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Morphological and molecular characteristics of adult worms of *Gnathostoma* Owen, 1836 (Nematoda) collected from domestic pigs in Dien Bien Province, northern Vietnam

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Abstract: Gnathostomes are of interest because of their unique appearance and medical importance. Among 13 valid species of the genus *Gnathostoma* Owen, 1836 (Nematoda: Spirurida), two species, *G. doloresi* Tubangui, 1925 and *G. hispidum* Fedtschenko, 1872, are parasites of pigs but their larvae can infect humans to cause gnathostomiasis. In this study, we collected adults of *Gnathostoma* sp. from the stomach of domestic pigs (*Sus scrofa domesticus* Linnaeus) from Dien Bien Province, northern Vietnam. Morphologically, nematodes found here are most similar to *G. doloresi* with a slight difference in the spicules of males. In contrast, they are genetically distinct from *G. doloresi* and other species of *Gnathostoma* in both ITS2 and *cox*1 sequences. The findings of the present study suggest that specimens of *Gnathostoma* sp. found in Dien Bien are likely a new species and emphasise the need of further studies on the taxonomy and phylogenetic relationship of species of *Gnathostoma*. Special attention should also be paid to swine and human gnathostomiasis in Dien Bien Province, Vietnam and the neighbouring areas of China and Laos.

Keywords: Gnathostomatidae, morphology, *cox*1, ITS2, South-East Asia.

Nematodes of the genus *Gnathostoma* Owen, 1836 are parasites of mammals, particularly carnivores, marsupials and pigs (Miyazaki 1991, Nawa et al. 2015). The advanced third-stage larvae of some species, such as *G. binucleatum* Almeyda-Artigas, 1991, *G. doloresi* Tubangui, 1925, *G. hispidum* Fedtschenko, 1872, *G. nipponicum* Yamaguchi, 1941 and *G. spinigerum* Owen, 1836, can infect humans causing a zoonotic disease, gnathostomiasis.

The most common manifestations of human infection are migratory swellings under the skin and peripheral blood eosinophilia. In addition, larvae of *Gnathostoma* spp. can migrate into other sites such as the liver to cause abscesses, into the eye, resulting in visual loss, or into the peripheral or central nervous system including spinal cord and brain, resulting in nerve pain, paralysis, coma and death (Waikagul and Diaz-Camacho 2007, Nawa et al. 2015). Given the medical importance, species of *Gnathostoma* have been intensively studied (Nawa et al. 2015).

So far, more than twenty species of *Gnathostoma* have been described as parasites of mammals, mainly in Asia and the Americas (Bertoni-Ruiz et al. 2011), of which 13 species are considered to be valid (Waikagul and Diaz-Camacho 2007, Nawa et al. 2015). Among them, two species, *G. doloresi* and *G. hispidum*, are parasites of domestic pigs and wild boars but their larvae can infect humans (Waikagul and Diaz-Camacho 2007, Nawa et al. 2015).

Although these two species have been reported from Vietnam (Le et al. 1996), there is a lack of the morphological and molecular data on these medically important swine parasites. Recently, while conducting an epidemiological survey on pig diseases, we found adult worms of *Gnathostoma* in the stomach of domestic pigs (*Sus scrofa domesticus* Linnaeus) from Dien Bien Province, northern Vietnam, where free-range pig production is common. The present paper describes the morphological and molecular characteristics of these specimens collected in Vietnam.

MATERIALS AND METHODS

A total of 50 stomachs of domestic pigs were purchased from local pig farmers in Dien Bien Province, northern Vietnam, for parasite examination. Adults of *Gnathostoma* sp. were collected from five (10%) out of 50 pigs. The worms were washed in physiological saline to remove food debris and mucus and fixed in 10% formalin for morphological study. Four worms (two males and two females) were fixed separately in 96% ethanol for molecular analyses. For morphological examination, sixteen adult worms

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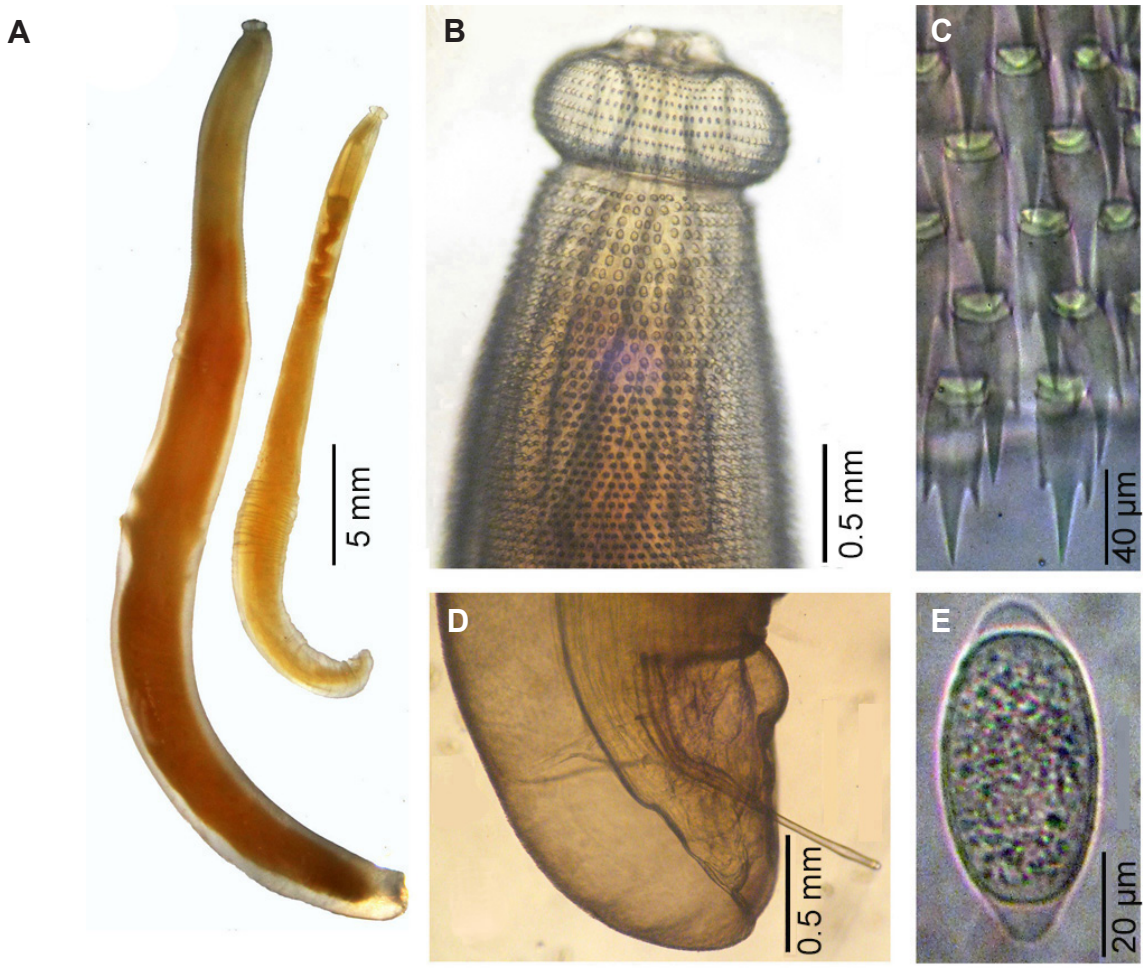

Fig. 1. *Gnathostoma* sp. collected from the stomach of pigs in Dien Bien Province, Vietnam. **A** – whole body of a female (left) and a male (right); **B** – anterior part of a male; **C** – region of tridentate spines; **D** – posterior part of a male showing two unequal spicules; **E** – egg with a polar bulge on both ends.

fixed in formalin were cleared in lactophenol for morphological and morphometric study. The worms were examined under a stereomicroscope and an Axio Lab A1 microscope (Carl Zeiss, Oberkochen, Germany).

For molecular analyses, genomic DNA from individual worms was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA and a partial region of the mitochondrial cytochrome C oxidase subunit 1 (*cox*1) were chosen to analyse because these genetic markers were used for identification of species of *Gnathostoma* (see Ando et al. 2006).

ITS2 region was amplified using polymerase chain reaction (PCR) with the primer pair NEWS2 and ITS2-RIXO (Almeyda-Artigas et al. 2000). A part of the *cox*1 gene was amplified using PCR with the primer pair JB3 and JB4.5 (Bowles et al. 1993). PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen). These products were primed using a Big-Dye Terminator Cycle Sequencing Kit and both strands were directly sequenced using an Ab3730 sequencer (Applied Biosystems, Foster City, CA, USA), using the PCR primers as sequencing primers.

Eight nucleotide sequences of the ITS2 and *cox*1 gene from four samples were obtained and deposited in the DNA data bank of Japan (DDBJ) with the accession numbers LC404124-LC404127 (*cox*1 sequences) and LC390038-LC390041 (ITS2 sequences). In addition, ITS2 and *cox*1 sequences of other species of *Gnathostoma* were obtained from the GenBank nucleotide database for analyses and for reconstruction of phylogenetic trees in MEGA6 using the Maximum likelihood method based on the best model (Tamura et al. 2013).

RESULTS AND DISCUSSION

The result of the morphological examination showed that the body of specimens of *Gnathostoma* sp. collected from pigs in Dien Bien is slightly widened posteriorly, mature females (30–42 × 2.4–4 mm in size) are bigger than males (22–25 × 1.8–2.2 mm) (Fig. 1A). The head bulb is armed with 9–11 rows of hooklets (Fig. 1B). The whole body is covered with cuticular spines which vary in the number of teeth: multi-dentate (4–6 teeth), tridentate (3 teeth), bidentate (2 teeth) and unidentate spines. Of these, the tridentate spines occupy a major part of the body (Fig. 1C). The unidentate region includes single-pointed spines with a gradual change of the size to more slender and hair-like spines towards the posterior end of the worms.

The tail of the males curves ventrally. Spicules of males are unequal, with blunt apex (Fig. 1D). Longer spicule measures 1.1–1.4 mm in length; shorter spicule is 0.6–0.7 mm long.

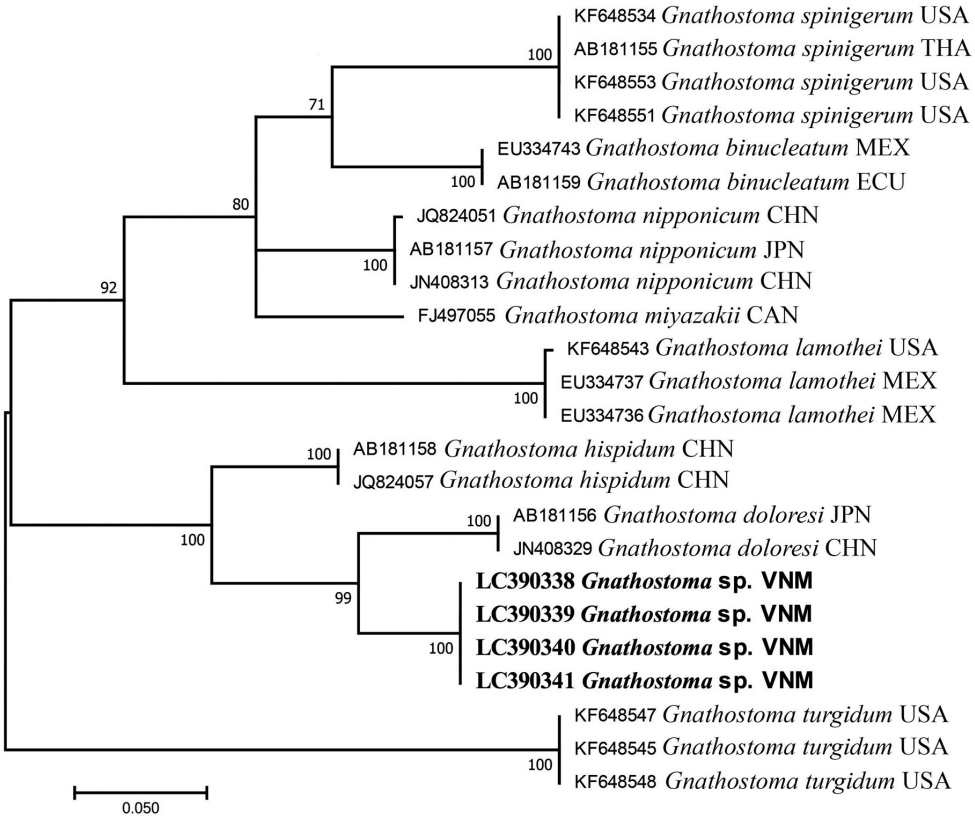

Fig. 2. Molecular phylogenetic tree reconstructed from ITS2 sequences using the Maximum Likelihood method based on the Kimura 2-parameter model. The bootstrap values are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The nucleotide sequences obtained in this study are printed in bold and others from the DNA database are shown with Accession No. and country code: CAN – Canada, CHN – China, ECU–Ecuador, JPN – Japan, MEX – Mexico, THA – Thailand, USA – United States of America, VNM – Vietnam.

The tail of females is blunt (Fig. 1A). Eggs are 64–70 × 30–32 µm in size, with two polar bulges (Fig. 1E). In these characteristics, specimens from Vietnam are similar to *Gnathostoma doloresi* (see Miyazaki 1991).

In contrast, the molecular data showed that *Gnathostoma* sp. of this study was genetically distinct from *G. doloresi* as well as the other congeners in both ITS2 and *cox*1 sequences. For ITS2, the nucleotide differences of *Gnathostoma* sp. from other *Gnathostoma* species were high: 8.6% *vs G. doloresi*, 12.9% *vs G. hispidum* and 26.5–31.6% *vs* other species, namely, *G. nipponicum, G. miyazaki* Anderson, 1964*, G. binucleatum*, *G. lamothei* Bertoni-Ruiz, Garcia-Prieto, Osorio-Sarabia et Léon-Règagnon, 2005*, G. turgidum* Stossich, 1902 and *G. spinigerum*. In the phylogenetic tree (Fig. 2), samples of *Gnathostoma* sp. made a distinct group close to *G. doloresi* and *G. hispidum*.

For *cox*1 sequences, the nucleotide differences between samples of *Gnathostoma* sp. and other species were also high (14.2–14.9% *vs G. binucleatum*, 14.3–18.1% *vs G. spinigerum*, 16.7– 18.5% *vs G. hispidum*, 17.2–20.1% *vs G. doloresi* and 21.5–22.9% *vs G. nipponicum*). In the phylogenetic tree, every species formed a separate clade. *Gnathostoma* sp. was markedly distant from *G. doloresi* and grouped with *G. hispidum,* but with a low bootstrap value (Fig. 3).

For traditional morphological identification of adults species of *Gnathostoma*, the most important characteristics are the pattern of cuticular spines and the number of polar bulges of eggs (Miyazaki 1991, Nawa et al. 2015). Till now, only two species, *G. doloresi* and *G. hispidum*, were found in the stomach of pigs and wild boars (Bertoni-Ruiz et al. 2011, Nawa et al. 2015). Both species have cuticular spines covering the whole body, but they are different from one another in the patterns of the cuticular spines and the number of polar bulges of eggs. Eggs of *G. hispidum* have only one bulge, whereas those of *G. doloresi* have a bulge at both ends (Miyazaki 1991, Nawa et al. 2015).

Gnathostoma sp. collected in Dien Bien Province, Vietnam, is most similar to *G. doloresi* in two important characteristics: the presence of spines on the whole body surface and bipolar-bulge eggs. They also show similarities in the pattern of cuticular spines. The difference between them is the ratio of long/short spicules of males. They both have a similar short spicule (0.6–0.7 mm in length), but the length of the long spicule of *Gnathostoma* sp. reported here (1.1–1.4 mm) is much shorter than that (1.9–2.1 mm) of *G. doloresi* (see Maplestone 1930).

In contrast to morphological similarity, specimens of *Gnathostoma* sp. are clearly distinct genetically from *G. doloresi* and the other congeners in both ITS2 and *cox*1 sequences. As can be seen in the phylogenetic trees

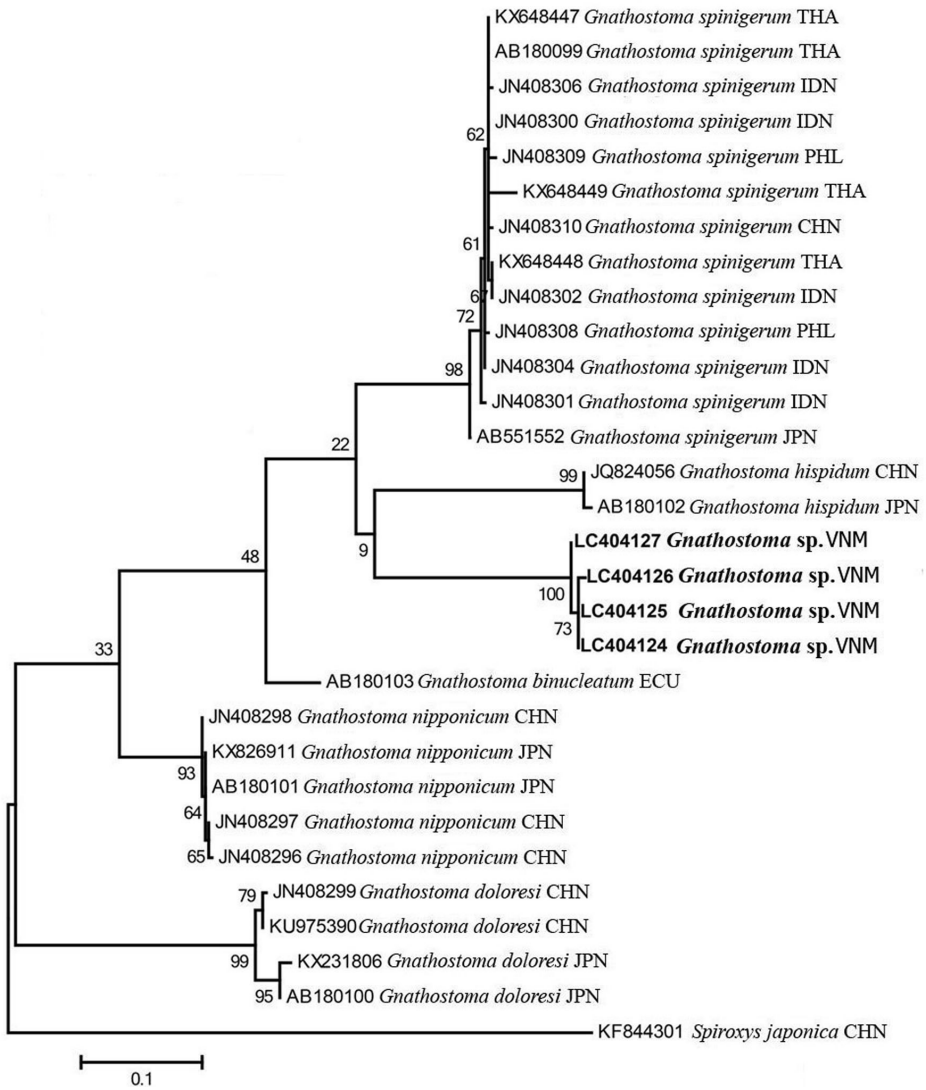

Fig. 3. Molecular phylogenetic tree of species of *Gnathostoma* reconstructed from *cox*1 sequences using the Maximum Likelihood method. The evolutionary history was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The bootstrap values are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The nucleotide sequences obtained in this study are printed in bold and others from DNA database are shown with Accession No. and country code: CHN – China, ECU–Ecuador, IDN – Indonesia, JPN – Japan, PHL – Philippines, THA – Thailand VNM – Vietnam.

(Figs. 2, 3), *G. doloresi* from two different countries, China and Japan, is identical (ITS2) or very similar (*cox*1). In contrast, *Gnathostoma* sp. from Vietnam belongs to a distinct group far distant from *G. doloresi*. Molecular data thus suggest that *Gnathostoma* sp. most likely represents a distinct species.

At present, it is difficult to find out any clear-cut morphological differences between *Gnathostoma* sp. and *G. doloresi*, because the morphological descriptions of *G. doloresi* were not detailed enough. The original description by Tubangui (1925) was very simple without description of the shape and size of cuticular spines. Eduardo (1989) described the spines of *G. doloresi* from the Philippines but did not mention the presence of bidentate spines. Koga and Ishii (1992) described body spines of *G. doloresi* from Japan including all four regions, but bidentate and tridentate spines were reported as narrower compared to those of *G. doloresi* from the Philippines.

Since the pattern of cuticular spines is an important taxonomic characteristic for identification of *Gnathostoma* spp., more detailed descriptions of the body spines as well as other characteristics, especially of females *G. doloresi* and *Gnathostoma* sp., are necessary. ITS2 and *cox*1 sequences have been commonly used for species identification and phylogenetic analyses of species of *Gnathostoma* (e.g. Almeyda-Artigas et al. 2000, Bertoni-Ruiz et al. 2005, Martínez-Salazar et al. 2005, Ando et al. 2006, Hernández-Gómez et al. 2010, Li et al. 2015, Jongthawin et al. 2016).

However, some lineages were not strongly supported in the phylogenetic trees, especially in the *cox*1 tree, generated in the present study. More sequence data on *Gnathostoma* spp. are, therefore, necessary to obtain a better res-

olution of phylogenetic relationships among the members of this genus. Since two previously known species from swine, *G. hispidum* and *G. doloresi*, are zoonotic parasites that affect the health of pigs and humans, special attention should be paid to swine and human gnathostomiasis in Dien Bien Province, Vietnam and the neighbouring areas of China and Laos.

In conclusion, *Gnathostoma* sp. of specimens collected from the stomachs of pigs in Dien Bien Province, northern Vietnam, are morphologically similar to *G. doloresi* with a slight difference in spicules of males, but are genetically far distant from *G. doloresi* and other congeners, representing a putative new *Gnathostoma* species. Further studies on the taxonomy and phylogenetic relationship of species of *Gnathostoma* are required to find out more taxonomic characteristics for their identification.

Ethical approval

The present study was approved by the Rector of Thai Nguyen University of Agriculture and Forestry (decision no. 225/QD-DHNL-DT dated February 28, 2018). Stomachs of domestic pigs were purchased from the local owners in Dien Bien Province where pork meat and visceral parts of pigs are sold in local markets. The use of the stomachs for research purpose was informed to the owners with their agreement.

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