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TECHNICAL NOTE

Simple and quick methods for nematode DNA preparation

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Abstract A DNA extraction kit, ISOHAIR® (Nippon Gene), which was originally developed for preparing DNA from hair and nail samples, was used to prepare nematode DNA for PCR and sequencing analyses. The methods provided here, which involved digesting (resolving) a single nematode individual in a tube containing the mixed enzyme solution, enabled the DNA to be prepared within 20 min. The prepared DNA was suitable for PCR amplification of near-full-length small subunit ribosomal RNA (ca 1.7 kb), of the D2/D3 expansion segments of large subunit RNA (ca. 0.7 kb), and of partial mitochondrial COI (ca. 0.7 kb) genes, followed by sequencing analysis. Furthermore, the prepared material could be preserved in a freezer (−30 °C) for at least 20 months, and more than 300 PCR reactions could be performed from a single individual nematode.

Keywords DNA · ISOHAIR® · Nematode · PCR · Sequencing

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Introduction

The morphology of nematodes is rather uniform within a family or genus compared with their species diversity (Blaxter et al. 1998; De Ley and Blaxter 2002). Because molecular identification techniques are indispensable for identifying species and conducting diversity surveys (Porazinska et al. 2010), several methods have been developed for preparing nematode DNA. For example, Floyd et al. (2002) used NaOH solution to dissolve nematodes; Iwahori et al. (2000) squashed a nematode using a small piece of filter paper and then used the material directly as a PCR template; and Williams et al. (1992) and Ye et al. (2007) used a "nematode digestion buffer", which was a mixture of buffer reagents and proteinase K solution.

In this study, we provide a quick and simple method for preparing nematode DNA using a DNA extraction kit, ISOHAIR® (Nippon Gene: http://nippongene.com/pages/products/extraction/isohair/hair01.html). The kit, which contains a mixture of buffer reagents and enzyme (proteinase) solution, was originally developed for preparing DNA from hair and nail samples, whose main components are barely degraded protein (keratin). Because keratin is also one of the main components of nematode exoskeletons (Bird and Bird 1991), we expected the body could be dissolved more easily by adding keratin degrading reagents to a normal degrading buffer and that the resolved nematode body could then be used directly as PCR template DNA unless the reagents inhibited the PCR reaction. This method has already been used for LAMP-based detection methods (Kikuchi et al. 2009) and applied in the "*Bursaphelenchus xylophilus* Detection Kit®" (Nippon Gene: http://nippongene-analysis.com/matsu-fs.htm). The method should also be useful for other nematode species besides the pinewood nematode, *B. xylophilus* (Steiner & Buhrer)

Nickle, although a detailed method has not yet been provided. Therefore, we report herein a simple and quick method for general nematode DNA preparation.

Materials and methods

DNA preparation

A single nematode was placed in 30 µl "nematode dissolving solution" which contained five parts "enzyme solution", four parts "lysis solution" (the enzyme and lysis solutions were provided as the contents of the ISOHAIR® extraction kit), and 100 parts TE buffer (pH 8.0), and incubated at 60 °C for 20 min. The resulting nematode solution was used directly in PCR reactions without purification. Adults and dauer juveniles of *Bursaphelenchus okinawaensis* Kanzaki, Maehara, Aikawa and Togashi, *Caenorhabditis elegans* (Maupas) Osche, and *Pristionchus pacificus* Sommer, Carta, Kim and Sternberg were used as sample nematodes. Four adult individuals of each of the three species, four dauer juveniles each for *B. okinawaensis* and *C. elegans,* and eight dauers of *P. pacificus* were used as replicates.

PCR amplification

PCR amplification of three genes was conducted: near-full-length ribosomal small subunit RNA (ca. 1.7 kb), D2/D3 expansion segments of the large subunit RNA (ca. 0.7 kb), and mitochondrial cytochrome oxidase subunit I (ca. 0.7 kb). PCR was performed using GoTaq Green Master Mix® (Promega: http://www.promega.com/resources/articles/pubhub/promega-notes-2005/gotaq-green-master-mix-from-amplification-to-analysis/) in accordance with the manufacturer's instructions, i.e., 1 µl template DNA solution (= dissolved nematode), 1 µl forward and reverse primers (10 µM), 12 µl distilled water, and 15 µl GoTaq Green kit. The PCR conditions were: 2 min of preheating at 95 °C; 30 cycles with 1 min of denaturation at 95 °C, 1 min of annealing at 52 °C, and 2 min of extension at 72 °C; and 10 min of post-extension at 72 °C. *C. elegans* and *P. pacificus* were used for PCR of all three genes, but *B. okinawaensis* was used for amplification of SSU and D2/D3 LSU only, because the universal primer set for mtCOI does not match *B. okinawaensis* (Kanzaki, unpublished observation).

The PCR products were electrophoresed in an agarose gel (1.0 %) with 2-Log DNA Ladder (New England BioLabs, http://www.neb.com/nebecomm/products/productn3200.asp) as molecular size marker at 100 V for 30 min, stained using ethidium bromide solution, and examined under a UV trans illuminator.

To assess the lower limit of DNA concentration, three different dilutions, ×1 (original solution), ×10, and ×100 solutions, were used as template DNA. To examine the storage stability of the DNA solution, sample solutions were stored in a laboratory freezer (−30 °C), and the experiment was repeated three and 20 months after sample preparation. Some of the PCR products were arbitrarily selected from the first experiment in accordance with the amplification conditions (different illumination strengths), purified, and sequenced to examine the applicability of using PCR products for sequence analysis using a Montage PCR Filter Unit (Millipore: http://www.Millipore.com/techpublications/tech1/6dljnb) and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems: http://www.ibt.lt/sc/files/BDTv3.1_Protocol_04337035.pdf).

Results and discussion

The study results are shown in Fig. 1 and Supplementary Table 1.

With the exception of one sample from a *C. elegans* adult which is assumed to have failed during the preparation of DNA material for some reason (e.g., the nematode was not properly dipped in the reagent) all of the sample material was suitable for PCR amplification of the two or three genes examined. Therefore, the single failed sample was excluded from the following discussion and remarks.

In the first experiment, which examined DNA amplification of freshly prepared material, DNA fragments were successfully amplified from ×1 (without dilution) and ×10-diluted template DNA samples. No or only weak amplification was observed for *B. okinawaensis* adults and dauers, and *C. elegans* dauers in the ×100 dilution treatment. On the other hand, DNA fragments were successfully amplified from ×100-diluted material from *C. elegans* adults and *P. pacificus* adults and dauers. One possible explanation for these differences involves the body size of the nematodes. *B. okinawaensis* specimens had smaller bodies than the other two species (i.e., <1/4 the volume; Sommer et al. 1996; Kanzaki et al. 2008), which suggests there was a smaller amount of DNA in the *B. okinawaensis* specimens. However, irrespective of the small body size, DNA fragments were successfully amplified from ×100-diluted template samples from *P. pacificus* dauers. This difference could be because of the suitability of the "universal" primer sets for the difference species. We used universal primer sets that were provided in previous reports (Kanzaki and Futai 2002; Ye et al. 2007; Zeng et al. 2007), however, as Porazinska et al. (2009, 2010) suggested, the designation of universal primers for nematodes is very difficult because of their high molecular substitution rate. Thus, parts of the primer sequence might not match the

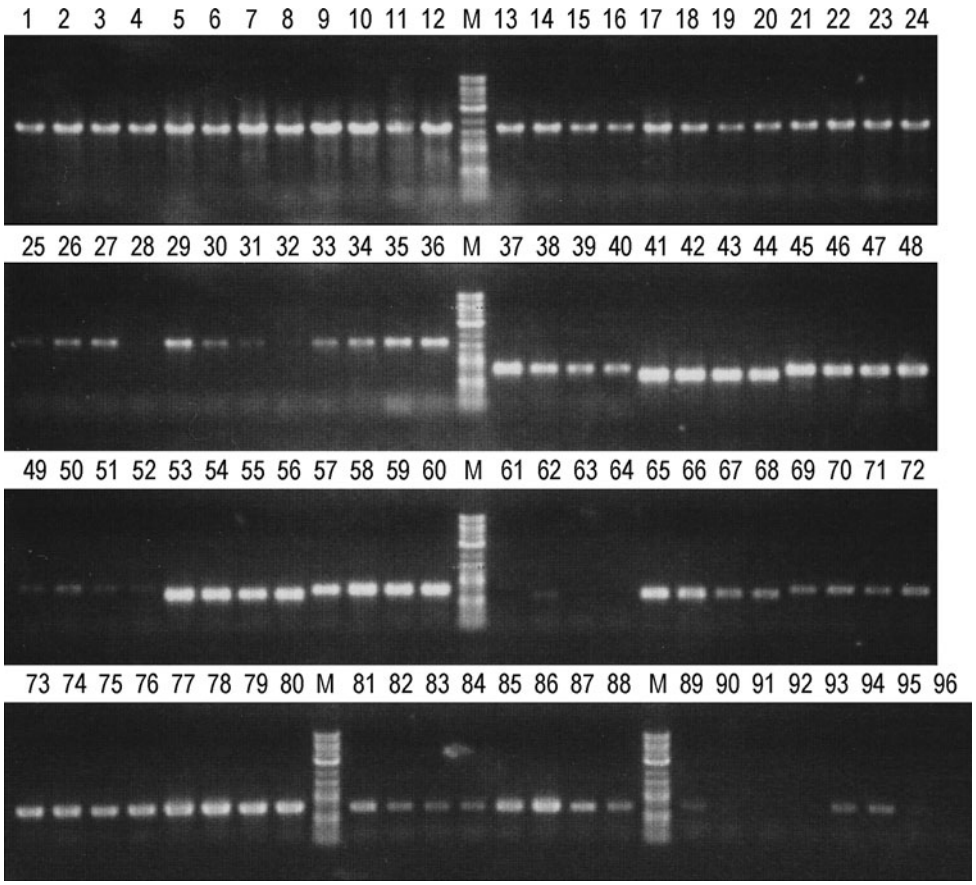

Fig. 1 DNA fragments amplified from nematode DNA material that was prepared by use of ISOHAIR® and stored in a laboratory freezer for 20 months. *M*: molecular size marker (2-Log DNA Ladder, NEB). *Lanes 1–12*, *13–24*, and *25–36* are near full length SSU amplified from DNA materials with ×1, ×10, and ×100 dilution, respectively. *Lanes 1*, *2*, *13*, *14*, *25*, *26* are *Bursaphelenchus okinawaensis* adults; *3*, *4*, *15*, *16*, *27*, *28* are *B. okinawaensis* dauers; *5*, *6*, *17*, *18*, *29*, *30* are *Caenorhabditis elegans* adults; *7*, *8*, *19*, *20*, *31*, *32* are *C. elegans* dauers; *9*, *10*, *21*, *22*, *33*, *34* are *Pristionchus paciticus* adults; *11*, *12*, *23*, *24*, *35*, *36* are *P. pacificus* dauers. *Lanes 37–48*, *49*, *60*, *61–72* are D2/D3 LSU amplified from DNA materials with ×1, ×10 and ×100 dilution, respectively. *Lanes 37*, *38*, *49*, *50*, *61*, *62* are *B. okinawaensis* adults; *39*, *40*, *51*, *52*, *63*, *64* are *B. okinawaensis* dauers; *41*, *42*, *53*, *54*, *65*, *66* are *C. elegans* adults; *43*, *44*, *55*, *56*, *67*, *68* are *C. elegans* dauers; *45*, *46*, *57*, *58*, *69*, *70* are *P. paciticus* adults; *47*, *48*, *59*, *60*, *71*, *72* are *P. pacificus* dauers. *Lanes 73–80*, *81–88*, *89–96* are partial mtCOI amplified from DNA materials with ×1, ×10 and ×100 dilution, respectively. *Lanes 73*, *74*, *81*, *82*, *89*, *90* are *C. elegans* adults; *75*, *76*, *83*, *84*, *91*, *92* are *C. elegans* dauers; *77*, *78*, *85*, *86*, *69*, *70* are *P. paciticus* adults; *79*, *80*, *87*, *88*, *95*, *96* are *P. pacificus* dauers). The fragment sizes are described in Supplementary Table 2

annealing sites of *B. okinawaensis* and *C. elegans*. All of the DNA fragments that were examined were suitable for sequence analysis; i.e., the expected molecular sequences were obtained from the materials. Also, under identical conditions (×1 or ×10 diluted materials), ribosomal RNA genes from more than 200 nematode species belonging to various phylogenetic clades (e.g., orders Mermithida, Monhysterida Mononchida and Dorylaimida, infraorders Tylenchomorpha, Panagrolaimomorpha and Cephalobomorpha, and families Diplogastridae and Rhabditidae in the order Rhabditida) were successfully sequenced (data not shown).

Amplification in the second experiment, using 3-month-old template material, was similar to the first. Although the amplification efficiency was slightly lower than in the first experiment when using diluted material, amplification was generally successful with ×1 and ×10 diluted template samples.

In the third experiment, using 20-month-old template material, amplification was generally successful with ×1 and ×10 diluted template samples. However, many of ×100 samples were no longer suitable for PCR amplification. This was probably because of degeneration of the DNA. The samples were stored in a laboratory freezer at −30 °C. Degradation of DNA seems to have occurred at some point during storage, probably during freeze/thaw cycles when preparing samples for PCR or during several electricity failures. Also, the PCR amplified regions used in

this study, ribosomal and mitochondrial genes, are thought to exist as multi-copies in nematode genomes. Therefore, the amplification efficiency would be lower when trying to amplify a single-copy gene compared with the genes used in this study. However, the use of a higher efficiency enzyme during PCR amplification could enable us to obtain DNA fragments of multi-copy genes from degraded DNA and/or single-copy genes from regular material.

Overall, the method of DNA preparation provided here, which enabled 300–3000 PCR reactions to be conducted from a single nematode individual over a span of at least 20 months after preparation, provided a relatively quick, easy, and inexpensive (less than 50 JPY for each material) method for conducting PCR and sequencing of nematode genes. The method also proved applicable to DESS-fixed materials (Yoder et al. 2006) in other experiments that were conducted by the authors (Kanzaki, unpublished observation), and so may have wider applicability than has been demonstrated here. This easy method could be extremely useful for high-throughput analysis for diagnosis and for studying the population genetics, taxonomy, and phylogeny of nematodes.

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