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Universal nematode detection by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) of purified nematode nucleic acids

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Abstract

In this study, we have described a modified degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) method for molecular typing of nematodes collected from wild birds. To design the DOP-PCR, we selected 50 nonamers as DOP-motifs referring the data from a nematode genome. Inverted repeats of nonamers with a 100–1,500 base interval in the reported nematode genomes on the selection of the 50 nonamers. In these nonamers, 5 nonamers showed to create ladder pattern by DOP-PCR on 9, 6, 1, 2 and 3 species of morphologically identified adult nematodes, respectively. Eleven species of nematodes were distinguishable by combining the results of the two primers. It was suggested that the nematode species could be distinguished by DOP-PCR with a combination of 2 nonamers.

Key Words: degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), inverted repeat, molecular typing PCR

Nematode includes over 26,000 species in its taxonomy[14]. Despite a uniform body plan, nematodes are more diverse at the molecular level comparing previously recognized[10]. Mitreva *et al.* analyzed 265,494 expressed-sequence tag sequences, defined 4,228 nematode-specific protein families from nematode-restricted genes interesting as drug and vaccine targets[11]. Although veterinary researchers have focused on parasitic nematodes, free-living nematodes reported to contribute or affect the status of parasitic nematodes[14,17,19]. Some parasitic species has a free-living generation, which affects epidemiological status[19]. In this context there is an increasing demand for species-identification of nematode[14].

Morphological observation has been main

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procedure for identification of species, which needed long experience and was a time-consuming step[14]. As a countermeasure, molecular methods of nematode identification provide accurate, alternative diagnostic approaches[14]. DNA sequence of target regions has been the main taxonomic marker for species-identification. The main region targeted for species-identification is 18S rRNA and cytochrome oxidase I (COI) genes[2,15]. Similar to rRNA and COI, other genes homologous in many species of nematodes, such as ATP synthase subunit 6, heat shock protein 70, cytochrome oxidase subunit I and calponin-like protein genes, have also been used for molecular typing of nematode species[12,13,20,23]. Although many organisms conserved these genes, one must design specific primer for each nematode species. On epidemiological research of parasites in wild animals and birds, researchers should determine so many species from a variety of animals and birds, which might be a time-consuming[8,14].

As gene-independent molecular typing methods, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) have been used for analyzing nematodes[4,7,21,22]. In RFLP method, one must determine the ideal restriction enzyme (s) before using the method, and there may be difficulties in selecting the appropriate enzymes and recognizing the sequences for the methods without genomic information. With RAPD and AFLP, selected semi-random oligomers were used for amplification of PCR fragments in a tube, and the sizes of the fragments vary according to the species[5,7]. Problem of RAPD was the low stability, and it requires a preliminary test for the inspection method determined for each target species[7]. When one got the sequences of RAPD products, one could design primers for species-identification (sequence characterized amplified region, SCAR)[24]. As a similar method to RAPD, semi-random amplification method using short (6–10 mer) motifs with 3' degenerate nucleotide and linker sequence, degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) could be used for amplification of molecular typing fragments RAPD[18]. The Difference between RAPD and DOP-PCR is that DOP-PCR can make use of known genomic information for primer design.

In the DOP-PCR, preset 6–10 nucleotide motif (DOP-motif) connecting with degenerate bases and linkers was used to amplify the regions flanked by two DOP-motifs[18]. A DOP primer (e.g. DOP-motif) amplifies several regions flanked by an inverted repeat of the DOP-motif. If one could select the common inverted repeat to DOP-motif, the DOP-PCR method amplifies several fragments on many species and has advantages of RAPD and SCAR. Although there are several tools to search inverted repeats on the genome, procedure for prediction of numbers of inverted repeat flanking sequences needs difficult and complex steps. An analysis that satisfies such a requirement can be completed within a practical time by writing them in the script language which has been used for bioinformatics programming a one of techniques for artificial intelligence. In this study, we used Ruby scripting language and regular expression to search DOP-motif and predict sizes of amplified products on nematode genomes. We found some DOP-motifs which can distinguish nematodes.

Nematodes were collected from wild birds in the Wild Animal Medical Center of Rakuno Gakuen University (WAMC); the nematodes were collected during pathological examinations of all the organs of the birds. The collected nematodes were fixed in 70% ethanol; became transparent in a lacto-phenol solution, and the species were identified on the basis of morphological characteristics[1,3]. Genomic DNAs were extracted from 11 species of nematodes (Table 1), which were adequately distinguished on the basis of morphological characteristics. The worms were crushed in liquid nitrogen, and DNA was extracted by using the Genomic DNA isolation Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocols.

Table 1. Nematode species and hosts analyzed in the molecular-typing experiments

Order	Superfamily	Family	Species	Host
Enoplida	Trichurioidea	Capillariidae	*Eucoleus contortus*	*Aans crecca*
Strongylida	Trichostrongyloidea	Trichostrongylidae	*Epomidiostomum uncinatum*	*Anas platyrhynchos*
		Amidostomatidae	*Amidostomum anseris*	*Cygnus cygnus*
			A. acutum	*Anas platyrhynchos*
Ascaridida	Ascaridoidea	Anisakidae	*Contracaecum rudolphii*	*Phalacrocorax capillatus*
	Heterakoidea	Heterakidae	*Heterakis gallinarum*	*Phasianus chlorchis*
		Ascaridiidae	*Ascaridia columbae*	*Columba livia*
Spirurida	Habronematoidea	Tetrameridae	*Tetrameres fissispina*	*Aythya marila*
	Diplotiraenoidea	Diplotriaenidae	*Diplotriaena bargusinica*	*Turdus naumanni*
			Serratospiculum tendo	*Falco peregrinus*
	Filarioidea	Onchocercidae	*Sarconema eurycerca*	*Cygnus colombianus*

We used single primers for DOP-PCR as original design of DOP-PCR[18]. In the original design of DOP-PCR, primers flanked microsatellite sequence to obtain PCR products of various sizes. While, in the microsatellite in nematode reported to have relatively small deviations and may to be used for markers for species[16]. Thus, we simply search nonamers-inverted repeat which flanking a 100–1,500 base-sequence, such as ATGGGCTAC (100 to 1,500 bases) GTAGCCCAT, throughout the known genomic sequences (Table S1). As the known genomic sequences, we selected the 10 target species based on the total length of the scaffold sequences (Table S1). A phylogenetic tree including the 10 species and the target species for DOP-PCR were shown (Fig. S1).

The reaction conditions for DOP-PCR were primarily in accordance with a previously described method[18]. Go-Taq Green (Promega, Madison, Wisconsin) was used for amplification. The products were electrophoresed on 2.0% agarose gels in Tris-borate-EDTA (TBE) buffer (pH 8) for 1 hour at a constant voltage (100 V). Then, the products were stained with ethidium bromide and photographed.

Among the 50 primers (Table 2), 5 primers could amplify any of the prepared DNA samples from 11 species (Fig. 1A ～ E). In the trials, P32 (Fig. 1A) showed well divided and distinguishable patterns in 9 in 11 species of nematodes. Similarly, other nonamers showed to create ladder pattern by DOP-PCR on 6, 1, 2 and 3 species of nematodes, respectively (Fig. 1B ～ E). Eleven species of nematodes were distinguishable by combining the results of the two primers (P32 and 33). It was suggested that the nematode species could be distinguished by DOP-PCR with a combination of nonamers.

DOP-PCR method utilizes PCR with random oligomers, which amplify of random regions surrounded by the DOP-motif and its complementary sequence[18]. Since such a small repetitive inverted pair were present scattered on the genome by chance, the sizes-pattern of the amplified products different between species and could be used as a marker for species-identification[9].

On the results, size patterns were different with DOP-Primers. In this study, species which genome has not been published was subjected to DOP-PCR (Fig. 1). On eight species in the eleven tested species, relatively closely related genome-published species were existed (Fig. S1). *Contracaecum rudolphii*, *Ascardia columbae* and *Heterakis gallinarum* belonged to order Ascaridida same as *Parascaris univalens* (Fig. S1). *Tetrameres fissispina*, *Diploriaena bargusinica*, *Serratospiculum tendo* and *Sarconema eurycerca* belonged to order Spirurina same as *Loa loa*. *Eucoleus contortus* belonged to order Dorylaimia same as *Trichuris suis* and *Trichinella*. The numbers of nonamers-inverted repeats within

Table 2. Designed DOP-Primer sequences

Name of primer	Sequence (5'→3')	Name of primer	Sequence (5'→3')
P1	CCGAGTGGAGNNNGAAACAGTG	P26	CCGAGTGGAGNNNTTCCAACTC
P2	CCGAGTGGAGNNNCACTGTTTC	P27	CCGAGTGGAGNNNCTCGATTTC
P3	CCGAGTGGAGNNNCTGAAACAG	P28	CCGAGTGGAGNNNATCCAGAAG
P4	CCGAGTGGAGNNNTTCCAGAAG	P29	CCGAGTGGAGNNNTTTCCAGTG
P5	CCGAGTGGAGNNNCTGTTTCAG	P30	CCGAGTGGAGNNNCATCTTCTG
P6	CCGAGTGGAGNNNTTCTGGAAG	P31	CCGAGTGGAGNNNCACTTTCAG
P7	CCGAGTGGAGNNNCAGTTTCAG	P32	CCGAGTGGAGNNNTTCAGATGG
P8	CCGAGTGGAGNNNTTTCAGCTC	P33	CCGAGTGGAGNNNGTCATCATC
P9	CCGAGTGGAGNNNCTGAAACTG	P34	CCGAGTGGAGNNNCGATTTCAG
P10	CCGAGTGGAGNNNTGTCAACTG	P35	CCGAGTGGAGNNNTCGATTTCC
P11	CCGAGTGGAGNNNATCGATTGG	P36	CCGAGTGGAGNNNTTGAAGCTC
P12	CCGAGTGGAGNNNTTTCCAGAG	P37	CCGAGTGGAGNNNTGGAAACAG
P13	CCGAGTGGAGNNNCTGGAAATG	P38	CCGAGTGGAGNNNTTCCAACTG
P14	CCGAGTGGAGNNNCATTTCCAG	P39	CCGAGTGGAGNNNTGTTTGCAG
P15	CCGAGTGGAGNNNTTCCAGATG	P40	CCGAGTGGAGNNNTTCTCCAAC
P16	CCGAGTGGAGNNNTCAGAACTG	P41	CCGAGTGGAGNNNTTCTCGAAG
P17	CCGAGTGGAGNNNTCCATCTTC	P42	CCGAGTGGAGNNNAAGAAGCTG
P18	CCGAGTGGAGNNNTGTTTCCAG	P43	CCGAGTGGAGNNNTTTCCACTG
P19	CCGAGTGGAGNNNTTCCAGTTG	P44	CCGAGTGGAGNNNTTTCTCTGG
P20	CCGAGTGGAGNNNTTCGATTCC	P45	CCGAGTGGAGNNNGAATTGCTC
P21	CCGAGTGGAGNNNCTCATTCTC	P46	CCGAGTGGAGNNNTTCCATCTC
P22	CCGAGTGGAGNNNCTTCATCTG	P47	CCGAGTGGAGNNNGGAATTTCC
P23	CCGAGTGGAGNNNCATTTCCTC	P48	CCGAGTGGAGNNNCAGAAACTG
P24	CCGAGTGGAGNNNTTTCAGGTC	P49	CCGAGTGGAGNNNAACTGCTTC
P25	CCGAGTGGAGNNNTTCCAGTTC	P50	CCGAGTGGAGNNNTTCAGTTCC

*CCGAGTGGAGNNN: Universal linker including degenerate nucleotides.

1,500 bases were predicted to be 4 to 34 in the genome of *Parascaris univalens*, *Loa loa*, *Trichuris suis* and *Trichinella* which numbers might be suitable for DOP-PCR (Table S1). As similar frequencies of nonamers-inverted repeats which sizes were less than 1,500 bases were also shown in many species which were DOP-PCR failed, appropriate frequency is not a sufficient condition but may be included in band-formation on DOP-PCR. It was suggested that frequency analysis of nonamers-inverted repeats in published genome was an efficient approach to design DOP-PCR primers for species-identification.

In this study, a number of DOP-motifs which could be practically used were restricted. Any of the polymorphic band-pattern on DOP-PCR could not be predicted from genome analysis of DOP-motifs. Frequency analysis for nonamers-inverted repeats suggested to be one of guides for primer design. Recently, the next-generation sequencing improve genome data. Ninety-three genomic data are now registered on NCBI genome database (Table S2). Release of genomic sequences suggested to be accelerated by Nanopore sequencer[6]. From these points, DOP-PCR with preliminary information of DOP-motifs is considered becoming a useful method for species-identification of nematode.

In conclusion, to design a DOP-PCR using genomic information of nematodes is a significant beneficial way in advancing species-identification.

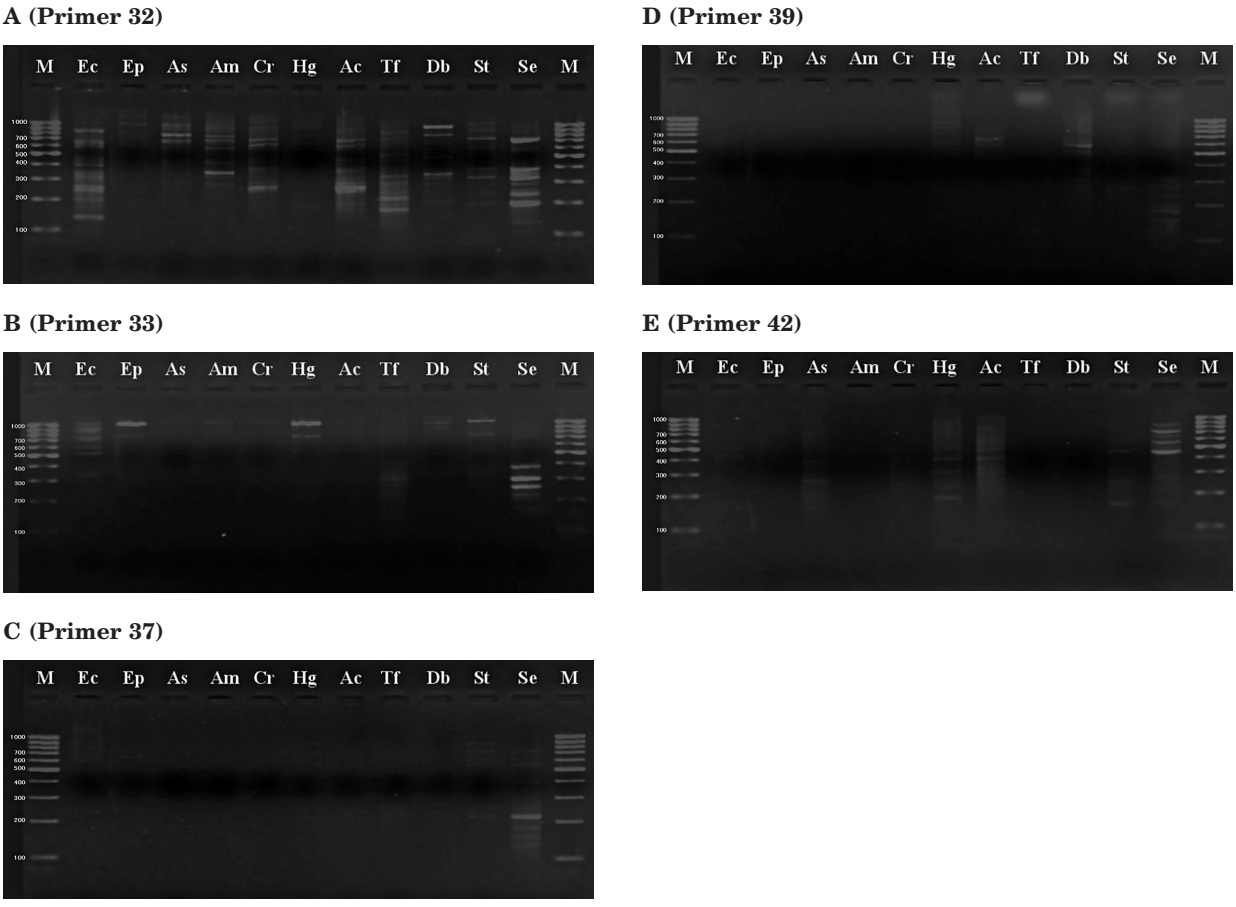

Fig. 1. Gel electrophoresis patterns of DOP-PCR products; primer 32 (A), primer 33 (B), primer 37 (C), primer 39 (D), primer 42 (E). M, marker (100 bp); Ec, *Eucoleus contortus*; Ep, *Epomidiostomum uncinatum*; As, *Amidostomum anseris*; Am, *Amidostomum acutum*; Cr, *Contracaecum rudolphii*; Hg, *Heterakis gallinarum*; Ac, *Ascaridia columbae*; Tf, *Tetrameres fissispina*; Db, *Diplotriaena bargusinica*; St, *Serratospiculum tendo*; Se, *Sarconema eurycerca*..

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.66.4.311

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