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Morphological and Molecular Characterization of *Pratylenchus hippeastri*, A New Record of Root-Lesion Nematode Associated With Apple in China

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Authors' Contribution

All authors conceived and designed the study, and wrote the article. HW collected the samples and identified the nematodes. KZ and HW analyzed the data.

Key words:

***Pratylenchus hippeastri, Malus pumila* root lesion nematode, apple tree.**

ABSTRACT

During October 2012, a survey of plant-parasitic nematodes associated with apple (*Malus domestica* Borkh.) was conducted in Shandong Province, China. One species of root-lesion nematodes (RLN) was isolated and analyzed. Morphological and morphometric data were obtained using light microscopy (LM) and scanning electron microscopy (SEM). After detailed examination, the species was identified as *Pratylenchus hippeastri*. LSU D2D3 segment and ITS-rDNA region of the species were amplified and sequenced; they showed 99.3% and 99.6% identity to that of the original population *P. hippeastri*, respectively. Bayesian inference (BI) method was employed to reconstruct the phylogenetic relationship of *P. hippeastri* in China and other populations, as well as other related *Pratylenchus* species. All sequences from populations of *P. hippeastri* formed a high supported clade. Therefore, both morphological characters and molecular phylogenetic analyses confirmed the species identity of *P. hippeastri* in China, and apple tree is a new host record of the nematode.

INTRODUCTION

The apple tree (*Malus domestica* Borkh.) is an important fruit tree that is cultivated in temperate zones all over the world (Goulão and Oliveira, 2001). China is the largest apple producer with almost half of world's output (Food and Agriculture Organization of the United Nations Statistical Database FAOSTAT, 2012). Apple replant disease is a serious problem which influences growth and production of apple trees in all major apple producing areas (Traquair, 1984). Plant-parasitic nematodes are considered as the most important biotic causal agents for apple replant disease (Traquair, 1984). Roots of affected apple trees may be weak, sparsely branched and discolored (Traquair, 1984). Root-lesion nematodes have great economic impact on various crops, ranking second only to root-knot and cyst nematodes (Castillo and Vovlas, 2007). It was found that *Pratylenchus penetrans* (Jaffee *et al*., 1982; Merwin and Stiles, 1989), *P. vulnus*, *P. brachyurus* and *P. coffeae* are important species of lesion nematodes on apple trees (Mai and Abawi, 1981). So far, 12 species of the genus *Pratylenchus* have been reported as potential pathogens on apple (Castillo and Vovlas, 2007).

P. hippeastri was originally described from amaryllis (*Hippeastrum* spp.) and then reported from bromeliads in Florida (Inserra *et al*., 2007; De Luca *et al*., 2012). Recently, it was also collected from *Acer palmatum* plant and *Hippeastrum vittatum* bulbs exported from Japan and Israel, respectively (Chen *et al*., 2014; Gu *et al*., 2014). During October 2012, a survey of plant-parasitic nematodes associated with apple was conducted in Shandong Province, China. A population of *P. hippeastri* was detected from both soil and root samples. The objective of our study was to characterize the Chinese population of *P. hippeastri* from apple and confirm the species identity.

MATERIALS AND METHODS

Nematode populations

Soil and roots of apple tree were collected from Qixia city, Shandong Province, China. Nematodes were isolated from both soil and root samples by Baermann funnel method (Feng, 2001). Single females of root-lesion nematodes were transferred to carrot discs at 25°C (Castillo *et al*., 1995). Purified nematodes maintained on carrot discs were used for further

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morphological and molecular analysis after 8 weeks.

Morphological studies

For light microscopy (LM) studies, nematodes were relaxed by gentle heat, fixed in a solution of 4% formaldehyde + 1% glycerin and processed by the glycerin-ethanol method (Feng, 2001). Nematodes were measured and photographed with the aid of a Nikon ECLIPSE Ni microscope equipped with a Nikon Digital Sight Camera and exclusive NIS-Elements BR software (Nikon, Tokyo, Japan).

Specimens for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2) at 4°C, dehydrated in a graded series of ethanol, critical point dried with CO_2 and sputter coated with gold palladium (Feng, 2001). Nematodes were observed by using a XL-30-ESEM microscope (Philips, Netherlands).

DNA extraction, amplification and sequencing

DNA was extracted from nematode individuals following the protocol mentioned in detail by Mundo-Ocampo *et al.* (2008). Two rDNA fragments, *i.e.*, LSU D2D3 segment and ITS region were amplified respectively. Primers for LSU D2D3 amplification were D2A (5'-ACAAGTACCGTGGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') (Nunn, 1992). Primers for ITS amplification were 18S (5'-TTGATTACGTCCCTGCCCTTT -3') and 26S (5'-TTTCACTCGCCGTTACTAAGG -3') (Vrain *et al.*, 1993). Detailed methods and PCR conditions were conducted as described by Tanha Maafi *et al.* (2003). DNA sequencing was performed according to Zhuo *et al.* (2010) and Yu *et al.* (2014). The obtained sequences were deposited in GenBank under the accession numbers KR029084 (LSU D2D3) and KR029085 (ITS), respectively.

Phylogenetic analyses

The LSU D2D3 and ITS sequences of *P. hippeastri* and related species from GenBank database, together with the sequences generated in this study, were used for phylogenetic analyses. Sequences were also aligned using the ClustalW implemention in MEGA 6.0 software (Tamura *et al.*, 2013). Bayesian inference (BI) was used to construct the phylogenetic trees. Models of nucleotide substitution for the BI analyses were evaluated using Modeltest 3.7 (Posada and Crandall 1998) combined with PAUP4.0 (Swofford, 1998). The Akaike-supported model, the base frequencies, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. The model selected as the best fit for LSU D2D3 and ITS dataset were GTR+I+G and TVM+I+G, respectively. For both LSU D2D3 dataset and ITS dataset, *P. japonicus* was selected as out-group. Bayesian inference analyses of each dataset were conducted separately using MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). Bayesian analyses were run with four chains for 1×10^6 generations, with sampling every 100 generations and 2500 trees discarded as burn in. The MCMC (Markov Chain Monte Carlo) method was used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) and generate a 50% majority-rule consensus tree.

RESULTS

Pratylenchus hippeastri Inserra, Troccoli, Gozel, Bernard, Dunn & Duncan, 2007 (Table I, Figs.1-2)

Description

Female: Body slender, usually slightly bend ventrally after gentle heat (Fig. 1A). Lip region flatten anteriorly with two annuli, and first annulus narrower than the second one (Fig. 1B-C; Fig. 2). A third lip annulus appears on one side occasionally. Under SEM, *En face* pattern plain and smooth, with labial disc and all lip sectors fused together (Fig. 2). Lateral field with four lines (Fig. 1E), oblique striae may present in middle band by accident. Stylet strong, knobs ellipsoidal or slightly concave (Fig. 1B-C). Excretory pore at the level of or just anterior to the pharyngeal-intestinal valve (Fig. 1B-C). Hemizonid located just anterior to excretory pore and two body annuli long. Pharyngeal glands overlapping intestine ventrally (Fig. 1B-C). Oocytes arranged in single row (Fig. 1D). Spermatheca rectangular with a big round internal cavity, not containing sperm (Fig. 1D). Vulval lips usually slightly raised in some specimens (Fig. 1D). Post-uterine branch with distinct cells occasionally visible in distal portion. Phasmids located in the distal half of tail. Tail conoid with bluntly pointed or subhemispherical, smooth terminus (Fig. 1F-G). Hyaline portion of tail distinct (Fig. 1F-G).

Male: Not found.

Host and distribution

It has been recorded from the type locality at Gainesville, Florida, USA, from roots of amaryllis (*Hippeastrum* sp.) (Inserra *et al.*, 2007). It has been also recorded at Goulds, Florida, USA, on bromeliads (*Guzmania* sp., *Neoregelia* sp. and *Vriesea* sp.) (De Luca *et al.*, 2012). Moreover, it has been detected in *Acer palmatum* plant and *Hippeastrum vittatum* bulbs exported from Japan and Israel, respectively (Chen *et al.*, 2014; Gu *et al.*, 2014). In this study, the species was collected from soil and roots of apple tree in Qixia, Shandong Province,

China.

Table I.- Morphometrics of females of *Pratylenchus hippeastri* populations.

Character [+,]	China Population	Inserra *et al*. (2007)	De Luca *et al*. (2012)	Gu *et al*. (2014)
n	10	22	10	16
L	447.8±28.2 (400.7-479.8)[§,]	590± 21.8 (545-627)	614±22.4 (585-651)	479.9±31.9 (418.0-526.0)
a	27.7±1.4 (25-29.1)	25.5±1.2 (23.2-27.9)	25.2±2.0 (23.7-26.5)	28.0±1.9 (25.5-32.2)
b	5.4±0.3 (5-5.8)	6.5 ± 0.4 (5.7-7.1)	6.6±0.4 (5.9-7.2)	6.2±0.3 (5.7-6.7)
b'	3.2±0.2 (2.8-3.5)	3.9 ± 0.2 (3.6-4.3)	4.5±0.4 (4.0-5.3)	3.9±0.3 (3.4-4.5)
c	17.9±1.5 (15.6-20.5)	16.1±1.0 (14.6-18.7)	18.6±2.0 (16.4-23.3)	16.5±1.3 (13.6-18.4)
c'	2.3±0.2 (1.9-2.5)	2.6 ± 0.2 (2.2-2.9)	2.2±0.2 (1.8-2.5)	2.7±0.3 (2.2-3.4)
V	78.2±1.3 (76.4-80.2)	77 ± 0.8 (75-78)	77.7±1.2 (75.7-79.4)	77.2±1.0 (75.0-79.6)
Stylet length	14.9±0.4 (14.4-15.6)	15.5 ± 0.4 (15-16)	15.8±0.4 (15.3-16.7)	15.8±0.8 (14.5-17.5)
Stylet shaft	7.4±0.2 (7.1-7.6)	—	—	—
Stylet knob width	3.2±0.2 (2.9-3.5)	4.7 ± 0.3 (4.0-5.0)	—	—
Stylet knob height	1.8±0.1 (1.6-1.9)	2.1 ± 0.3 (1.5-3.0)	—	—
DGO from stylet base	2.8±0.2 (2.5-3.2)	2.9 ± 0.2 (2.5-3.0)	2.5±0.3 (2.0-2.7)	2.7±0.4 (2.1-3.3)
Anterior end to:				
Centre of metacorpus	53.7±2.4 (50-57)	63 ± 1.9 (59-66)	62 ± 1.9 (58-65)	52.6±2.6 (48.9-58.0)
Cardia	83.3±3.2 (79.4-89.9)	92 ± 3.3 (83-98)	93.0 ± 5.3 (87-106)	—
End of pharyngeal gland lobe	138.9±6.1 (126.3-148)	134 ± 6.6 (116-145)	137± 8.8 (123-147)	—
Secretory/excretory pore	79.2±2.7 (74.9-83.1)	91 ± 2.5 (85-95)	94 ±2.9 (89-99)	78.1±4.6 (70.9-84.8)
Pharyngeal overlap	54.9±5.4 (44.5-64)	43 ± 5.4 (32-51)	44.5 ±7.6 (33-58)	47.1±6.7 (37.6-57.9)
Max. body diam.	16.2±0.9 (14.6-17.6)	23.2 ± 1.4 (21-27)	24.4±0.7 (23.3-25.7)	17.2±1.7 (14.3-20.0)
Vulval body diam.	14.9±1 (13.3-16.2)	20.5±1.1 (18.0-23.0)	21.6 ± 1.6 (19.3-24)	15.6±1.7 (12.7-18.5)
Anal body diam.	11±0.7 (9.5-12.1)	14.4 ± 0.8 (13-16)	15.3 ± 0.4 (14.7-16)	11.4±1.2 (10.2-13.2)
Spermatheca-vagina	28.6±2.5 (26.5-34.1)	45 ± 9.5 (32-71)	—	42.2±9.6 (33.4-58.4)
Tail length	25±1.9 (21.3-27)	36.8±2.2 (32.0-42.0)	33.3 ± 3.0 (28-37.3)	29.5±3.1 (25.0-34.4)
No. of tail annuli	22.8±1.6 (21-26)	22 ± 2.1 (19-26)	24 ± 1.9 (21-26)	21.3±2.2 (17-25)
Vulva to anus distance	74.4±8.6 (61.5-85.1)	98 ± 6.1 (88-112)	103 ± 5.4 (92-109)	77.0±9.8 (65.393.6)
Post-uterine sac	22.2±3.1 (19.5-27.6)	30 ± 4.9 (21-45)	34.5 ±3.0 (30-39.3)	24.0±2.8 (19.0-27.2)
Lateral field width	5.3±0.4 (4.8-5.6)	—	—	—
Phasmids from tail terminus	11.9±1 (10.5-14.2)	—	—	—
E.P. (%)	17.7±0.7 (16.9-19.1)	—	—	—
Lip width	7.1±0.3 (6.6-7.4)	—	—	—
Lip height	2±0.2 (1.6-2.2)	—	—	—

[+,] All other abbreviations used are defined by Siddiqi (2000).
[§,] All measurements are in µm and in the form: mean ± standard deviation (range).

Fig. 1. Light micrographs of female of *Pratylenchus hippeastri* of China population. A, Entire body; B-C, Anterior region including the entire pharyngeal glands; D, Post-vulval region and ovaries (arrow shows spermatheca); E, Lateral lines near mid-body; F-G: Lateral view of tail (Scale bar: A, 50 µm; B-G, 20 µm).

Molecular characters and phylogenetic analyses

LSU D2D3 segment of *P. hippeastri* China population was 787 bp in length. A Blastn search of the LSU D2D3 sequence revealed high matches with the original reported sequences of *P. hippeastri* (DQ498829 and DQ498831). The identities of the sequences of the Qixia population and the original population were 99.3% (692/697) without insertions/deletions. The BI tree based on LSU D2D3 (Fig. 3) showed that the sequence of *P. hippeastri* obtained in this study and all the reported *P. hippeastri* sequences formed a high supported clade (PP=100). ITS region of *P. hippeastri* China population was 1185 bp in length. A Blastn search of the ITS sequence revealed high matches with the original reported sequences of *P. hippeastri* (FN554888). The identities of

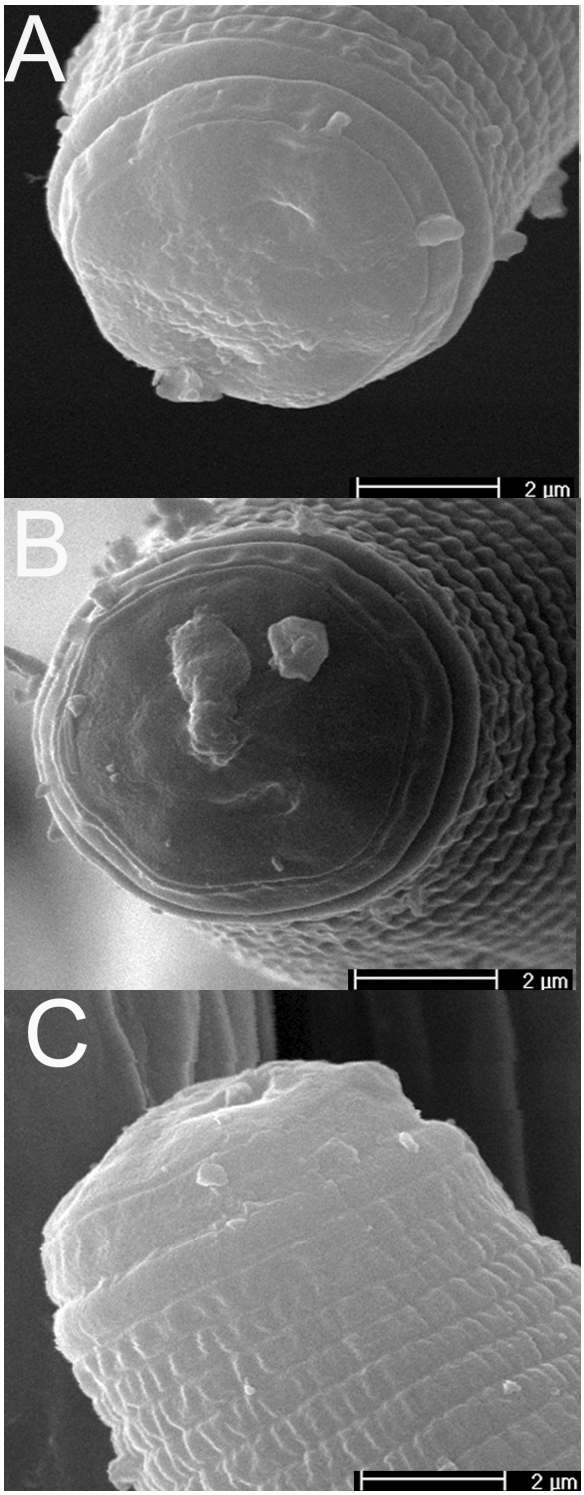

Fig. 2. Scanning electron micrographs of female of *Pratylenchus hippeastri* of China population A-B: *En face* view of lip pattern; C: Lateral view of lip region.

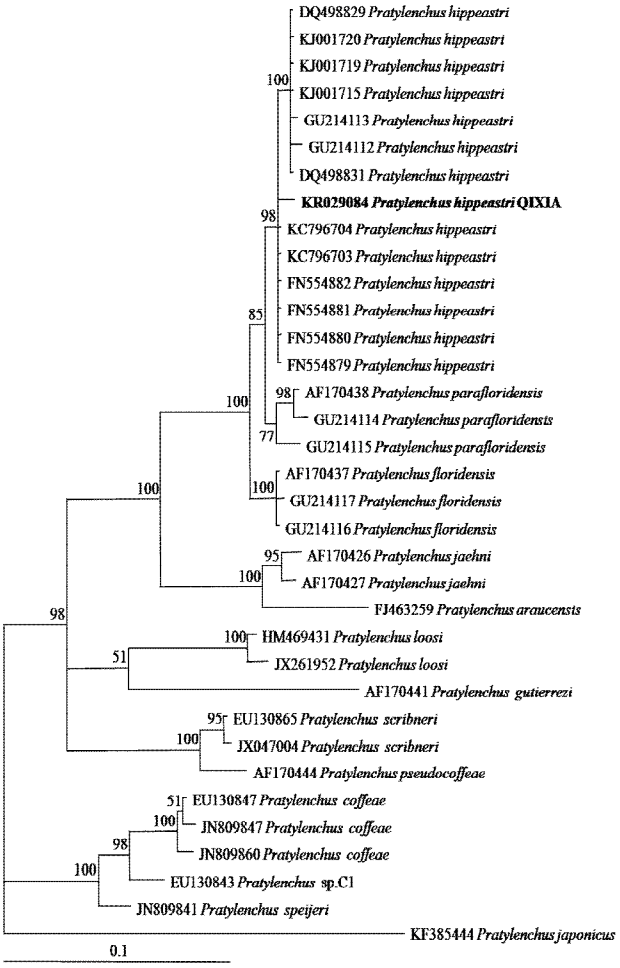

Fig. 3. Bayesian consensus tree inferred from LSU D2D3 segments of *Pratylenchus hippeastri* populations and related species under model (lnL=2738.0295; AIC=5496.0591; freqA=0.1965; freqC=0.2371; freqG=0.3251; freqT=0.2413; R(a)=1.7037; R(b)=3.6372; R(c)=1.9163; R(d)=0.3963; R(e)=5.0957; R(f)=1; Pinva=0.362; Shape=0.7453). Posterior probability values exceeding 50% are given on appropriate clades. Newly obtained sequence in this study are in bold. Fig. 4. Bayesian consensus tree inferred from ITS regions of *Pratylenchus hippeastri* populations and related species under model (lnL=8687.5947; AIC=17393.1895; freqA=0.2548; freqC=0.2091; freqG=0.2374; freqT=0.2988; R(a)=1.5728; R(b)=3.2438; R(c)=1.5324; R(d)=0.8127; R(e)=3.2438; R(f)=1; Pinva=0.2063; Shape=1.8298). Posterior probability values exceeding 50% are given on appropriate clades. Newly obtained sequence in this study are in bold.

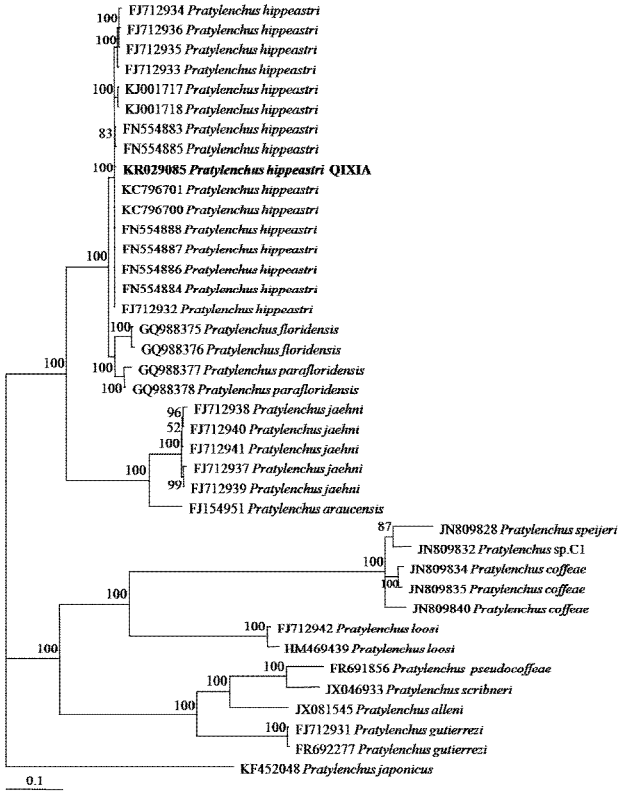

Fig. 4. Bayesian consensus tree inferred from ITS regions of Pratylenchus hippeastri populations and related species under model (lnL=8687.5947; AIC=17393.1895; freqA=0.2548; freqC=0.2091; freqG=0.2374; freqT=0.2988; R(a)=1.5728; R(b)=3.2438; R(c)=1.5324; R(d)=0.8127; R(e)=3.2438; R(f)=1; Pinva=0.2063; Shape=1.8298). Posterior probability values exceeding 50% are given on appropriate clades. Newly obtained sequence in this study are in bold.

the sequences from the Qixia population and the original population were 99.6% (989/994) without insertions/ deletions. The BI tree based on ITS (Fig. 4) showed that the sequence of *P. hippeastri* obtained in this study formed a high supported clade (PP=100) with all the reported *P. hippeastri* sequences.

DISCUSSION

Root-lesion nematodes are difficult to identify because of interspecific overlap and intraspecific variation in conventional morphological characters

(Roman and Hirschmann, 1969; Castillo and Vovlas, 2007). *P. hippeastri* was described as a closely-related species of *P. scribneri* with minor differences under light microscopy (Inserra *et al*., 2007). *P. hippeastri* differed from *P. scribneri* Steiner, 1943 by longer tail (36.6 *vs* 26.7 μm), slightly longer stylet (15.4 *vs* 14.7 μm), and shape of tail terminus (often bluntly pointed and smooth *vs* the consistently hemispherical and smooth tail terminus) (Inserra *et al*., 2007). However, interspecific overlap occurred in these characters between *P. hippeastri* and *P. scribneri* when more *P. hippeastri* populations were found.

In this study, morphometric and morphological characteristics of *P. hippeastri* from China were consistent with the original report except for shorter body and tail (401-480 and 21-27 μm *vs* 545-627and 32-42 μm, respectively) (Inserra *et al*., 2007). But the length of body and tail of Chinese population was similar to Japan population (Gu *et al*., 2014), which were 418-526 and 25-34 μm, respectively. Body length within a species may be influenced by some factors, like nutrient or environmental conditions (Castillo and Vovlas, 2007). Duncan *et al*. (1998) found female body length of *P. coffeae* on Florida citrus to be seasonal and correlated with concentration of starch in the fibrous roots of the host. It is difficult to reliably separate *P. hippeastri* from *P. scribneri* based only on morphology under LM. Therefore, *en face view* under SEM and molecular characters become more indispensable for distinguishing these two species. In this study, lip pattern and molecular characters confirm that the root-lesion nematode on apple in China is a population of *P. hippeastri*. This is the first report of this species in China and apple is a new host record of this species.

It was assumed that *P. hippeastri* was better adapted to warm tropical regions because amaryllis and bromeliads were tropical ornamentals (Inserra *et al*., 2007; De Luca *et al*., 2012). In this study, *P. hippeastri* was collected in Qixia City, Shandong Province, China. Qixia is located at the center of Jiaodong Peninsula in Shandong Province, well known as the Home of Apple in China (Qu *et al*., 2008). It is mainly covered by hills with temperate continental monsoon climate, average annual temperature is *ca* 11.3 (Qu *et al*., 2008). Therefore, *P. hippeastri* can also live in template regions. Further research on pathogenicity and host-parasite relationships of *P. hippeastri* on apple in China should be conducted.

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Competing interests

The authors have declared that no competing interests exist.

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