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NOTE

Parasitology

Development of conventional multiplex PCR method for discrimination between *Dispharynx nasuta* and *Cheilospirura hamulosa* (Nematoda: Acuariidae) parasitizing poultry

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ABSTRACT. The poultry infections caused by *Dispharynx nasuta* and *Cheilospirura hamulosa* nematodes are difficult to be diagnosed by fecal examination because of their egg similarity. In this study, we analyzed DNA sequences of nuclear ribosomal 18S-ITS1-5.8S-ITS2-28S region of *D. nasuta* and *C. hamulosa* and developed conventional multiplex PCR method using species-specific primers for discriminating between the two species. The method amplified 455-bp and 319-bp fragments specific to *D. nasuta* and *C. hamulosa*, respectively, and did not produce them against the other chicken nematode species, *Ascaridia galli*, *Oxyspirura mansoni*, *Heterakis gallinarum*, *Heterakis beramporia*, and *Heterakis indica*, suggesting that the multiplex PCR is sensitive and available for species diagnosis.

KEY WORDS: *Cheilospirura hamulosa*, *Dispharynx nasuta*, multiplex PCR, nuclear ribosomal 18S-ITS1-5.8S-ITS2-28S DNA, poultry

The infections caused by the family Acuariidae nematodes have been fear problems in poultry, especially in local chickens, and present the digestive disorder in the bird hosts [3]. The main causative agents of the infections are *Dispharynx nasuta* and *Cheilospirura hamulosa* parasitizing the proventriculus and the gizzard, respectively, of the definitive hosts, and particularly severe gross lesions of the gizzard infected with *C. hamulosa* have been reported [12, 16]. These two species have indirect life cycle and their third stage larvae develop in the intermediate hosts which are a terrestrial arthropod such as grasshoppers, beetles, and weevils for *D. nasuta* [16], and isopods for *C. hamulosa* [2]. The *D. nasuta* and *C. hamulosa* infections in birds are diagnosed by detecting eggs in fecal examination; however, the morphological characteristics of their eggs are very similar to each other: the eggs are ellipsoid and thick-shelled, and the size is 33–40 × 17–26 µm in *D. nasuta*, and 36–51 × 22–29 µm in *C. hamulosa*. Therefore, it is difficult to morphologically distinguish their eggs, and the eggs of *D. nasuta* cannot be also distinguished from those of the other spirurid nematodes [2].

Recently, molecular techniques using PCR targeting nuclear ribosomal 18S, ITS1, ITS2, and 28S regions have been developed for discriminating between morphologically related parasite species [7, 13]. Unfortunately, no reliable nucleotide sequences of *D. nasuta* and *C. hamulosa* available for molecular technique have been elucidated.

The purpose of this study is to determine the nuclear ribosomal DNA sequences of *D. nasuta* and *C. hamulosa*, and to develop multiplex PCR method for discriminating between the two species.

Nine *D. nasuta* nematodes (2 males and 7 females) and eight *C. hamulosa* nematodes (2 males and 6 females) were collected from seven domestic chickens in Rangamati (21°50'N–23°45'N and 91°45'E–92°53'E), Sylhet (24°36'N–25°11'N and 91°38'E–92°30'E), Rajbari (23°35'N–23°55'N and 89°09'E–89°55'E), Rajshahi (24°70'N–24°43'N and 88°19'E–88°58'E) and Dinajpur (25°10'N–26°04'N and 88°05'E–88°28'E), Bangladesh in 2016 and 2018, and then preserved in 70% ethanol. For species identification of these nematodes, the anterior and posterior parts of individual nematodes were removed and treated with lacto-phenol solution. They were accurately confirmed based on their morphological characteristics according to Anderson *et al.*

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[1], Zhang *et al.* [18] and Gomes *et al.* [5], and comparison of morphometrical measurements with the related species [3–5, 18].

Total DNA of individual nematodes was extracted from the remaining middle parts of the body using a High-pure PCR Template preparation Kit (Roche, Mannheim, Germany). DNA fragments covering nuclear ribosomal 18S–ITS1–5.8S–ITS2–28S gene (18S-28S amplicon) were amplified in standard PCR mixture including Tks Gflex DNA polymerase (TaKaRa, Kusatsu, Japan) and a pair of primers, ITS1-F [8] and ITS2-R [9]. Standard cycle condition with annealing temperature of 55°C was used for PCR amplification. The 18S-28S amplicons were purified using NucleoSpin Plasmid QuickPure Gel and PCR clean up kit (TaKaRa), ligated into a pUC118 plasmid vector, and subsequently introduced into *Escherichia coli* DH5α using the Mighty Cloning reagent set kit (Blunt end) (TaKaRa). Four to five clones per individual nematode were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in both directions using the provided vector primers, and newly designed DPF1 (forward) (5′-GTACCGCATGTGTATGTGTG-3′) and DPR1 (reverse) (5′-AGAGCCGCTACTCCATTATC-3′) for *D. nasuta*, and CHF1 (forward) (5′-GTGATTGTGGATGGTGATGC-3′) and CHback1 (reverse) (5′-AATACACTGATTGCGGCCAC-3′) for *C. hamulosa*, on a 3500-Genetic Analyzer (Applied Biosystems). Nucleotide sequences obtained were assembled using ATGC version 6.0.3 (Genetyx Co., Tokyo, Japan) and aligned using MAFFT [10]. The sequence homology was estimated by using MEGA X [11].

To design PCR primers specific to *D. nasuta* and *C. hamulosa*, the 18S-28S sequences of the two species as well as that (HE793715) of *Stegophorus macronectes*, which belongs to the same Acuariidae family as the two species, were aligned using MAFFT [10] and compared on their sequence specificity. The following forward and reverse primer sets inside the ITS2 sequence were designed as specific primers to each species: DnI-2F2 (5′- AGCGGCTCTCTATTGTCATC -3′) and DnI-2R2 (5′- ATGTGTGACTGCAAGTGCTG -3′) for *D. nasuta*, and ChI-2F2 (5′- CATCGTCGTCGTCATTATGC -3′) and ChI-2R2 (5′- AACACGCTTGCTCTCTCTC -3′) for *C. hamulosa* (Fig. 1).

Conventional multiplex PCR was performed in a final volume of 10 µl containing 0.5 µl of template DNA (6.5−11.5 ng), 10 µM of each primer (DnI-2F2, DnI-2R2, ChI-2F2, and ChI-2R2), 0.2 µl of Tks Gflex DNA polymerase (TaKaRa), and 5 µl of manufacturer's supplied reaction buffer. Total DNAs extracted from the nine *D. nasuta* and eight *C. hamulosa* nematodes, and from

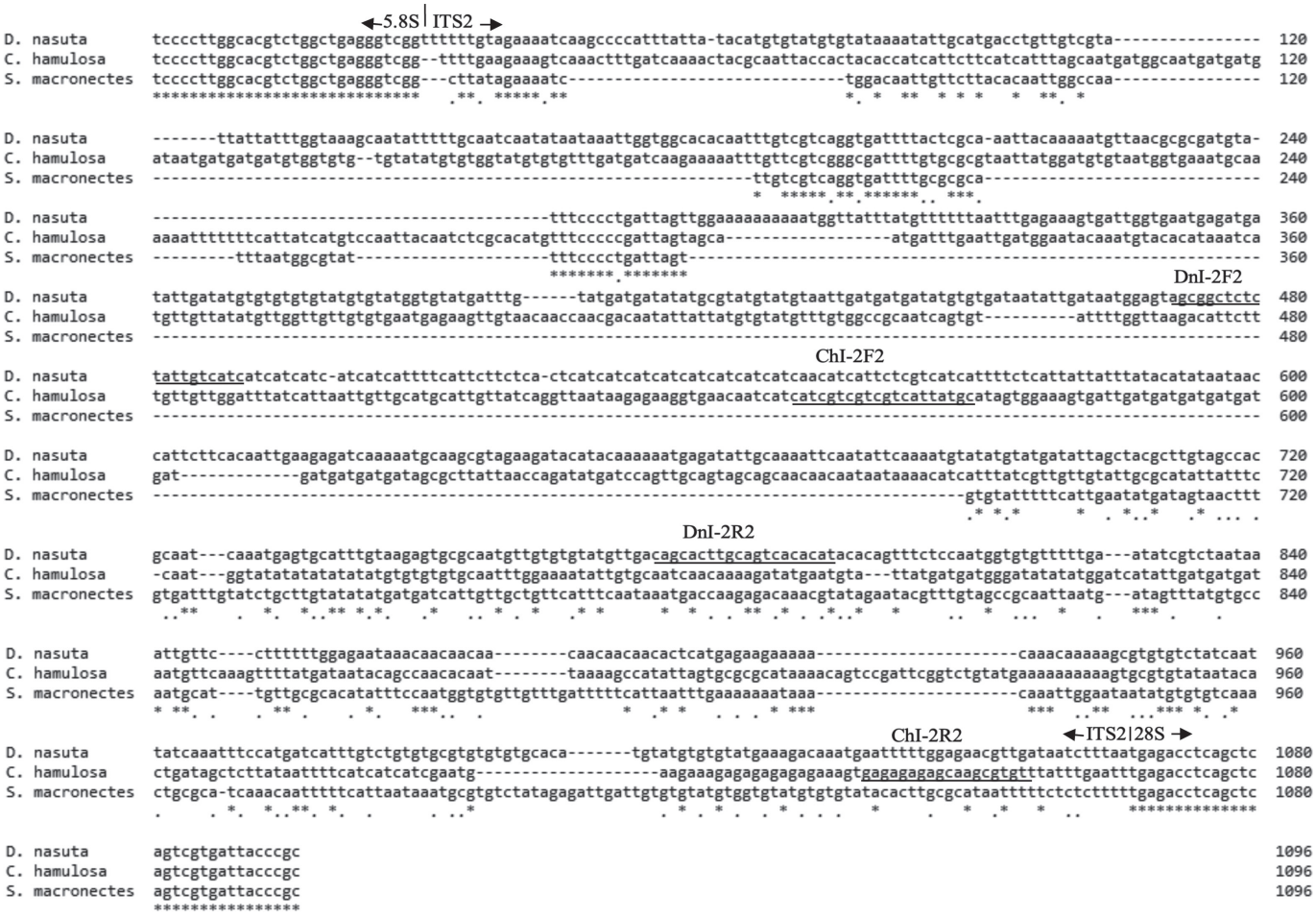

Fig. 1. DNA sequence alignments in ITS2 region of *Dispharynx nasuta, Cheilospirura hamulosa* and *Stegophorus macronectes*. Sequences are displayed at 5′-3′. The primers sites are indicated by underline. Nucleotide identity and gaps are indicated by "*" and by "-", respectively. *D. nasuta*: LC594428, *C. hamulosa*: LC594466, *S. macronectes*: HE793715.

three *Ascaridia galli*, three *Oxyspirura mansoni*, three *Heterakis gallinarum*, three *H. beramporia*, and three *H. indica* nematodes as negative control, which were collected from domestic chickens in Bangladesh, were used for the template DNA. Thermal cycling condition was an initial 1 min at 94°C, followed by 35 cycles at 98°C for 10 sec, 55°C for 15 sec, and 68°C for 30 sec. The amplicons were separated by 1.8% agarose gel electrophoresis and stained with ethidium bromide.

A total of 38 distinct sequences (2,435–2,462 bp) in the 18S–28S amplicons were obtained in 38 clones (4–5 clones per nematode) from nine *D. nasuta* nematodes, and deposited to GenBank as accession numbers, LC594428−LC594465. The 18S (207 bp), 5.8S (157 bp), and 28S (63 bp) sequences showed no intraspecific variation. The ITS1 (1,097–1,120 bp) and ITS2 (912–917 bp) sequences showed intraspecific variation of 0.1–0.7% and 0.1–0.7%, respectively. There was no variation within the primers, DnI-2F2 and DnI-2R2 sequences. A total of 36 distinct 18S–28S sequences (2,513–2,547 bp) were determined in 36 clones from eight *C. hamulosa* nematodes and registered as LC594466−LC594501. The 18S (183 bp), 5.8S (157 bp) and 28S (84 bp) sequences showed no intraspecific variation.The ITS1 (1,136–1,167 bp) and ITS2 (949–959 bp) sequences showed intraspecific variation of 0.1–0.8% and 0.1–0.6%, respectively. There was no variation within the primers, ChI-2F2 and ChI-2R2 sequences.

The complete 5.8S, ITS1 and ITS2 sequences between *D. nasuta* and *C. hamulosa* showed the identity of 99.4%, 51.4–51.7% and 52.0–52.3%, respectively, including the size difference due to nucleotide deletion/insertion. In addition, the ITS1 and ITS2 sequences of the two species showed the similarity of 56.8–60.5% and 49.9–53.0%, respectively, with that (HE793715) of *S. macronectes* which is the only GenBank sequence available for Acuariidae nematodes.

In multiplex PCR, a 455-bp fragment was amplified by all of the *C. hamulosa* DNAs, but not amplified by all of the *D. nasuta* DNAs and negative controls (*A. galli*, *O. mansoni*, *H. gallinarum*, *H. beramporia*, and *H. indica* DNAs). A 319-bp fragment was amplified by all of the *D. nasuta* DNAs, but not by all of the *C. hamulosa* and the negative control DNAs. Moreover, the templates containing *C. hamulosa* and *D. nasuta* DNAs yielded both 455-bp and 319-bp fragments (Table 1 and Fig. 2).

Dispharynx nasuta and *C. hamulosa* nematodes have worldwide distribution, and actually have been reported to coexist in some countries such as India, Morocco, Tanzania, and Brazil, although their prevalence differs among the countries [2, 6, 12, 14, 16]. The common definitive hosts of the two species are poultry belonging to Galliformes as chickens, turkeys, and guinea fowls, and severe gross lesions such as hemorrhages, ulcers, thickening of the mucosa and cuticle, and yellowish nodules have been reported in the gizzard of chicken infected with *C. hamulosa* [12, 16]. Their species discrimination has been achieved based on morphological characteristics of adult nematodes recovered from the alimentary tracts of dissected birds, however the conventional multiplex PCR method developed in this study would enable their discrimination using total DNA extracted from their worms as well as their eggs that are difficult to be morphologically discriminated, and would result in reduction of time consuming for examination. Therefore, this molecular technique would provide easily and efficient protocols for analyzing multiple samples such as host feces, and should considerably contribute to the epidemiological study of these two species. Furthermore, this method would enable the discrimination of their morphologically unidentifiable third larvae detected in insect intermediate host, and would progress the study on intermediate host of these nematode species. In addition, this technique would also contribute to prepare the control strategy of these infections by preventing insect hosts different in each nematode species.

The multiplex PCR showed no amplification with DNAs from *A. galli*, *O. mansoni*, *H. gallinarum*, *H. beramporia*, and *H. indica* nematodes that also parasitize poultry, suggesting its high sensitivity. However, the genera *Dispharynx* and *Cheilospirura* consist of about 15 and 13 species, respectively [2, 15], and intra-genus cross-reaction should therefore be evaluated for the multiplex PCR in the future. Moreover, host feces would contain numerous compounds that may inhibit the PCR amplification [17]

Table 1. Amplification of 455-bp and 319-bp fragments by the multiplexPCR using DNAs of 7 nematode species

DNAs (sample numbers)	Fragments	
	455-bp	319-bp
Dispharynx nasuta (9)	0	9
Cheilospirura hamulosa (8)	8	0
D. nasuta + *C. hamulosa* (8)	8	8
Ascaridia galli (3)	0	0
Oxyspirura mansoni (3)	0	0
Heterakis gallinarum (3)	0	0
Heterakis beramporia (3)	0	0
Heterakis indica (3)	0	0

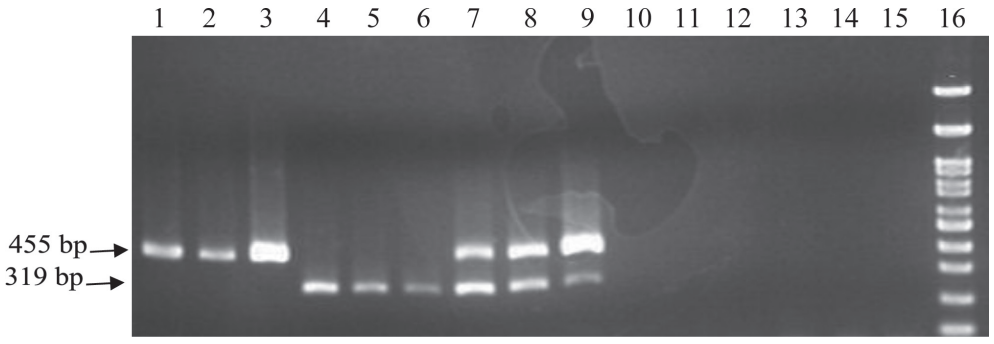

Fig. 2. A representative amplification pattern of *Dispharynx nasuta* and *Cheilospirura hamulosa* DNA using conventional multiplex PCR. 1-3: *C. hamulosa* DNAs, 4-6: *D. nasuta* DNAs, 7-9: *C. hamulosa* DNAs and *D. nasuta* DNAs, 10: No template DNA, 11: *A. galli* DNA, 12: *O. mansoni* DNA, 13: *H. gallinarum* DNA, 14: *H. beramporia* DNA, 15: *H. indica* DNA, 16: 100 bp DNA ladder.

and contaminated DNAs from hosts that may cross-react with the primers; therefore, the effect on these matters that may hamper the results of the multiplex PCR using DNA from fecal eggs should be also solved in the future study.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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