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OPTIMIZATION OF DNA EXTRACTION PROTOCOL FOR DNA ISOLATION FROM AIR-DRIED COLLECTION MATERIAL FOR FURTHER PHYLOGENETIC ANALYSIS (COLEOPTERA: CARABIDAE)

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Different DNA extraction protocols exist and a lot of companies are developing diverse manufactory protocols for DNA isolation from various samples. In our investigation we have chosen Qiagen manufactory protocol. The main goal was to obtain DNA isolation protocol from air-dried collection material. In addition we wanted to make up the non-destructive DNA extraction method for samples from pinned collections.

Key words: DNA extraction, air-dried collection material, PCR amplification.

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INTRODUCTION

Biosystematics is a complicated science field in which a lot of different methods for species cladistics are developed. The most popular have become molecular systematics which provides a big yield of statistical information for study (Mauro & Agorreta 2010).

A lot of different species are held in air-dried Entomology collections around the world, which provides a lot of researches for morphological and molecular analysis. These species provides information for phylogenetic analysis and are valid for evolutionary researches (Gandhi 2005; Gilbert et al. 2007).

All phylogenetic researches are starting with DNA isolation from investigated object. Quality and quantity of DNA is extremely important in phylogenetic analysis, species identification and systematic group relationships detection (Ober 2002).

Qualitative DNA extraction from air-dried collection material is one of the most difficult parts in molecular analysis. In some cases DNA may be so fragmentated, that it is impossible to get sufficient amount of product. DNA fragmentation depends on many factors, such as, specimen collection, fixation methods and storage (Gilbert et al. 2007, Thomsen et al. 2009). DNA isolation protocols should be accessible for phylogenetic analysis

and safety for specimens. Biosystematics can exist only if several methods are used for species identification and relationships understanding (Caterino 2000, Bybee et al. 2010).

Carabidae is one of the biggest families in order Coleoptera represented with high amount of specimens. Ground beetles inhabit all terrestrial habitat types from the subarctic to wet tropical regions (Howland & Hewitt 1995, Arndt 2005). This diversity and wide distribution, has resulted in a considerable interest in many aspects of their biology, including systematics, phylogeny, biogeography, ecology and evolution (Maddison 2008, Kotze et al. 2011).

The main objective of present research is to adapt and improve DNA extraction protocol for Carabidae air-dried collection material.

EXPERIMENTAL PROCEDURES

Study species

Samples used in our research were collected in various parts of Europe, Asia, USA and Japan in different time periods, from 1934 to 2008. In most cases, species collection and fixation methods were unknown. Three genus of Carabidae family for this study were selected: *Agonum* Bonelli, 1810, *Omophron* Latreille, 1802 and *Notiophilus* Duméril, 1806. From genus *Agonum* Bonelli, 1810 were analyzed 60 specimens, *Omophron* Latreille, 1802 – 24 and *Notiophilus* Duméril, 1806 – 21 specimens. More details about collection time and geographic region of samples used in our study are in Table 1.

Samples pre-treatment and DNA extraction procedure

Before all extraction procedures, specimens were washed with ethanol (96, 6%) and warm distillate water. Wash procedures were performed in sterile Petri-dishes. Specimens were rinsed from small syringe filled with ethanol (96, 6%), thereafter with warm (30°C) distillate water.

For DNA extraction DNeasy Blood & Tissue Kit (Qiagen, Germany) with some modifications was used. Modifications in extraction protocol were based on proportionally changing of the amount of tissue lysis buffer and proteinase K, increasing incubation time, changing final elution volume. In some cases final elution volume were divided in two stages. During the research, different combinations of these parameters were analyzed.

Whole specimens or their legs were used for DNA extraction procedures. Some species in our research were crushed before adding the tissue lysis buffer with proteinase K. Before the beginning of main extraction procedure, specimens were placed in 2 mL microcentrifuge tubes and soaked in tissue lysis buffer with proteinase K for incubation (56°C). Using the non-destructive methods the whole specimens after incubation time were washed in ethanol (96, 6 %) and returned back to the collection.

Details of protocol modifications are presented in Table 2.

DNA quantity and purity

DNA quantity and purity were checked using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). DNA quantity of each sample was checked for concentration (ng/µl). DNA purity was analyzed using ratio of sample absorbance at 260 and 280 nm (260/280) and at 260 and 230 nm (260/230).

PCR amplification

PCR reactions for all samples were performed using Taq PCR Core Kit (Qiagen, Germany). For PCR reactions were used 0.2 ml strip tubes with caps. PCR reactions were performed following the manufacturer's protocol in Taq PCR Handbook, (Protocol: PCR Using Taq DNA Polymerase) proportionally counted for 25 µl reaction. The amount of template DNA for each reaction was counted using data of concentration from NanoDrop 1000 Spectrophotometer (Thermo Scientific).

mtDNA cytochrome c oxidase gene I (COI)

Table 1. Details of species used in the research

Species	Number of specimens	Pinned	Geographic Origin	Year Collected
Agonum fuliginosum Panzer, 1809	26	Yes	Latvia	1995
Agonum fuliginosum Panzer, 1809	7	Yes	Latvia	1994
Agonum fuliginosum Panzer, 1809	2	Yes	Latvia	1993
Agonum fuliginosum Panzer, 1809	1	Yes	Latvia	1992
Agonum fuliginosum Panzer, 1809	7	Yes	Latvia	1991
Agonum fuliginosum Panzer, 1809	4	Yes	Latvia	1990
Agonum fuliginosum Panzer, 1809	3	Yes	Latvia	1989
Agonum fuliginosum Panzer, 1809	2	Yes	Latvia	1986
Agonum gracile Sturm, 1824	1	Yes	Latvia	1995
Agonum gracile Sturm, 1824	1	Yes	Latvia	1993
Agonum gracile Sturm, 1824	2	Yes	Latvia	1991
Agonum thoreyi Dejean, 1828	1	Yes	Latvia	1995
Agonum thoreyi Dejean, 1828	1	Yes	Latvia	1991
Agonum thoreyi Dejean, 1828	1	Yes	Latvia	1989
Agonum thoreyi Dejean, 1828	1	Yes	Latvia	1986
Notiophilus aquaticus Linnaeus, 1758	2	Yes	Russia	2007
Notiophilus aquaticus Linnaeus, 1758	1	Yes	Russia	2006
Notiophilus aquaticus Linnaeus, 1758	1	Yes	Russia	2004
Notiophilus aquaticus Linnaeus, 1758	1	Yes	Mongolia	2003
Notiophilus aquaticus Linnaeus, 1758	2	Yes	Latvia	2002
Notiophilus aquaticus Linnaeus, 1758	4	Yes	Russia	2002
Notiophilus aquaticus Linnaeus, 1758	1		Russia	2000
Notiophilus aquaticus Linnaeus, 1758	1	Yes	Belarus	1998
Notiophilus aquaticus Linnaeus, 1758	1	Yes	Kazakhstan	1990
Notiophilus aquaticus Linnaeus, 1758	2	Yes	USA	1934
Notiophilus semistriatus Say, 1823	1	Yes	Russia	1996
Notiophilus semistriatus Say, 1823	1	Yes	USA	1952
Notiophilus semenovi Tschitscherine, 1903	2	Yes	Kirgizstan	1991
Notiophilus semenovi Tschitscherine, 1903	1	Yes	Kirgizstan	1987
Omophron aequale aequale Morawitz, 1863	12	Yes	Japan	2001
Omophron aequale jacobsoni Semenov, 1922	12	Yes	Russia	2008

barcoding universal 10 µl primers were used: LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'; HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 49°C for 30 s, 72°C for 1 min and final extension 72°C for 10 min. In cases than PCR was not successful we used double PCR with the same primers and conditions.

Amplifications were confirmed by standard submarine gel electrophoresis, using 2% agarose, TAE gels, stained with ethidium bromide.

RESULTS AND DISCUSSION

During the study, DNA was extracted from 105 specimens using eight different methods. In most cases it is impossible to improve quality of isolated DNA from air-dried collection material. Protocol modifications can help improve quantity and purity of extracted DNA.

Of course, DNA extraction procedure modifications can be varied. In our opinion the best is non-destructive method V (see Table 2), this protocol in most cases let to have a necessary amount of template for DNA amplification for further sequencing. This non-destructive protocol allows save the sample from external damage. Non-destructive method idea was based on earlier Gilbert et al. (2007) and Thomsen et al. (2009) studies. Figure 1 shows an example of specimen before incubation, but Figure 2 is the same specimen after 16 h (56°C) incubation time in 175 mkl tissue lysis buffer with 25 mkl proteinase K. Incubation does not affect the specimen morphological structure, and after using this method, specimen could be returned to the collection and may also be useful for morphological studies.

Results of DNA quantity and purity are represented in Table 3. In most cases 30-40 ng/mkl is the concentration of DNA for successful PCR amplification. Ratio of 260/280 indicates the level of phenol, protein and other contaminants that absorb at 280 nm. Ratio of 1.8 is represented as pure DNA. For DNA Ratio of 260/230 indicates the level of co-purified contaminants. Range of 1.8-2.2 is acceptable for pure DNA. In our case the best results of DNA quality and purity was using non-destructive method V.

Normally, the yield of isolated mitochondrial DNA perceptually is higher than amount of isolated nuclear DNA (Gusarov, 2011, pers. comms). To test principles of better DNA extraction protocol were used universal mtDNA barcoding primers. Figure 3 shows some examples of successful PCR amplification. In this agarose gel image are presented PCR products, which were observed using four different specimens. In this application DNA was extracted using non-destructive method V. Samples were amplified out the first time. In some cases it was not possible to get

Table 2. DNA extraction methods with modifications

Method	Proteinase K (mkl)	Tissue lysis buffer (mkl)	Incubation time, 56°C (h)	Final elution volume (mkl)
Destructive method I (complete specimen)	20	180	6	100
Destructive method II (legs)	20	180	6	100
Destructive method III (legs)	25	175	16	100
Non-destructive method I	20	180	6	100
Non-destructive method II	25	175	8	(50+50)*
Non-destructive method III	30	170	20	(25+25)*
Non-destructive method IV	25	175	16	(25+25)*
Non-destructive method V	25	175	16	(50+25)*

* Volumes of final elution divided in two stages are presented in brackets

Table 3. Results of DNA quantity and purity

Method	Number of analyzed specimens	Mean concentration (ng/µl)	Mean 260/280 ratio	Mean 260/230 ratio
Destructive method I (complete specimen)	10	0.98	0.58	0.42
Destructive method II (legs)	12	1.13	0.64	0.51
Destructive method III (legs)	12	4.56	1.24	1.01
Non-destructive method I	15	6.99	1.77	0.65
Non-destructive method II	12	19.79	1.56	1.69
Non-destructive method III	12	23.39	1.42	0.44

Table 4. Results of DNA amplification

Method	Number of analyzed specimens	Number of sample with amplifiable DNA	Number of sample with amplifiable DNA after double PCR	Number of successful DNA amplification samples (%)**
Destructive method I (complete specimen)	10	1	3	40
Destructive method II (legs)	12	3	5	67
Destructive method III (legs)	12	4	6	83
Non-destructive method I	15	6	5	73
Non-destructive method II	12	4	6	83
Non-destructive method III	12	5	4	75
Non-destructive method IV	12	6	5	92
Non-destructive method V	20	9	10	95

** Number of successful DNA amplification samples (%) = (number of sample with amplifiable DNA + number of sample with amplifiable DNA after double PCR) / number of analyzed specimens

any product, in that course we used double PCR. More contaminations are after reamplification, but it does not influence on sequence analysis so much, if use well cleaned up products.

Comparative data analyses were done which represent number of analyzed specimens using one of the eight methods, number of sample with amplifiable DNA and number of sample with amplifiable DNA after double PCR. Numbers of successful DNA amplification samples in percent were counted for each used method. Results of successful PCR using different methods are represented in Table 4. Also were specimens which were impossible to amplify. Figure 4 shows some unsuccessful examples of amplification. Percentage of unsuccessful DNA amplification depends on method what was used for DNA extraction procedure. The worst results of amplification was using destructive method I (complete specimen), there were a lot of contaminations.

Certainly, for molecular biology needs, it's better to use material which was specially prepared, for example initially collected in ethanol (96, 6 %) or special buffers to prevent DNA fragmentation (King et al. 2008). It is possible to use air-dried material in cases when a big collection of pinned samples already exists. There will always be the percentage of unsatisfactory reactions regardless of sample that is used.

Fig. 1. Photo of *Omophron aequale jacobsoni* Semenov, 1922 before incubation.

Fig. 2. Photo of *Omophron aequale jacobsoni* Semenov, 1922 after 16 h (56°C) incubation time in tissue lysis buffer with proteinase K.

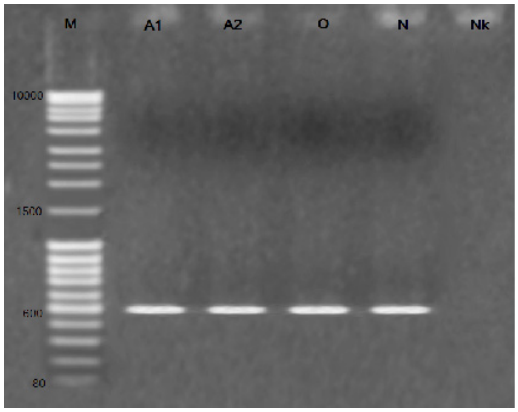

Fig. 3. Agarose gel image
(successful PCR amplification)
M: marker (bp)
A1: *Agonum fuliginosum* Panzer, 1809
A2: *Agonum thoreyi* Dejean, 1828
O: *Omophron aequale aequale* Morawitz, 1863
N: *Notiophilus semistriatus* Say, 1823
Nk: negative control.

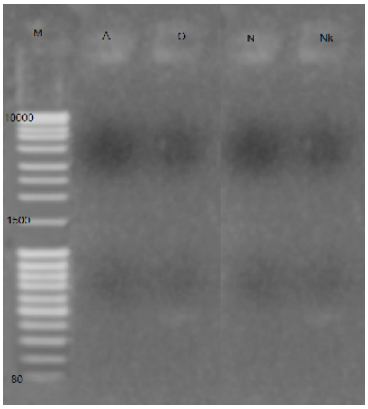

Fig. 4. Agarose gel image
(failed PCR amplification)
M: marker (bp)
A: *Agonum fuliginosum* Panzer, 1809
O: *Omophron aequale aequale* Morawitz, 1863
N: *Notiophilus semistriatus* Say, 1823
Nk: negative control.

CONCLUSIONS

105 specimens were analyzed. The non-destructive method for DNA extraction from air-dried collection material was performed. The method mentioned above could be successfully used in future. Observed PCR products may be used in sequencing for further phylogenetic analyses. It is the first time when it will be possible to get sequences for the most of the analyzed specimens.

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