Advances in Research

12(3): 1-4, 2017; Article no.AIR.37016
ISSN: 2348-0394, NLM ID: 101666096

Determination of mtDNA Sequences by Using Novel Primers to Establish Intraspecific and Phylogenetic Relations of Hungarian Isophya (Orthoptera, Tettigoniidae) Species

E. Vadkerti[1*], P. Putnoky[2], G. Hoffmann[2], F. Antal[2], M. Boros[2], K. Götzer[2], P. Kisfali[2], I. Mészáros[2], K. Nyárády[2], S. Stranczinger[2], A. Szloboda[2], S. Farkas[3], T. Karches[1], I. Matrai[1], Z. Varga[4] and K. Pecsenye[4]

[1]Faculty of Water Science, National University of Public Service, H-6500 Baja, Bajcsy-Zsilinszky Str. 12-14, Hungary.
[2]Faculty of Sciences, University of Pécs, H-7601 Pecs, Ifjúsag Str. 6, Hungary.
[3]Faculty of Agricultural and Environmental Sciences, Kaposvár University, H-7400 Kaposvár, Guba Sándor Str. 40, Hungary.
[4]Department of Evolutionary Zoology and Human Biology, Faculty of Sciences, University of Debrecen, H-4010 Debrecen, Egyetem Sq. 1, Hungary.

Authors' contributions

This work was carried out in collaboration between all authors. Authors EV, PP, GH, ZV and KP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EV, GH, SS, FA, MB, KG, PK, I. Mészáros, KN and AS managed the analyses of the study. Authors EV, SF, TK and I. Mátrai managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AIR/2017/37016
Editor(s):
(1) Farzana Khan Perveen, Founder Chairperson & Associate Professor, Department of Zoology, Shaheed Benazir Bhutto University (SBBU), Main Campus, Pakistan.
Reviewers:
(1) Manoel Fernando Demétrio, Universidade Federal da Grande Dourados, Brazil.
(2) Jeffrey M. Marcus, University of Manitoba, Canada.
Complete Peer review History: http://www.sciencedomain.org/review-history/21927

Short Research Article

Received 27th September 2017
Accepted 11th November 2017
Published 16th November 2017

ABSTRACT

Novel gene specific primers were designed for coding sequences of a part of 16S ribosomal RNA encoding gene and a piece of ND1 gene, separated by a short stretch of DNA that encodes a tRNA-Leu gene for *Isophya* species.

**Corresponding author: E-mail: Vadkerti.Edit@uni-nke.hu;*
E-mail: Karches.Tamas@uni-nke.hu;

Keywords: Isophya; mtDNA; ND1 gene; PCR primers; 16S RNA gene.

1. INTRODUCTION

From a geological point of view, the Carpathian basin belongs to the youngest areas of Europe, regarded as diversity hot-spot [1].

The endemic insects, as a rule, short-winged, flightless forms which have been isolated in "massifs de refuge" e. g. *Isophya* katydid species [2]. These endemic species are under local and/or international protection. From the Hungarian Isophya species *I. costata* and *I. stysi* are listed in the European Natura 2000 Network, Appendix II.-IV., and are under special local protection. Except of *I. kraussi* three additional Hungarian species *I. camptoxypha*, *I. modesta* and *I. modestior* are under local protection.

Unfortunately, the taxonomic identification of *Isophya* species based only on single characteristics is highly doubtful. That is why the phylogenetic relationship of these species is frequently contradicted. Although the phylogeographycal connection between the postglacial relict *I. costata* and the *I. modesta* steppic species have been verified by genetic analyses, the *I. stysi* and *I. modestior* Illyrian-Dacian vicarious species pair connection had not been confirmed by allozyme analysis [3]. Morphometric and allozyme analysis on level of both species and populations were in conflict [4].

In general, the evolutionary root and phylogeographycal history of this genus remains unclear, and much research is needed on additional characters such as DNA based approaches [2] and [5].

This research is intended to develop a primer for future phylogenetic investigations of Hungarian *Isophya* species based on ND1 *16S RNA gene* mtDNA region.

2. MATERIALS AND METHODS

The investigated parts of specimen were storage from previously studies no animal were captured only for this research [3] and [4]. The same species had been included into mtDNA analysis than morphometric and allozyme analysis [3] and [4].

Whole adult bushcrickets were placed into 80% ethanol or stored at -20℃. Total DNA was isolated from about 20 mg of hindleg muscles using a chloform extraction protocol [6]. The integrity of the total DNA was checked by agarose gel electrophoresis, and the quantity was assessed by UV spectrophotometry. Polymerase chain reaction (PCR) amplifications of mtDNA segments were carried out in a volume of 25 µl in MJ Research PTC-100 thermal cycler. PCR reactions included: 20 ng DNA, 20 pmoles of each primer and 2.5 U of Taq polymerase in 1X PCR mix (Fermentas).

Initial PCR reactions were performed using a pair of 16S rRNA- [7] and ND1 [8] primers based on literature data (Table 1). Each PCR reaction was started with denaturation at 95ºC for 3 min followed by a total of 33 cycles that consisted of denaturation at 95ºC for 30 sec, annealing at 55ºC for 30 sec and extension at 72ºC for 30 sec. The reaction was terminated with a final extension at 72ºC for 5 min.

Amplifications were successful with *I. modesta* and *I. costata* DNA (539 bp in size), however reactions from other species gave very weak signals, probably due to mismatches between primer and template sequences.

To give a frame for a manageable mtDNA fragment, we investigated an alignment among a wide range of insect sequences such as *Gryllotalpa orientalis* (AY660929), *Locusta migratoria* (X80245), *Periplaneta fuliginosa* (AB126004), *Sclerophasma paresisensis* (DQ241798), *Tamolanica tamolana* (DQ241797) and *Timema californicum* (DQ241799). Two conserved regions (900 bp apart) were considered and 16S-ORT and ND1-ORT primers were designed. The newly designed primers (mentioned as outer) named ORT-ND1 and ORT-16S (Table 2, Fig. 1).

Table 1. Initial primers for amplification of the 16rRNA-ND1 region of mtDNA in *Isophya* species

Code	Locus	Primer sequenc (5'- 3')	Tm (°C)	PCR product (bp)	Reference
16S	16S rRNA	acatgatctgagttcaaaccg	55,2	539	Vogler 1993 [7]
ND1	ND1	tagaattagaagatcaaccag	49,9		Pashley 1992 [8]

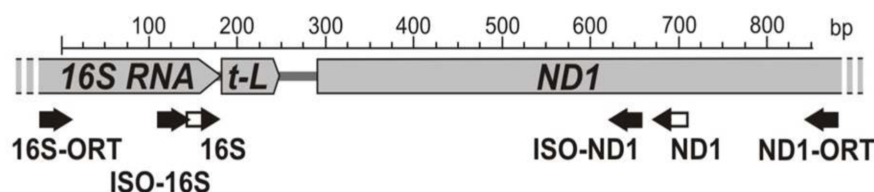

Fig. 1. Primer positions applied in this work. Rooler shows the largest amplified and sequenced region of the mtDNA. 16S RNA: large subunit (16S) ribosomal RNA gene; t-L: transfer RNA-Leu coding region; ND1: NADH dehydrogenase subunit 1 coding region. Black arrows represent new primers (see Tables 1 and 2)

Table 2. Newly designed primers for amplification of the 16rRNA-ND1 region of mtDNA in *Isophya* species

Code	Locus	Primer sequenc (5'- 3')	Tm (°C)	PCR product (bp)	Reference
ORT-ND1	ND1	attcaccttcagcaaaatcaaaag	53.3	900	Present paper
ORT-16S	16S rRNA	gcgacctcgatgttggatta	54.4		Present paper
ISO-ND1	ND1	tgaccatcctgcaattattactg	53.2	535	Present paper
ISO-16S	16S rRNA	cctttaaatccttacatgatctgag	51.8		Present paper

By using the outer primers c.a. 850 bp long fragments were amplified. PCR conditions: 3 min at 95℃, followed by 4 cycles of 30 sec at 95℃, 30 sec at 57℃ and 50 sec at 72℃; followed by 4 cycles of 30 sec at 95℃, 30 sec at 55 ℃ and 50 sec at 72℃; followed by 25 cycles of 30 sec at 95℃, 30 sec at 53℃ and 50 sec at 72℃.

Even though PCR amplification results showed that all of the tested *Isophya* species were positive for these primers, quality of the bands and reproducibility of the PCR amplification was frequently weak. In order to aid sequencing of the amplified fragments, a cloning step was inserted into the process. In the cloning procedure, a single specimen of each *Isophya* species was used.

3. RESULTS AND DISCUSSION

DNA sequences obtained in this study were deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the following accession numbers: AM779087-AM779092.

Determined sequences one from each species have been registered in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the following accession numbers: AM779087 (*I. costata*), AM779088 (*I. camptoxypha*), AM779089 (*I. stysi*), AM779090 (*I. modestior*), AM779091 (*I. kraussi*), AM779092 (*I. modesta*).

After aligning these Isophya sequences new "inner" Isophya specific primers (ISO-16S, ISO-ND1) were designed (Table 2) to complete the sequence of the cloned regions (Fig. 1). After sequence of Isophya species comparison, ISO-ND1 and ISO-16S nested *Isophya* specific primers were designed (Table 2, Fig. 1).

Based on the results, we decided to use the new "inner" primers (ISO-16S, ISO-ND1) for amplification of the region described above. For future research the phylogenetic relations will be established.

This work was supported by the Hungarian Government Grant (NKFP B023/2004.) and we have followed all of the current regulations for sampling these species.

4. CONCLUSION

In conclusion, simple utilization of published primers are unsatisfactory for amplification of *Isophya* mtDNA. Based on these results, we expect that these *Isophya* specific primers can be used in future studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Williams PH, Humphries C, Araújo MB, Lampinen R, Hagemeijer W, Gasc JP, Mitchell-Jones T. Endemism and important

areas for representing European biodiversity: a preliminary exploration of atlas data for plants and terrestrial vertebrates. Belgian Journal of Entomology. 2000;2:21–46.
2. Varga Z. Post-glacial dispersal strategies of Orthoptera and Lepidoptera in Europe and in the Carpathian basin. Proceedings of the 13. Colloquium of EIS, Leiden. 2003; 93-105.
3. Orci KM, Pecsenye K, Szövényi G, Vadkerti E, Nagy B, Rácz IA, Varga Z. The *Isophya* genus in Hungary In: Forró L. ed. Genesis of the fauna of the Carpathian Basin. 2007;47-56.
4. Vadkerti E. Populationbiology and morphometrics of Hungarian *Isophya* species. PhD thesis. University of Debrecen, Hungary; 2005.
5. Heller KG, Orci KM, Grein G, Ingrisch S. The *Isophya* species of Central and Western Europe (Orthoptera: Tettigonioidea: Phaneropteridae). Tijdschrift Voor Entomologie. 2004;147: 237-258.
6. Lodhi MA, Ye G, Weeden NF, Reisch BI. A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species. Plant Molecular Biology Reporter. 1994;12:6-13.
7. Vogler AP, DeSalle R. Phylogeographic patterns in costal North American tiger beetles (*Cicindela dorsalis* Say) inferred from mitochondrial DNA sequences. Evolution. 1993;47(4):1192-1201.
8. Pashley DP, Ke LD. Sequence evolution in mitochondrial ribosomal and ND-1 genes in lepidoptera: Implications for phylogenetic analyses. Mol Biol Evol. 1992;9(6):1061-75.

© 2017 Vadkerti et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21927