PRIMER NOTE

Nematode-specific PCR primers for the 18S small subunit rRNA gene

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Abstract

A set of polymerase chain reaction primers were designed, which amplify a *c.* 1 kb fragment of the 18S ribosomal DNA gene, and are specific to the phylum Nematoda. These have proven useful in isolating nematode genes from samples mixed with other biological material, particularly with application to DNA barcoding. Optimal reaction conditions are described. These primers have successfully amplified the correct fragment from a wide phylogenetic range of nematodes, and have so far generated no sequences from any other organismal group.

Keywords: Nematodes, 18S ribosomal RNA (SSU), specific amplification, DNA barcoding

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There is currently much interest in the use of DNA sequences as markers for taxonomic identification and biodiversity surveys, an approach also known as DNA barcoding (Hebert *et al.* 2003). The 18S small subunit ribosomal RNA gene has been shown to be an effective marker for barcoding of nematode worms (Floyd *et al.* 2002). However, a common difficulty faced by researchers in this field is that of contamination. When isolating DNA from small organisms in complex natural environments such as soil or marine sediment, it is often impossible to avoid coextracting DNA from a multitude of other biological material that may not be of interest to the study at hand, such as microorganisms and fungal spores. The polymerase chain reaction (PCR) can be used to isolate and amplify a gene of interest from a mixed sample of DNA. However, the 18S gene, like all ribosomal genes, is universally present in eukaryotes. Commonly used PCR primers bind to highly conserved regions of the gene, and will potentially amplify any 18S homologue, regardless of its organism of origin. We have been carrying out a DNA-based survey of nematodes from deep-sea sediment from the equatorial Pacific Ocean, but found that standard 18S primers frequently amplified fungal sequences, rather than the nematode genes that were the intended target. We

have therefore designed a new set of oligonucleotide primers, which will specifically amplify nematode 18S genes.

Using CLUSTALX (Thompson *et al.* 1997), an alignment was constructed containing all available full-length nematode 18S sequences, and a selection of sequences from fungi related to those taxa which had been identified as contaminants. The alignment was examined for regions conserved among all nematodes but not present in fungi, and which would generate a fragment of at least 500 bp in length, so as to provide sufficient information to resolve individuals into distinct taxa. Candidate regions were examined using the program, PRIMER 3 (Rozen & Skaletsky 2000), which calculates parameters such as annealing temperature and stability to determine which might be suitable as PCR primer sites.

In total, two forward and three reverse primers were found which appeared as suitable candidates. When these were synthesized and tested, it was found that one particular pair reliably generated amplification products from nematode DNA extracts. The 5'–3' sequence of the forward primer (designated Nem_18S_F) is CGCGAATRGCTCAT-TACAACAGC (23 bases); the reverse primer (Nem_18S_R) is GGGCGGTATCTGATCGCC (18 bases). A standard reaction volume was 20 µL, comprising: QIAGEN PCR buffer at 1 × concentration (including $MgCl_2$ at 1.5 mM); dNTPs at a concentration of 0.2 mM for each nucleotide; primers at 0.5 µM each and QIAGEN *Taq* DNA polymerase

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Laxus oneistus (Nematoda, Desmodorida) Y16919          ..TATGGTGAAGCGCGGAATGGCTCATTACAACAGCCGTTGTTTC[...........]AGTTAGAGGTTCGAAGGCGATCAGATACCGCCTAGTTCTAA..
Xiphinema rivesi (Nematoda, Dorylaimida) AF036610      ..TACGGTGAAGCGCGAATAGCTCATTACAACAGCCACCGTTTA[...........]AGTTAGAGGTTCGAAGGCGATCAGATACCGCCTAGTTCTAA..
Monhystera riemanni (Nematoda, Monhysterida) AY593938  ..AACAGTGAAGCGCGCGAATAGCTCATTACAACAGCCGTTGTTTC[...........]AGTAACCGGTTCGAAGGCGATCAGATACCGCCTAGTCGTCA..
Plectus acuminatus (Nematoda, Araeolaimida) AF037628   ..AATGGTGAAGCGCGGAATGGCTCATTACAACAGCCACTGTTTA[...........]AGTCAGAGGTTCGAAGGCGATCAGATACCGCCTAGTTCTGA..
Prismatolaimus intermedius (Nematoda, Enoplida) AY284729 ..AATGGTAAAGCGCGAATGGCTCATTACAACAGCCATAGTTTA[<--~900bp-->]AGTCTGAGGTTCGAAGGCGATCAGATACCGCCTAGTTCAGA..
                                Forward primer, Nem_18S_F  CGGGAATRGCTCATTACAACAGC          Reverse primer, Nem_18S_R   GGCGATCAGATACCGCC
Trichosporon cutaneum (Fungus) X60182                  ..TACCGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTA[...........]GGTTAGGGGGATCAAAAACGATTAGATACCGTTGTAGTCTTAA..
Dioszegia aurantiaca  (Fungus) AB049615                ..TACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTA[...........]GGTTAGGGGGATCAAAAACGATTAGATACCGTTGTAGTCTTAA..
Antrodia camphorata (Fungus) AJ496290                  ..TACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTA[...........]GGTTAGGGGGATCGAAAAACGATCAGATACCGTTGTAGTCTTAA..
Cladosporium cladosporioides (Fungus) AF548071         ..TACGGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTA[...........]AGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAA..
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Fig. 1 Part of an alignment constructed from a selection of nematode and fungal 18S sequences, showing regions where the new primers bind. GenBank Accession nos are given for each sequence.

at 0.025 units/μL. To each reaction, 1 μL of extracted nematode DNA template was added, typically containing around 2–10 ng of genomic DNA. The optimal thermocycling conditions were found to be: an initial denaturation at 94 °C for 5 min; 35 cycles of amplification (94 °C for 30 s; 54 °C for 30 s; 72 °C for 1 min); followed by a final extension at 72 °C for 10 min. The thermocycling machine used was an MJ Research DNA Engine Tetrad.

The amplified DNA fragments were sequenced using an Amersham Biosciences DYEnamic ET sequencing kit, and run on a MegaBACE 500 DNA analysis system. The nucleotide sequences obtained were compared using BLAST (Altschul *et al*. 1990) against known sequences in GenBank, and it was found that all sequences generated using these primers showed close matches to nematodes.

The primers amplify an internal fragment of the 18S, approximately 900 bp in length (the full-length gene is approximately 1700 bp): the forward primer binds at a site around 100 bp inward from the 5′ end of the gene, and the reverse primer at around 700 bp inward from the 3′ end (see Fig. 1). The fragment therefore covers most of the 5′ half of the 18S gene, where much sequence variability tends to be found, making it a useful region for barcoding. In the course of our marine nematode survey, using the previously discussed protocol, sequences from a wide range of taxonomic groups have been identified, including Enoplida, Araeolaimida, Monhysterida, Dorylaimida and Chromadorida, suggesting the broad utility of these primers across the phylum Nematoda.

Acknowledgements

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