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Short note

PCR amplification of a rRNA gene fragment from formalin-fixed and glycerine-embedded nematodes from permanent slides

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Formalin has been used as fixative in nematology for many years. For long-term storage, nematodes, after fixation, are usually transferred to glycerine via a dehydration step. Using this technique, several large slide collections of nematodes have been established worldwide. They are consulted by taxonomists keen to examine paratypes for their faunistic and systematic studies.

Recent advances in molecular biology and, in particular, the polymerase chain reaction (PCR) have opened alternatives for the identification and characterisation of nematode fauna. Development of bar-coding techniques is a promising tool for nematode identification and estimation of species content, abundance and diversity in samples. Currently, information useful for species identification based on DNA fragments is scarce in nematode databases. However, the increasing number of deposition of DNA sequences in GenBank and NemATOL databases will synergistically be beneficial for diagnostics in the near future (Powers *et al.*, 2004).

It was assumed that the effects of formalin fixation caused fixed specimen to be unsuitable for DNA analyses. However, several methods of DNA extraction from formalin-fixed material stored for many years were developed and tested. It was shown that their success depends on a number of factors including composition of fixation buffer, fixation time, DNA extraction protocols, the length of the PCR target, the concentration of target DNA and the PCR protocol itself (France & Kocher, 1996; Marchetti *et al.*, 1998; Coombs *et al.*, 1999; Sato *et al.*, 2001; Bucklin & Allen, 2004). De Giorgi *et al.* (1994) and Thomas *et al.* (1997) attempted to amplify DNA fragments from formalin-fixed nematodes. Although these studies were successful at both DNA recovery and PCR amplification, they faced some problems and limitations. The first team detected artefacts in sequences after amplification from fixed formalin nematodes, whereas the second one did only a limited number of experiments with samples fixed no more than 48 h before processing for PCR.

In this paper we describe a simple method of DNA extraction and amplification of an rDNA fragment from nematodes of the genus *Longidorus*. These specimens were fixed in formalin or TAF (containing formalin and triethanolamine) and embedded in glycerine for various periods on permanent slides.

DNA extraction. Permanent slides were opened carefully and TAF-fixed nematodes were transferred into a small Petri dish half filled with phosphate-buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4; Sambrook & Russel, 2001). The Petri dishes were placed on a shaking platform at slow speed. After 2 h the PBS was renewed and the plates were kept shaking over night. Freshly formalin-fixed specimens of *L. leptocephalus* were treated in the same way.

Single nematodes were put into 13 µl ddH_2O, cut into 2-5 pieces with a sterilised scalpel and transferred to an Eppendorf tube. Ten µl of 2 X Worm Lysis Buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3.0 mM Mg_2Cl, 2.0mM DTT, 0.9% Tween 20) and 0.1 µl Proteinase K from stock solution (20mg l^{-1}) were added to the tube, which was briefly spin and stored at −70°C for at least 10 min. Subsequently, the tube was incubated at 65°C for 1 h and at 95°C for 10 min. Finally, the DNA suspension was cooled to 4°C and stored at −70°C for further applications. No additional purification was made for following PCR procedure.

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                                  *        20        *        40        *        60        *        80
L. artemisiae*                  : CGGAATCAGTCGTA-CGACGCGC-GATGGCGCGT-TTTG--TCAGAGCG--AAAGCCTGG-CG-GGCGTGTTGG-AGTTAGTTGC : 75
Longidorus sp.* (Belgium)       : ..............-........-.........-....--.TG.....--......C..-..-.........-......... : 75
L. intermedius (AF480074)       : ..............-........-.........-..C.--..G.....--....T.C..-..-.......C--------..A.. : 68
L. elongatus (AF480075)         : ..............-........-.........-....--..G.....--....T.C..-..-....C.....G..A....A.. : 76
L. leptocephalus (AY601580)     : ..............-........-.........C-..C.--..G.....--....T.C..-..-..........T.AA....A.. : 76
L. leptocephalus**              : ..............-........-.........C-..C.--..G.....--....T.C..-..-..........T.AA....A.. : 76
L. piceicola (AY601577)         : ..............-...T....-.........-..C.--..G.....--....T.C..-..-.........--------..A.. : 68
Longidorus sp.* (Israel)        : T.............C-........-...AAT.T..-.....--CT......A-..-.T..A.CT.--.T.CA..A.G.AA....A.. : 76
L. athesinus (AY601574)         : ..............T-........-...AAT.....-..C.--C.......A-.-..T....TG.--....A..A.G.AA....A.. : 76
L. juvenilis (AY601579)         : ..............-..G.....-.........-..C.--C.C.....--....T.G..-..-....C.....G.AA....A.. : 76
L. apulus (AY601571)            : T.............T-........-..CA.T..AC-CC..--C.......A-.-..T....TG.--.T..A....G.AA....A.. : 76
L. goodey (AY601581)            : ..............-..T.....-..CT.G...C-..G.--.........--....T...AT..----.C..CC.GCTAC----.. : 70
L. goodey* (Ukraine)            : ..............-..T.....-..CT.G...C-..G.--.........--....T...AT..----.C..CC.GCTAC----.. : 70
L. arthensis (AY601570)         : ..............T-..AA..A-...AAT...C-.-..--.........A-.-..T....CG---.T.....A.G.AAC..---- : 70
L. sturhani (AF480071)          : ..............GT-..AA..T-...AAT...C-.-..--.........A-.-..T....CG---.T.....A.G.AAC..---- : 70
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Fig. 1. Fragment of D2 expansion sequence alignment for several species of the genus *Longidorus*.
* - sequence obtained from TAF fixed and glycerine embedded materials;
** -sequence obtained from formalin fixed materials.

PCR amplification, cloning and sequencing. After centrifugation (1 min; 16 000 *g*), 4 µl of the resulting DNA suspension was added to the PCR reaction mixture containing: 2.5 µl of 10 X PCR buffer, 5 µl Q solution (Qiagen, Hilden, Germany), 0.5 µl dNTPs mixture, 1.5 µl of each primer (1.0 µM), 0.2 µl of *Taq* Polymerase (*Taq* PCR Core Kit, Qiagen, Germany) and double distilled water to a final volume of 25 µl. Forward D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and reversed Fix2R (5'-CRG AAA TGC ATC GAA YGA AC-3') primers were synthesized by Eurogentec, Merelbeke, Belgium. These primers amplify the short fragment of the D2 expansion segment of the 28S rRNA gene. The cycling profile was 94°C for 3 min, 35 cycles 94°C for 30 s, 54°C for 40 s, and 72°C for 1 min followed by an extension at 72°C for 10 min. The PCR was performed in a PTC-100 Thermocycler (MJ Research, San Diego, USA). After DNA amplification, 3 µl of the product was run on a 1% agarose gel. The remainder was stored at −20°C and used for cloning and/or sequencing. PCR products were purified using a QIAquick Gel Purification Kit (Qiagen, Germany), cloned into pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega, Leiden, The Netherland). Several clones of each sample were isolated and submitted to PCR with vector primer. PCR product from both clones and DNA fragments obtained directly from nematode samples, were sequenced in both directions with Fix1F (5'-AAGGGCGTGAAACCGCTTAG-3') or Fix2R primers with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Sequences were run on a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

DNA was successfully extracted, amplified and sequenced from individual specimens recovered from paratype slides of *Longidorus artemisia* Rubtsova, Chizhov & Subbotin 1999, prepared in 1995 (Rubtsova *et al.*, 1999), from slides of *L. goodeyi* Hooper 1961, prepared in 1991, from two slides with unidentified species of the genus *Longidorus* from Belgium and Israel, prepared in 1999 and 2000, respectively, and from females of *L. leptocephalus* Hooper 1961, collected in 1997 and fixed and stored in formalin. At the time of analysis, these samples were 7, 11, 3, 2, and 5 years old, respectively.

When working with formalin-fixed tissue, one of the main concerns is contamination with exogenous DNA. To eliminate this problem, we included negative and positive controls for all DNA extractions and PCR experiments and designed and used a specific primer. For sequence analyses, we considered only sets that did not show any band in negative controls. A set of primers was developed along the following principles: (i) primers should produce a amplicon not longer than 250 bp to increase efficiency of amplification from fragmented DNA; (ii) one of the primers should be group-specific to avoid amplification of exogenous nematode DNA; (iii) primers should amplify most variable DNA region having enough nucleotide differences within the nematode group being studied to distinguish closely related species. Based on these principles and using information of rRNA gene sequences deposited in GenBank for longidorids (Rubtsova *et al.*, 2001; He *et al.*, 2005), we designed the primer Fix2R that, in combination with the universal primer D2A, amplifies a short amplicon (202-217 bp) of the 28S rRNA gene. This fragment corresponds to the most variable helix C2 of D2 expansion segment of the LSU molecule. We believe that this gene region can be used for molecular bar-coding of longidorids.

PCR products of about 210 bp were obtained from each of the five samples in several replications. Sequences of a fragment of the D2 expansion segment are aligned in Fig. 1. Comparison of sequences obtained from *L. artemisiae* or two putative new *Longidorus* species with those deposited in GenBank showed their uniqueness and difference from known sequences.

It has been shown that several factors, e.g. low pH of formalin solution, can induce extensive DNA-degradation and fragmentation, resulting in inappropriate DNA for PCR. This might be the explanation for those experiments in which we failed to obtain a PCR product from longidorid specimens taken from permanent slides with no information on the fixation procedure.

Although PCR amplification from fixed materials may be successful, artificial mutations (one per 500-600 bases) could be present at a higher frequency than found with PCR from undamaged DNA. Artifacts could be the consequence of formalin damaging or cross-linking cytosine nucleotides on either strand, so that the *Taq* DNA polymerase would not recognize them and instead of guanosine incorporate adenosine, thereby creating an artificial C-T or G-A mutation (Williams *et al.*, 1999). De Georgi *et al.* (1994) reported artificial mutation in sequences recovered from formalin-fixed materials. We did not observe any evidence for such type of sequence artefacts.

Comparison of DNA sequences from formalin preserved and glycerine embedded specimens of *L. goodey* or *L. leptocephalus* with DNA sequences of the same species obtained from fresh materials showed their sequences were identical and unmodified.

In this paper we report for the first time the successful amplification of DNA from nematode specimens recovered from permanent slides after long storage. Success in amplification of DNA from formalin-fixed and glycerine-embedded materials allows increased use of archived collections for population genetics and molecular systematic studies in nematology. Detailed studies are required to identify the effects of fixation conditions on DNA. Various DNA extraction methods need to be compared to determine the most effective and less laborious method producing high yield and quality of DNA.

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