Molecular characterization of *Ascaridia galli* from Bangladesh and development of a PCR method for distinguishing *A. galli* from *Heterakis* spp.

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ABSTRACT. We analyzed the nuclear ribosomal internal transcribed spacer (ITS) 1 and ITS2 sequences for Bangladesh isolates of *Ascaridia galli*, and we determined that the sequences were unreliable as molecular markers for distinguishing *A. galli* from other *Ascaridia* species, because the sequences showed high identity with that of *A. columbae*. However, the ITS1 sequences were available for designing PCR primers distinguishable between *Ascaridia galli* and *Heterakis* spp. Bangladesh isolates of *A. galli* constituted a monophyletic clade along with other geographical isolates in the cytochrome c oxidase subunit I (COI) phylogenetic tree, however, we could not clarify the phylogenetic relationships between *A. galli* and other *Ascaridia* spp., because their available sequences in GenBank were very few. The developed PCR method using DNA from *A. galli* and *Heterakis* spp. eggs would enable differential diagnosis of the individual infections in the future.

KEY WORDS: *Ascaridia galli*, cytochrome c oxidase subunit I (COI), internal transcribed spacer (ITS) 1, internal transcribed spacer (ITS) 2, PCR method
and 89°39'E–91°15'E), Bangladesh in 2016 and 2018. The nematodes were preserved in 70% ethanol. Anterior and posterior parts of each nematode were removed and treated with lacto-phenol solution for morphological observation. The remaining middle parts of the body were used for DNA extraction. Males were identified as A. galli according to morphological descriptions by Kajerova et al. [13], particularly of the caudal alae, spicules, precloacal sucker, and caudal papillae. Females which exhibit no accurate morphological characteristics for species identification, were identified molecularly as A. galli based on the ITS1, ITS2 and COI sequences.

Total DNAs were extracted from 51 individual nematodes using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. DNA fragments were amplified by PCR on a GeneAmp PCR System 2700 (Applied Biosystems, Tokyo, Japan) in a standard mixture including Tks Gflex DNA polymerase (TaKaRa, Kusatsu, Japan) and three primer sets: ITS1-F and ITS1-R for the ITS1 region [11], ITS2-F and ITS2-R for the ITS2 region [12], and JB3 and JB4.5 for the COI gene [3]. The cycle conditions consisted of an initial 1 min at 94°C, followed by 35 cycles at 98°C for 10 sec, 55°C for ITS1 and ITS2 or 40°C for COI, and 68°C for 30 sec. PCR amplicons were directly sequenced in both directions with the respective primers using BigDye Terminator v3.1 Cycle Sequencing Kit and the 3500-Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). ITS2 fragments that showed heterogeneous nucleotides at some base sites were purified with NucleoSpin Plasmid QuickPure (Macherey-Nagel, Duren, Germany), ligated into a pUC118 plasmid vector, and subsequently introduced into E. coli DH5α using the Mighty Cloning Reagent Set Kit (Blunt End) (TaKaRa). Five clones per fragment were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in both directions using the kit-provided vector primers. The obtained sequences were assembled using ATGC version 6.0.3 (Genetyx Co., Tokyo, Japan) and the ITS1 and ITS2 sequences were concatenated for phylogenetic analysis. Phylogenetic trees were constructed on MEGA X [15] using the maximum likelihood method with the Tamura-3 Parameter model (T92) used as the best model for the concatenated ITS tree, while the Hasegawa-Krishino-Yano model with discrete gamma distribution (HKY+G) was used for the COI phylogenetic tree. The ITS tree included the reference sequences of A. galli (KX683286, KY789470, KY789472), Ascaridia columbae (JQ995321), Ascaridia nymphii (MF375321), and Brugia malayi (EU373615) as an out-group. The COI tree included the reference sequences of A. galli (KT388439, KT388440, KT613889, FM178545, GU138668, GU138669 and KP982856), A. columbae (JX624729), and Anisakis simplex (NC007934) as an out-group. The trees were evaluated using bootstrap tests with 1,000 replications. The Ascaridia galli sequences that were determined in this study have been registered in GenBank with accession numbers, LC592810-LC592844.

To design PCR primers specific to A. galli, in addition to the ITS1 sequences analyzed in this study, the reference sequences of A. galli, Heterakis gallinarum, H. beramporia and H. indica were aligned using the multiple-sequence alignment program MAFFT [14] to determine their identity. The forward primer AgI1-F1 (5′- ACTGGGTGATATACACTGCAAC-3′) and reverse primer, AgI1-R (5′- TTCTCTGTGGCACTGCACAC-3′) were designed as specific primers (Fig. 1). The PCR was performed in a final volume of 10 µl containing 0.5 µl of the template DNA (6.5–11.5 µg) measured by BioSpec-nano (Shimadzu, Kyoto, Japan), 10 µM of each primer (AgI1-F1 and AgI1-R1), 0.2 µl of Tks Gflex DNA polymerase (TaKaRa), and 5 µl of the manufacturer’s supplied reaction buffer. Thermal cycling was performed with an initial 1 min at 94°C, followed by 35 cycles at 98°C for 10 sec, 52°C for 15 sec, and 68°C for 30 sec. Total DNAs from 20 A. galli nematodes used in this study were used for template DNA in the PCR. As a control, the total DNAs from 3 A. galli DNAs, and the representative fragments are 99.8–100% identity with those of A. nasuta (KX683286, JQ995321), and 72.–80.6% identity to those of Ascaridia compar (FM177755) and A. nymphii (MF375321). Similar to the ITS1 analysis, eight ITS2 sequences (342, 344, 345, 347, 350, and 354 bp) were obtained, and they showed 99.8–100% sequence homology, with differences in base substitution at one site and base insertion/deletion mutations at 12 sites. The ITS2 sequences showed 100% identity with those of A. galli (KX683286, KT388439, KT388440, KT613889, KT613901, FM178545, GU138668, GU138669 and KP982856) and A. columbae (JX624729), respectively. The haplotypes formed a monophyletic clade in the tree with those of A. galli isolates from South Africa, China, Denmark, Brazil, and Italy, which were separated from the clade of A. columbae (Fig. 3).

In the PCR targeting the ITS1, a 223-bp fragment was amplified for all 51 A. galli DNAs, and the representative fragments are shown in Fig. 4. The fragment was not amplified for the DNAs of H. gallinarum, H. beramporia, H. indica, O. mansoni, D. nasuta and C. hamulosa (Fig. 4).

Ascaridia galli and A. columbae showed high ITS1 and ITS2 sequence identity and were separated in the same clade with a high bootstrap value in the ITS-concatenated tree. Similarly, Urbanowicz et al. [18] reported that the ITS1-5.8S-ITS2 sequences of A. galli (KX683286) showed high identity of 99% with that of A. columbae (JQ995321). These two valid Ascaridia species
exhibit different morphological features, such as caudal alae, spicules, precloacal sucker and caudal papillae in male worms [13], and they parasitize different avian hosts which are Galliformes birds including chickens in *A. galli* and Columbiformes birds in *A. columbae*. However, the results indicated that the two species are genetically closely related to each other, and the ITS1 and ITS2 sequences are unreliable markers for discriminating between *A. galli* and *A. columbae*, because they belonged to the same clade in the ITS1 and ITS2-concatenated tree. In contrast, *A. nymphii* differs in male morphological features, parasitizes Psittaciformes birds, and was positioned in a distinct clade. Mitochondrial COI sequences can be used to analyze phylogenetical relationships between closely related nematode species within a genus [10]. However, we could not clarify the phylogenetical relation between *Ascaridia* spp. using COI sequences from Bangladesh isolates of *A. galli*, because information on the COI sequences of *A. galli* and *A. columbae* is limited, and very few available COI sequences of the other *Ascaridia* spp., which include 40 species, were present. Additional COI sequences for other *Ascaridia* species and their different isolates are needed to elucidate the genetic relation among *Ascaridia* species.

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**Fig. 1.** Sequence alignments of internal transcribed spacer (ITS) 1 for *Ascaridia galli* (LC592810), *Heterakis gallinarum* (LC592776), *Heterakis beramporia* (LC592776), and *Heterakis indica* (LC592806). Sequences are displayed at 5'-3'. The primer sites are indicated by underline. Nucleotide identity and gaps are indicated by "*" and by "-", respectively.

**Fig. 2.** The maximum likelihood tree inferred from internal transcribed spacer (ITS) 1 and ITS2-concatenated sequences (747 bp) of *Ascaridia galli* genotypes. Scale bar shows genetic variation.
The PCR method developed in this study could distinguish *A. galli* from *Heterakis* spp. Their discrimination is important not only for controlling individual infections but for preventing fatal bacterial and protozoan infections from spreading. Although *A. galli* and *H. gallinarum* are the most common nematodes of domestic chickens in the world, the diagnosis specific for the infections has not been conducted, because morphological discrimination between their eggs was difficult in fecal examination [5]. Recently, protocols available for extracting DNA from nematode eggs in fecal samples have been developed [1, 8], and the PCR method using DNA from *A. galli* and *Heterakis* spp. eggs would enable the diagnosis of individual infections in the future.
POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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