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NOTE

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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

Nematodes of the genus *Ascaridia* parasitize the intestine of domestic and wild birds and cause digestive disorder in hosts [7]. *Ascaridia galli* is one of the most common and highly pathogenic nematodes in domestic chickens and has substantially damaged the poultry industry worldwide by causing reduced growth rate, weight loss, low egg production, and some fatalities in the infected chickens [2, 17]. Additionally, this species is also known as a mediator and enhancer of *Salmonella* spp., *Escherichia coli,* and *Pasteurella moltocida* infections to poultry [4, 6, 16].

Heterakis species are parasitic nematodes in the caeca of mainly bird hosts, and *H. gallinarum*, *H. beramporia,* and *H. indica* infect domestic chickens. Infections by the three species occasionally lead to a declining health but are usually regarded as non-pathogenicity to the host. *Heterakis gallinarum* is also a well-known vector of flagellate protozoan *Histomonas meleagridis*, which causes a fatal histomonosis in chickens [9].

Ascaridia galli and *Heterakis* spp. infections in chickens are diagnosed by detecting their eggs in fecal examination; however, morphological discrimination between their eggs is difficult because of their morphological similarities [5]. Therefore, no diagnostic tool exists for discrimination between both the infections. Accurate diagnosis between the infections is important for preparing a control strategy for these nematode infections and for preventing the protozoan and bacterial infections. Recently, DNA sequences of nuclear ribosomal internal transcribed spacer (ITS) 1 and 2, and mitochondrial cytochrome c oxidase subunit 1 (COI) regions have been reported as available markers for discriminating and identifying nematode species [10]. However, no molecular study for distinguishing *A. galli* from *Heterakis* spp. has been carried out, which might be caused by the lack of sequence data determined in the species including Bangladesh isolates.

The purpose of this study was to determine ITS1, ITS2 and COI sequences of *A. galli* isolates from Bangladesh and to develop a PCR method for discriminating *A. galli* from *Heterakis* spp.

A total of 51 nematodes (19 males and 32 females) were isolated from the intestines of 26 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), Mymensingh (24°02'N–25°25'N

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and 89°39'E–91°15'E), Dinajpur (25°10'N–26°04'N and 88°05'E–88°28'E), Rajshahi (24°70'N–24°43'N and 88°19'E–88°58'E), Rajbari (23°35'N–23°55'N and 89°09'E–89°55'E), Jhenaidah (23°15'N–23°45'N and 88°45'E–89°15'E), and Khulna (22°49'N 89°33'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 bootstraps 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′- TTTCTTGTTGGCAGCGCAC -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 *H. gallinarum*, 3 *H. beramporia*, 3 *H. indica*, 3 *Oxyspirura mansoni*, 3 *Dispharynx nasuta* and 3 *Cheilospirura hamulosa* nematodes that had been collected from chickens in Bangladesh were included in the study. The amplified fragments were separated by 1.8% agarose gel electrophoresis and stained with ethidium bromide.

Three distinct ITS1 sequences (477 and 480 bp) were obtained from 51 *A. galli* nematodes and showed 99.8–100% sequence homology, with differences in base substitution at a single site and base insertion/deletion mutations at three sites. The ITS1 sequences showed 99.8–100% identity to those of *A. galli* (KX683286, KY789470, KY789472, AM408550 and AJ007451) and *A. columbae* (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 a 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, KY789470, KY789472, AM408551 and AJ007452) and *A. columbae* (JQ995321), and 51.8% identity with that of *A. nymphii* (MF375321). The ITS1 and ITS2-concatenated sequences (819–831 bp) were attributed to the 10 genotypes (Ag-1 to Ag-10) with 99.9–100% identity. The genotypes formed a single clade in the tree along with the genotypes of *A. galli* (KX683286, KY789470 and KY789472) and *A. columbae* (JQ995321), which was separated from the *A. nymphii* clade (Fig. 2). The COI sequences (483 bp) yielded 24 distinct haplotypes (Ag-COI-1 to Ag-COI-24) with an identity of 97.5–99.8%, and showed 96.7–99.5% and 84.4–85.7% identities with the reference sequences of *A. galli* (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, Urbanowicw *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

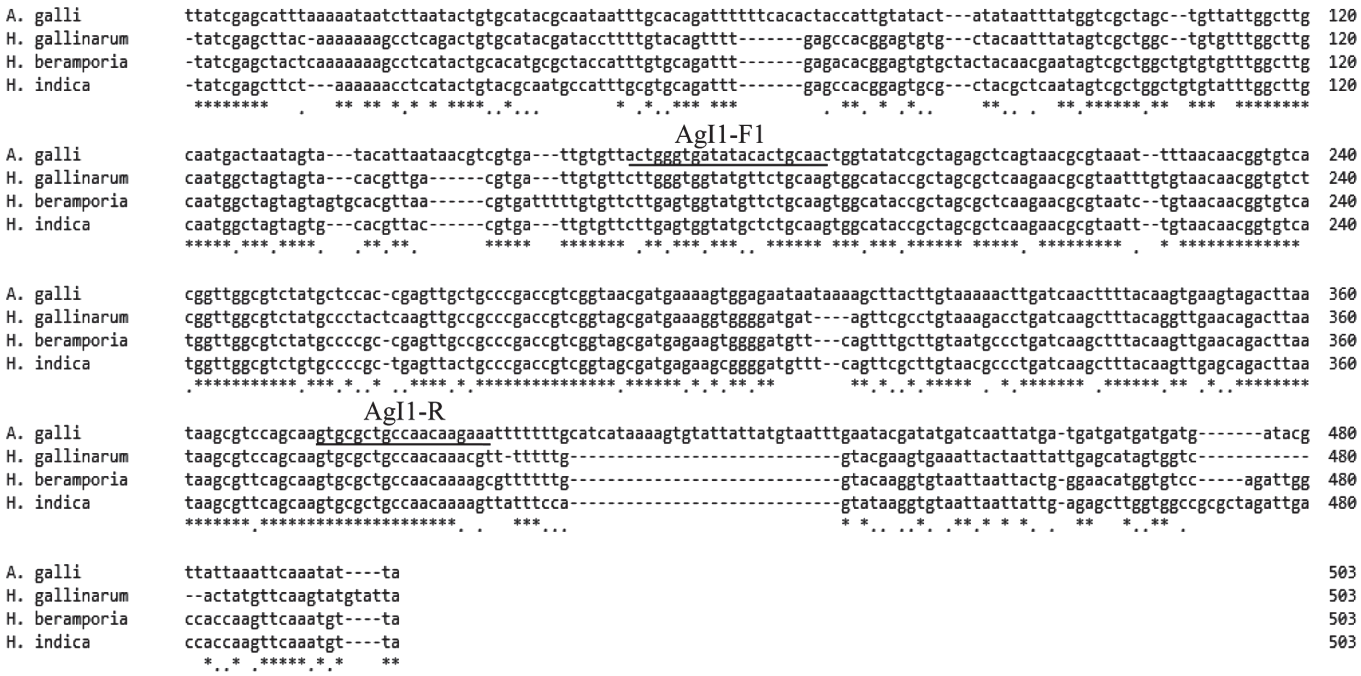

Fig. 1. Sequence alignments of internal transcribed spacer (ITS) 1 for *Ascaridia galli* (LC592810), *Heterakis gallinarum* (LC592776), *Heterakis beramporia* (LC592776), and *Heterakis indica* (LC592806). Sequences are displayed at 5′-3′. The primer sites are indicated by underline. Nucleotide identity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

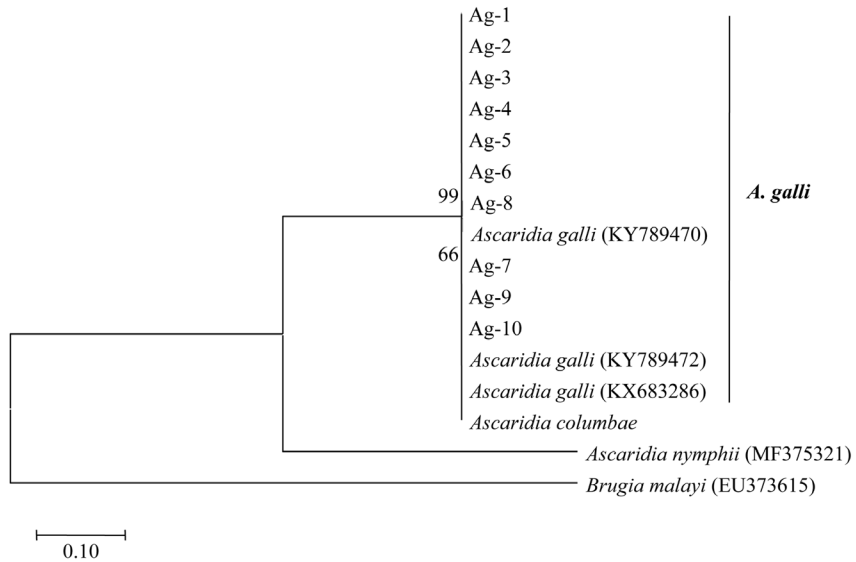

Fig. 2. The maximum likelihood tree inferred from internal transcribed spacer (ITS) 1 and ITS2-concatanted sequences (747 bp) of *A*scaridia *galli* genotypes. Scale bar shows genetic variation.

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 COIsequences of *A. galli* and *A. columbae* is limited, and very few available COIsequences 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.

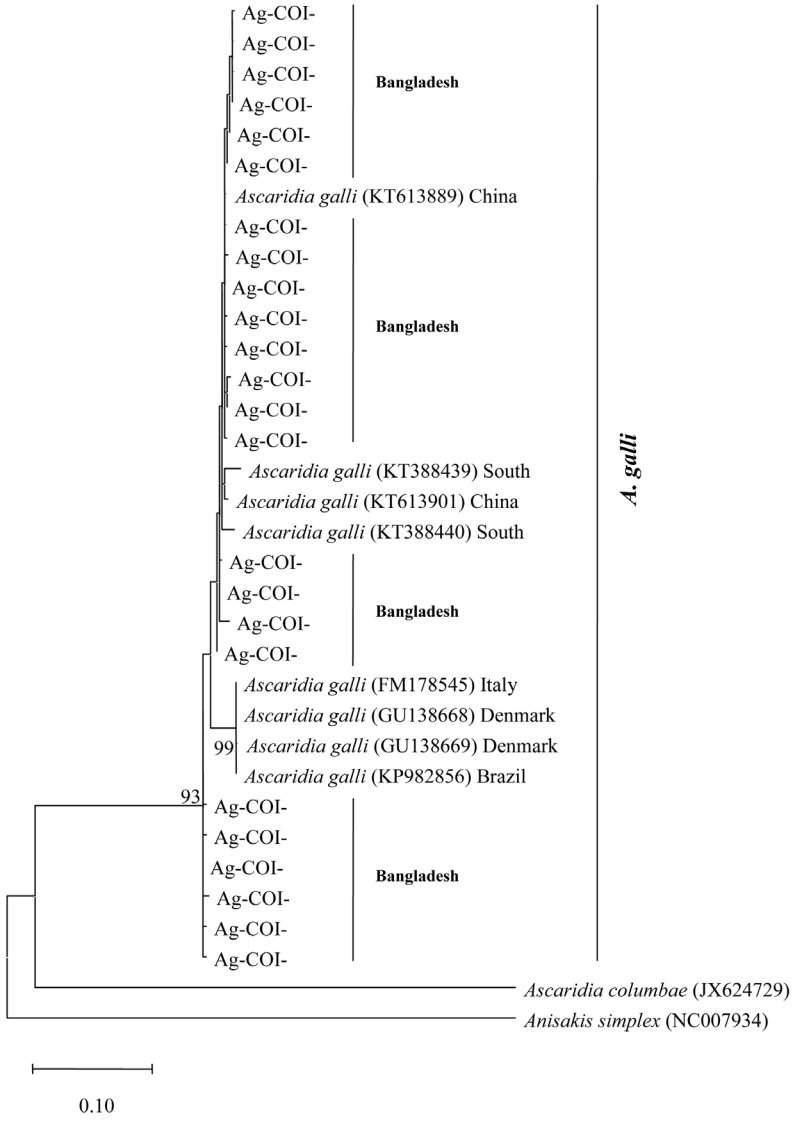

Fig. 3. The maximum likelihood tree inferred from cytochrome c oxidase subunit I (COI) sequences (393 bp) of *Ascaridia galli* haplotypes and other *Ascaridia* species. Scale bar shows genetic variation.

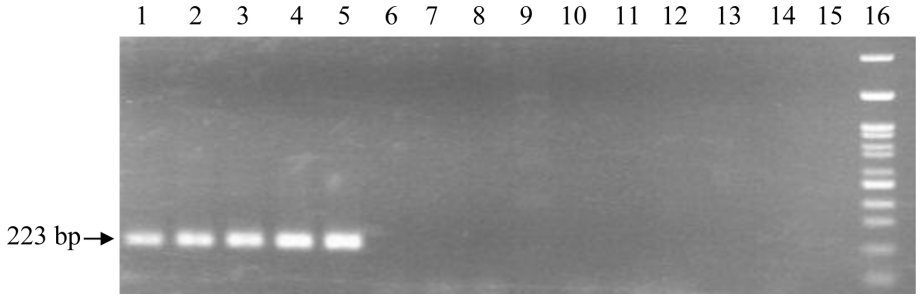

Fig. 4. A representative amplification of 223-bp fragments for *A*scaridia *galli* DNAs using PCR targeting the ITS1. 1–5: *A*scaridia *galli* DNAs, 6: No DNA, 7–8: *Heterakis gallinarum* DNA, 9–10: *Heterakis beramporia* DNA, 11–12: *Heterakis indica* DNA, 13: *Oxyspirura mansoni* DNA, 14: *Cheilospirura hamulosa* DNA, 15: *Dispharynx nasuta* DNA, 16: 100 bp DNA ladder.

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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