Short note

A new primer set for amplification of COI mtDNA in parasitic nematodes

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The mitochondrial gene cytochrome oxidase subunit I (COI) is currently a popular genetic marker, used for the barcoding of live organisms. DNA barcoding underlies such large research projects as the Consortium for the Barcode of Life (CBOL, http://www.barcodeoflife.org/) and the Barcode of Life Data Systems (BOLD, http://www.barcodinglife.org/). There are also some specialised genetic databases describing the diversity of various ecosystems as the Moorea Biocode Project (http://mooreabiocode.org/) or the Polar Barcode of Life (http://www.polarbarcoding.org/). As a marker, COI has become popular since the development of universal primers, researched by a group of scientists headed by Dr O. Folmer that enabled amplification of a 710 bp COI region from a wide range of metazoan invertebrates (Folmer *et al.*, 1994). At present, the so-called 'Folmer' partition is the most commonly used barcode region. Unfortunately, despite their great success Folmer primers are not very effective to work with such a large and important (from biological and medical aspects) group as nematodes, especially marine and parasitic species (De Ley *et al.*, 2005; Bhadury *et al.*, 2006; Derycke *et al.*, 2010). The main goal of our research was to develop a primer set for the successful amplification of the Folmer partition in wider range of parasitic nematodes.

A thorough analysis of COI sequences from mostly parasitic nematodes available from Genbank database (http://www.ncbi.nlm.nih.gov/genbank/) revealed the high variability in the Folmer's forward (LCO1490) primer annealing region that may cause the failure of the whole fragment amplification. Based on these data we modified the present primer by replacing the most variable nucleotide positions by degenerate ones. The development of reverse primer was based on the search for a new and more conservative COI gene region. Analysis of the multiple alignments of the nucleotide sequences generated using Clustal X (Thompson *et al.*, 1997) revealed the presence of a short conserved area shifted for 107 nucleotides to the 3' side from the reverse Folmer primer position, which was used for the development of a new reverse primer. In primer design we followed the recommendations of Dieffenbach *et al.* (1993), Kwok *et al.* (1994), Linhart & Shamir (2005) and Compton (1990). OligoAnalyzer 3.1 (http: //eu.idtdna.com/analyzer /Applications/OligoAnalyzer/default.aspx) was used for the analysis of primer properties. The check of primers' complementarity was carried out using Primer-BLAST program (http: //www.ncbi.nlm.nih.gov/tools/primer-blast). As a result, the new primer set has been developed (Table 1). Analysis of the thermodynamic characters and the secondary structure of the developed primers has shown that they do not form any heterodimers or homodimers and also do not form stable hairpin structures at temperatures above 35°C.

Nematode specimens used to verify the efficiency of the developed primers were collected in different locations on the territory of Russia, Mongolia, Indonesia and Philippines and represent the major parasitic groups such as Rhabditida, Ascaridida, Spirurida, Rhigonematida, Oxyurida and Mermithida. Specimens of *Haemonchus contortus* (Rudolphi, 1803), *Panagrolaimus* sp., *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, *Steinernema arenarium* (Artyukhovsky, 1967), *Baylisascaris* sp., *Toxocara canis* Werner, 1782, *Ichthyobronema hamulatum* (Moulton 1931), *Skrjabillanus tincae* Schigin & Schigina, 1958, *Carnoya philippinensis* Malysheva, Mohagan & Spiridonov, 2015, *Pseudonymus islamabadi* (Basir, 1941) and unidentified Mermithidae were stored at –18ºC until the DNA extraction. The DNA was extracted from the single nematode of each studied species according to Holterman *et al.* (2006). Usually 1 µl of homogenate was used as a template for a 25 µl PCR reaction.

Table 1. Analysis of thermodynamic properties and secondary structure of selected primers.

ID	Primer sequence (5'-3')	nt	GC%	Tm mean (min-max), °C	An%	Hairpins n/ΔG
NEM_COI_F	GGWSMAMMAAATCATAAAGATATTGG	26	32.7	51.8 (49.2-54.7)	19	2/-0.44; -0.32
NEM_COI_R	GTAATAGCMMCHGCYAAHACMG	22	48.5	55.1 (48.8-62.6)	27	–

Table legends: nt, number of nucleotides; GC%, percent of G and C nucleotides in sequence; Tm, melting temperature; An%, percent of ambiguous nucleotides; Hairpins n/ΔG, number and energy (kcal/mole) of hairpin.

PCR reactions were performed using Encyclo Plus PCR kit (Evrogen®, Moscow, Russia) according to the manufacturer's manual. PCR cycling parameters with NEM_COI_F/R primers included primary denaturation at 94°C for 3 min followed by 34 cycles 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, followed by post-amplification extension at 72°C for 7 min. For comparative purposes, amplification of the Folmer region with the original primer sets LCO1490/HCO2198 was also performed in accordance with the PCR protocol published on http://barcoding.si.edu/. PCR products were loaded on 1% agarose gels containing 0.003% EtBr and visualised using UV transilluminator ECX-15.M (Vilber Lourmat, Torcy, France) and Gel Imager-2 System (Helicon, Moscow, Russia). Bands were excised for DNA extraction with Wizard® SV Gel and PCR Clean-Up System (Promega®, Madison, USA). Samples were directly sequenced using the same primers as used for primary PCR reactions. DNA sequencing was performed by the Centre of Collective Usage "Genome" using a set of reagents as shown at http://www.genome-centre.ru/.

The developed DNA primers NEM_COI_F/R enabled amplification of a 600 bp region of the mitochondrial cytochrome oxidase subunit I gene from all nematode specimens studied. That is certainly a better result than that obtained using universal LCO1490/HCO2198 primers, which succeeded only in one specimen (*i.e.*, *C. philippinensis*). All obtained sequences have been deposited into GenBank database as KX365894-KX365904 (for newly developed primers) and KX365893 (for Folmer primers), respectively. The fact that the sequences obtained by our primers overlap sequences obtained by Folmer primers allows comparative analysis with data deposited earlier in international databases, which is convenient for research in the field of phylogeny and population genetics. We hope that the newly designed primers will become a useful tool for the study of the various groups of parasitic nematodes.

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REFERENCES

BHADURY, P., AUSTEN, M.C., BILTON, D.T., LAMBSHEAD, P.J.D. & ROGERS, A.D. 2006. Development and evaluation of a DNA-barcoding approach for the rapid identification of nematodes. *Marine Ecology Progress Series* 320: 1-9.

COMPTON, T. 1990. Degenerate primers for DNA amplification. In: *PCR Protocols: A Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White Eds). pp. 39-45. San Diego, USA, Academic Press.

DE LEY, P., DE LEY, I.T., MORRIS, K., ABEBE, E., MUNDO-OCAMPO, M., YODER, M., HERAS, J., WAUMANN, D., ROCHA-OLIVARES, A., JAY, B.A.H, BALDWIN, J.G. & THOMAS, W.K. 2005. An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences* 360: 1945-1958.

DERYCKE, S., VANAVERBEKE, J., RIGAUX, A., BACKELJAU, T. & MOENS, T. 2010. Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. *PLoS ONE* 5: e13716.

DIEFFENBACH, C.W., LOWE, T.M. & DVEKSLER, G.S. 1993. General concepts for PCR primer design. *Genome Research* 3: S30-S37.

FOLMER, O., BLACK M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

HOLTERMAN, M., VAN DER WURFF, A., VAN DEN ELSEN, S., VAN MEGEN, H., BONGERS, T., HOLOVACHOV, O.,

BAKKER, J. & HELDER, J. 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution towards crown clades. *Molecular Biology and Evolution* 23: 1792-1800.

KWOK, S., CHANG, S.-Y., SNINSKY, J.J. & WANG, A. 1994. A guide to the design and use of mismatched and degenerate primers. *Genome Research* 3: S39-S47.

LINHART, C. & SHAMIR, R. 2005. The degenerate primer design problem: theory and applications. *Journal of Computational Biology* 12: 431-456.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

URL: http://eu.idtdna.com/analyzer/Applications/ Oligo-Analyzer/default.aspx/ (accessed: Mach 14, 2016).

URL: http://www.ncbi.nlm.nih.gov/tools/primer-blast/ (accessed: Mach 14, 2016).

URL: http://www.ncbi.nlm.nih.gov/genbank/ (accessed: Mach 14, 2016).

URL: http://www.barcodeoflife.org/ (accessed: Mach 14, 2016).

URL: http://www.barcodinglife.org/ (accessed: Mach 14, 2016).

URL: http://mooreabiocode.org/ (accessed: Mach 14, 2016).

URL: http://www.polarbarcoding.org/ (accessed: Mach 14, 2016).

URL: http://barcoding.si.edu/ (accessed: Mach 14, 2016).

URL: http://www.genome-centre.ru/ (accessed: Mach 14, 2016).