolve and cool (gel should be 60_0 C). Add gel red. Pour the gel into the mould and add comb (s).
5.2 Prepare DNA samples as drops on a film strip. Each drop contains 3 μl loading buffer and 5 μl DNA sample or 2 μl ladder DNA (ladder in the first drop only).
5.3 Place mould with gel in electrophoresis bath. Remove combs. Add DNA drops to wells. Set voltage to about 80–90 V and run for 20–30 min or so.
5.4 After gel electrophoresis, images were taken to verify the correctness of DNA extraction (Picture 3).



Picture 3 – Photo of agarose gel after DNA electrophoresis

In the photo on the left (left track) – DNA fragments of known length, the rest – DNA in test samples.

6. Clearance of samples from by-products. Further work was continued with samples whose DNA was depicted in the photographs. ExoStar is sensitive to temperature. We stored at –20C and kept on ice during use. Prepared PCR machine for instance the program Exosap45.



Picture 4 – The scheme of PCR machine of the program Exosap45

Then we calculated the total amount of ExoStar needed (2 μl per 5 μl PCR product). Added diluted ExoStar to each well, then added PCR product to the well. Incubated the products using the PCR machine. Marked the strips to show they have been ExoStarred.

7. Preparing samples for sequencing at StarSEQ.

After the cleaning of samples prepared a table showing which samples and primers are in each well (Table 1).

Table 1 – A table showing which samples and primers are in each well

1	1	2	3	4	5	6	7	8
	B12	B13	B14	B15	B16	B24	44191	44193
2	9	10	11	12	13	14	15	16
	B12	B13	B14	B15	B16	B24	44191	44193
3	17	18	19	20	21	22	23	24
	44194	44201	44202	44339	44400	44401	44403	44406
4	25	26	27	28	29	30	31	32
	44194	44201	44202	44339	44400	44401	44403	44406

In accordance with the table, primers were added to the specific sample. For example, we use lCO 1490 и Cl 2416 ra for Staphylinidae.

In the process of sequencing, two DNA sequences of each sample were arranged from different ends under the action of certain primers.

Further study of sequenced samples was made in programs CodonCodeAligner [https://www.codoncode.com] and MEGA [https://www.megasoftware.net]. CodonCode Aligner is a versatile, powerful and easy-to-use DNA and RNA sequence assembler, aligner, and editor. The MEGA software has been to provide tools for exploring, discovering, and analyzing DNA and protein sequences from an evolutionary perspective.

The results of work in these programs will be highlighted in the next publication.

3, 4 Results and discussion

46 samples were processed in the DNA laboratory of Natural History Museum in 7 stages: 1) morphological determination, 2) Extraction part 1, 3) Extraction part 2, 4) PCR, Taq Polymerase, 5) Gel electrophoresis, 6) Cleaning, 7) Preparing samples for sequencing at StarSEQ.

All 46 samples after the results of gel electrophoresis were prepared and sent for sequencing:

1 Staphylinidae (23 specimens):
- 1.1 Philonthus (?) sp.
- 1.2 Bledius sp. (5)
- 1.3 Tachyporus sp.
- 1.4 Xantholinus sp.

1.5 *Philonthus politus* (2)
1.6 Philonthus sp. (3)
1.7 Aleochara sp.
1.8 Oxytelus sp. (2)
1.9 Paederus littoralis
1.10 Anotylus sp.
1.11 Athetini gen. sp.
1.12 Platystethus sp. (2)
1.13 Carpelinus sp. (2)
2. Elateridae (16):
2.1 Selatosomus latus
2.2 *Selatosomus sp.* (14)
2.3 *Agroites sp.*
3. Tenebrionidae (2 objects)
3.1 Opatrum sabulosum
3.2 Tentyria nomas
4. Coccinelidae (1 object)
4.1 Coccinella semipunctatum
5. Chrysomelidae (3)
5.1 *Cassida nebulosa* (2)
5.2 Chrisomella sp.
6. Curculionidae (1)
6.1 *Phyllobius gabrius*

Identification of the structural organization of the gene and other DNA sequences and further processing through special programs will allow confirming or refining the definition of species by morphological characters.

5 Conclusions

In the process of working in the laboratory, the participants mastered such methods as the isolation of total genomic DNA from arthropod samples, PCR amplification of selected markers (for example, CO1), visualization of PCR products using agarose gel electrophoresis, optimization and diagnosis of PCR, purification of PCR enzymatic products Sample Preparation For Individual Sanger Sequencing.

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БРАГИНА, Т.М., БОБРЕНКО, М.А., РУЛЁВА, М.М.
ЖӘНДІКТЕРДІҢ ТҮРЛІК ТИІСТІЛІГІН АНЫҚТАУ ҮШІН МОЛЕКУЛАЛЫҚ-ГЕНЕТИКАЛЫҚ ӘДІСТЕРДІ ҚОЛДАНУ ТӘЖІРИБЕСІ – ДНҚ СЕКВЕНИРЛЕУГЕ ҮЛГІЛЕРДІ ДАЙЫНДАУ

Мақала Ө. Сұлтанғазин атындағы Қостанай мемлекеттік педагогикалық университетінде жүзеге асырылған бірқатар халықаралық жобалар аясында жәндіктер түрлерін молекулалық-генетикалық сәйкестендіру үшін үлгілерді дайындау тәжірибесіне арналған. Зерттеу нысаны – Қостанай облысында жиналған қоңыздардың әртүрлі тұқымдастарының түрлері.

Қостанай облысының омыртқасыздарында алғаш рет ДНҚ-ны штрих-кодтау әдістері қолданылды.

***Кілт сөздер:** молекулалық генетикалық әдістер, гель электрофорезі, жәндіктер, ДНҚ штрих-кодтау. ПТР машинасы.*

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ОПЫТ ПРИМЕНЕНИЯ МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИХ МЕТОДОВ ДЛЯ ОПРЕДЕЛЕНИЯ ВИДОВОЙ ПРИНАДЛЕЖНОСТИ НАСЕКОМЫХ – ПОДГОТОВКА ОБРАЗЦОВ К СЕКВЕНИРОВАНИЮ ДНК

Статья посвящена опыту подготовки образцов для молекулярно-генетической идентификации видов насекомых в рамках ряда международных проектов, реализуемых в Костанайском государственном педагогическом университете имени У. Султангазина. Объектом исследования были особи разных семейств жесткокрылых, собранные на территории Костанайской области.

Впервые для беспозвоночных Костанайской области были использованы методы ДНК-штрих кодирования.

***Ключевые слова:** молекулярно-генетические методы, гель-электрофорез, насекомые, ДНК-баркодинг, ПЦР машина.*

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СТУДЕНТ ЖАСТАРДЫҢ ЖЕМҚОРЛЫҚҚА ҚАРСЫ МӘДЕНИЕТІН ҚАЛЫПТАСТЫРУДЫҢ ӘЛЕУМЕТТІК-ПСИХОЛОГИЯЛЫҚ ЕРЕКШЕЛІКТЕРІ

Түйін

Мақалада қазіргі кездегі жоғарғы оқу орнында оқитын студенттердің жемқорлыққа қарсы және құқықтық мәдениетін қалыптастыру мәселесі қарастырылған. Тұлғаның жемқорлыққа қарсы және құқықтық мәдениеті мәселесі отандық және шетел ғалымдарының еңбектеріне сүйене отырып, теориялық жағынан талданды және арнайы құрастырылған әлеуметтік-психологиялық бағдарлама арқылы студенттердің жалпы құқықтық мәдениетін көтеру жұмыстары жүргізіліп, психологиялық диагностика жұмыстары жүргізілді. Нәтижесінде, зерттеу тобына енген студенттердің құқықтық және жемқорлыққа қарсы мәдениеті деңгейінің көтерілгені анықталды.