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Morphological and molecular characterizations of *Pratylenchus coffeae* infecting Ming aralia and coffee in Vietnam

H. D. NGUYEN[1], H. T. NGUYEN[2,3], T. M. L. LE[2,3], D. D. HOANG[4], T. D. NGUYEN[2,3], Q. P. TRINH[2,3,*]

[1]Department of plant pathology, Faculty of Agronomy, Vietnam National University of Agriculture, Trau Quy, Gia Lam, 100000 Hanoi, Vietnam; [2]Institute of Ecology and Biological Resources, Vietnam Academy of Sciences and Technology, 18 Hoang Quoc Viet, Cau Giay, 100000 Hanoi, Vietnam, *E-mail: *tqphap@gmail.com*; [3]Graduate University of Science and Technology, Vietnam Academy of Sciences and Technology, 18 Hoang Quoc Viet, Cau Giay, 100000 Hanoi, Vietnam; [4]Center for experiment and vocational training, Vietnam National University of Agriculture, Trau Quy, Gia Lam, Hanoi, Vietnam

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Summary

Pratylenchus coffeae, belonging to the root-lesion nematode group, is a highly prevalent and destructive plant-parasitic nematode that is able to infest a wide range of host plants. Although this species' devastating impacts on coffee plantations across the world are widely known on other host plants, its association with Ming aralia has never been reported. Our study characterized two populations of *P. coffeae* (associated with Ming aralia and coffee) and compared them with other populations from previous studies in Vietnam and other countries in the world. The identification of *P. coffeae* in our study was confirmed by the comprehensive analysis encompassing morphological examination, morphometric data, and molecular characterizations of the *COI* mtDNA and D2D3 of 28S rRNA regions. The cluster and MDS analyses revealed that the two populations of *P. coffeae* from Vietnam are closely related to those from Japan and Indonesia. The D2-D3 sequences of 28S rRNA and *COI* mtDNA regions exhibited high similarity among these populations, indicating a stable genetic profile. Our research contributes to a better understanding of the distribution and genetic characterizations of *P. coffeae* by offering new morphological and molecular insights into the presence of this nematode in Vietnam. Additionally, this nematode species was found to be associated with host plant's symptoms such as chlorotic leaves, stunted growth and root lesion in both hosts. Given the economic significance of both Ming aralia and coffee crops in Vietnam, as well as the damaging potential of *P. coffeae*, this study emphasizes the need of proactive nematode management measures to control this destructive pest.
Keywords: Cluster analysis; COI; D2-D3; Lam Dong; MDS; systematics; taxonomy; variation; Vinh Phuc

Introduction

Pratylenchus coffeae (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941, a species of root-lesion nematode, is one of the most damaging plant-parasitic nematodes in the world (Castillo & Vovlas, 2007; Jones *et al.*, 2013). This species has a wide host range, infecting various cash crops such as coffee, banana, yam, potato, and several citrus species (Castillo & Vovlas, 2007). The damaging effects of *P. coffeae* on a wide range of crops have been well-known to the world. For instance, the infestation of *P. coffeae* in coffee seedlings has been shown to have rapid detrimental effects on carbon fixation and distribution of photoassimilates (Mazzafera *et al.*, 2004). In citrus orchards and nurseries, both *P. coffeae* and *P. brachyurus* have been reported, with *P. coffeae* exhibiting higher

* – corresponding author

virulence (Perry & Moens, 2013). Infected red clover experienced a reduction in plant growth up to 41 % by *P. coffeae* (Chapman, 1958). In coffee plants, the presence of *P. coffeae* leads to stunted growth, chlorotic leaves, yellowing and browning of roots, and rotting of lateral roots (Sikora *et al.*, 2018; Souza, 2008; Villain *et al.*, 2018). Infestation of banana cultivars has also resulted in reduced bunch weight and fewer fingers (Sikora *et al.*, 2018). Furthermore, *P. coffeae* has been associated with "spreading decline" in citrus trees, causing severe damage to the fibrous roots and leading to weakened root systems incapable of supporting the above-ground portion of the tree. The consequences include sparse foliage, small fruit, weakened and dead branches, and reduced yield of citrus plants (O'Bannon *et al.*, 1976). In regions such as Florida, Japan, and India, *P. coffeae* has been reported as a major factor contributing to citrus decline (O'Bannon & Tomerlin, 1973; Siddiqi, 1964; Yokoo & Ikegemi, 1966).

Accurate identification of *P. coffeae* is of crucial importance for understanding its distribution and devising effective management strategies. However, there have been variations in host preferences observed among different isolates of *P. coffeae*, suggesting genetic diversity within the species (Silva & Inomoto, 2002). Furthermore, study of De Luca *et al.* (2012) has revealed the presence of cryptic species in the *P. coffeae* species complex that is indistinguishable from *P. coffeae* using morphological characteristics alone. Therefore, a combination of morphological examination and molecular analyses is needed to ensure for an accurate identification of species within this complex group.

In Vietnam, *P. coffeae* has been recorded in 30 different host plants, including banana (*Musa paradisiaca* L.), bean (*Phaseolus vulgaris* L.), black pepper (*Piper nigrum* L.), brassica (*Brassica* spp.), cabbage (*Brassica oleracea* var. *capitata* L.), carrot (*Daucus carota* L.), cassava (*Manihot esculenta* Crantz), citrus (*Citrus limon* (L.) Burm. f.), coffee (*Coffea arabica* L.), cucumber (*Cucumis sativus* L.), eggplant (*Solanum melongena* L.), green tea (*Camellia sinensis* (L.) Kuntze), groundnut (*Apios americana* Medik.), jackfruit (*Artocarpus heterophyllus* Lam.), lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), ming aralia (*Polyscias fruticosa* (L.) Harms), mung bean (*Vigna radiata* (L.) R. Wilczek), orange (*Citrus aurantium* L.), pak choi (*Brassica chinensis* L.), pea (*Pisum sativum* L.), pineapple (*Ananas comosus* (L.) Merr.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.), soybean (*Glycine max* (L.) Merr.), spring onion (*Allium cepa* var. *cepa*), sugar beet (*Beta vulgaris* L.), sugarcane (*Saccharum officinarum* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.) (Nguyen *et al.*, 2023; Nguyen & Nguyen, 2000). However, the presence of *P. coffeae* was confirmed by the combination of morphological characters and molecular data of D2-D3 of 28S rRNA region in only few host plants, inluding banana and coffee (Nguyen *et al.*, 2014). The objective of our study was to investigate and compare the morphological and molecular characteristics of *P. coffeae* populations associated with Ming aralia and coffee, as well as to compare them with populations from previous studies conducted in Vietnam and other countries in the world.

Materials and Methods

Sampling and nematode extraction

Prior to sampling, the debris layer was carefully removed. Subsequently, soil and root samples were collected from the upper 25cm soil layer in the rhizosphere of Ming aralia (Vinh Phuc province) and coffee (Lam Dong province) in Vietnam. Soil and root samples from the same sampling site were put together in a nylon bag and transported to laboratory for nematode extraction. Nematodes were extracted from the soil samples using the modified Baerman tray method, as described by Whitehead and Hemming (1965). Root samples were sectioned into small pieces measuring 0.5 cm in length, and nematodes were extracted from sectioned roots using the same method employed for the soil samples. Two populations were selected for further molecular and morphological analyses (one each from growing area of Ming aralia and coffee).

Morphological characterization

The extracted nematodes were killed by immersing them in hot water (60 – 70 °C) for 30 seconds. After that, they were transferred to a fixative solution containing 8 % formalin and 2 % triethanolamine in distilled water and left for 4 – 5 days for fixation, following the method described by Courtney *et al.* (1955). Fixed nematodes were then transferred to glycerin for slide preparation, following the technique outlined by Seinhorst (1959). Measurements and photographs of the nematodes were taken using a Carl Zeiss Axio Lab.A1 microscope.

Molecular characterization

For DNA extraction, live nematodes were cut into small pieces and transferred to PCR tubes, after adding a total of 20µl of WLB solution (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl2, 0.45 % NP-40, 0.45 % Tween-20). The samples were then incubated at -20 degrees Celsius for a minimum of 10 minutes. Following this, 1 µl of proteinase K (1.2 mg ml^{-1}) was added, and the samples were incubated in a PCR machine at 65°C for 1 hour, followed by 10 minutes at 95°C.

The D2-D3 region of the 28S rRNA and the *COI* mtDNA gene regions were amplified using the primers DP391/501 (5'-AGCGGAGGAAAAGAAACTAA-3'/5'-TCGGAAGGAACCAGCTACTA-3') and JB3/JB4 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3' / 5'-TAAAGAAAGAACATAATGAAAATG-3'), respectively (Derycke *et al.*, 2010; Nadler *et al.*, 2006). The amplification was carried out with the following thermal profile: 1 cycle at 94°C for 4 minutes; 5 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes; 45 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute; and a final step at 10°C for 10 minutes. Later on, the PCR products were sent to Macrogen (Korea) for sequencing.

The obtained forward and reverse sequences were assembled using Geneious R11 software (www.geneious.com). A Blast search (Altschul *et al.*, 1997) was conducted to look for closely related

Table 1. Measurements of *Pratylenchus coffeae* from Ming aralia and coffee in Vietnam.

Characters	*Pratylenchus coffeae* (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941			
Host plant	Ming aralia		Coffee	
Sex	Female	Male	Female	Male
n	10	10	10	10
a	23 ± 2.4 (22 – 28)	28.6 ± 1.2 (27.6 – 30.7)	27 ± 1.4 (25.5 – 29.1)	31 ± 2.9 (25.9 – 36.4)
b	6.0 ± 0.3 (5.7 – 6.5)	5.9 ± 0.2 (5.7 – 6.2)	6 ± 0.3 (5.7 – 6.5)	6 ± 0.3 (5.7 – 6.4)
b'	4.2 ± 0.2 (4.1 – 4.5)	4.2 ± 0.2 (4 – 4.6)	4 ± 0.2 (3.8 – 4.4)	4.2 ± 0.2 (3.8 – 4.5)
c	18.8 ± 0.5 (18.3 – 19.6)	19.5 ± 1.4 (18.3 – 21)	19.2 ± 1.4 (17.4 – 22)	20 ± 1.5 (18.5 – 23)
c'	1. 9 ± 0.1 (1.7 – 2.1)	2.3 ± 0.2 (2 – 2.7)	2.2 ± 0.2 (1.9 – 2.5)	2.1 ± 0.2 (1.9 – 2.4)
Body length (L)	519 ± 25 (485 – 550)	497 ± 24 (476 – 542)	473 ± 28 (440 – 531)	494 ± 26.7 (454 – 533)
V	79 ± 3 (74 – 82)	–	80 ± 2 (78 – 84)	–
Stylet length	15.9 ± 0.2 (15.7 – 16.1)	14.5 ± 0.3 (13.9 – 14.9)	15.9 ± 0.4 (15.3 – 16.6)	14.7 ± 0.8 (13.8 – 16)
Dorsal gland opening from stylet base (DGO)	3.2 ± 0.7 (2.2 – 3.9)	3.3 ± 0.3 (2.9 – 3.6)	2.9 ± 0.4 (2.3 – 3.3)	3.1 ± 0.4 (2.5 – 3.7)
Anterior end to valve of median bulb	56 ± 3 (52 – 58)	53 ± 1.4 (52 – 55)	50 ± 3.4 (46 – 58)	52 ± 1.8 (49 – 55)
Anterior end to nerve ring	70 ± 3.3 (64 – 72)	65 ± 3 (63 – 69)	61 ± 4 (57 – 70)	64 ± 2 (61 – 68)
Anterior end to secretory-excretory pore	80 ± 2 (78 – 82)	76 ± 3 (72 – 79)	75 ± 5 (69 – 81)	76 ± 2 (72 – 79)
Anterior end to pharyngo-intestinal junction	87 ± 5 (79 – 91)	84 ± 2 (81 – 87)	79 ± 5 (74 – 89)	82 ± 4 (74 – 86)
Anterior end to end of pharyngeal gland	123 ± 8 (109 – 129)	118 ± 6 (108 – 124)	117 ± 5 (109 – 123)	119 ± 7 (109 – 127)
Pharyngeal gland overlap	36 ± 4 (30 – 39)	34 ± 5 (27 – 42)	38 ± 4 (31 – 44)	37 ± 4 (31 – 43)
Max body diam.	22 ± 1.5 (20 – 24)	17.4 ± 0.7 (16.3 – 18.5)	17.5 ± 1 (16.2 – 19.3)	16.1 ± 2.1 (13.6 – 19.6)
Vulval body diam.	19.9 ± 1 (19 – 22)	–	16.3 ± 1.4 (14.4 – 19.4)	–
Post-uterine sac (PUS)	21 ± 4 (16.4 – 26)	–	27 ± 2 (24 – 30)	–
Anal body diam.	14.7 ± 0.3 (14.3 – 15)	11 ± 0.4 (10.3 – 11.6)	11.3 ± 0.4 (10.7 – 12)	11.4 ± 1 (10.1 – 12.9)
Tail length	28 ± 2 (25 – 30)	26 ± 2 (23 – 30)	25 ± 2 (21 – 27)	24 ± 2 (21 – 28)
Spicule length	–	18.4 ± 1 (17.2 – 19.9)	–	17.8 ± 1 (16.1 – 19.3)
Gubernaculum length	–	4.5 ± 0.3 (3.9 – 4.8)	–	4.3 ± 0.6 (3.5 – 5.5)

All measurements are in µm (except for ratio) and in the form: mean±s.d. (range)

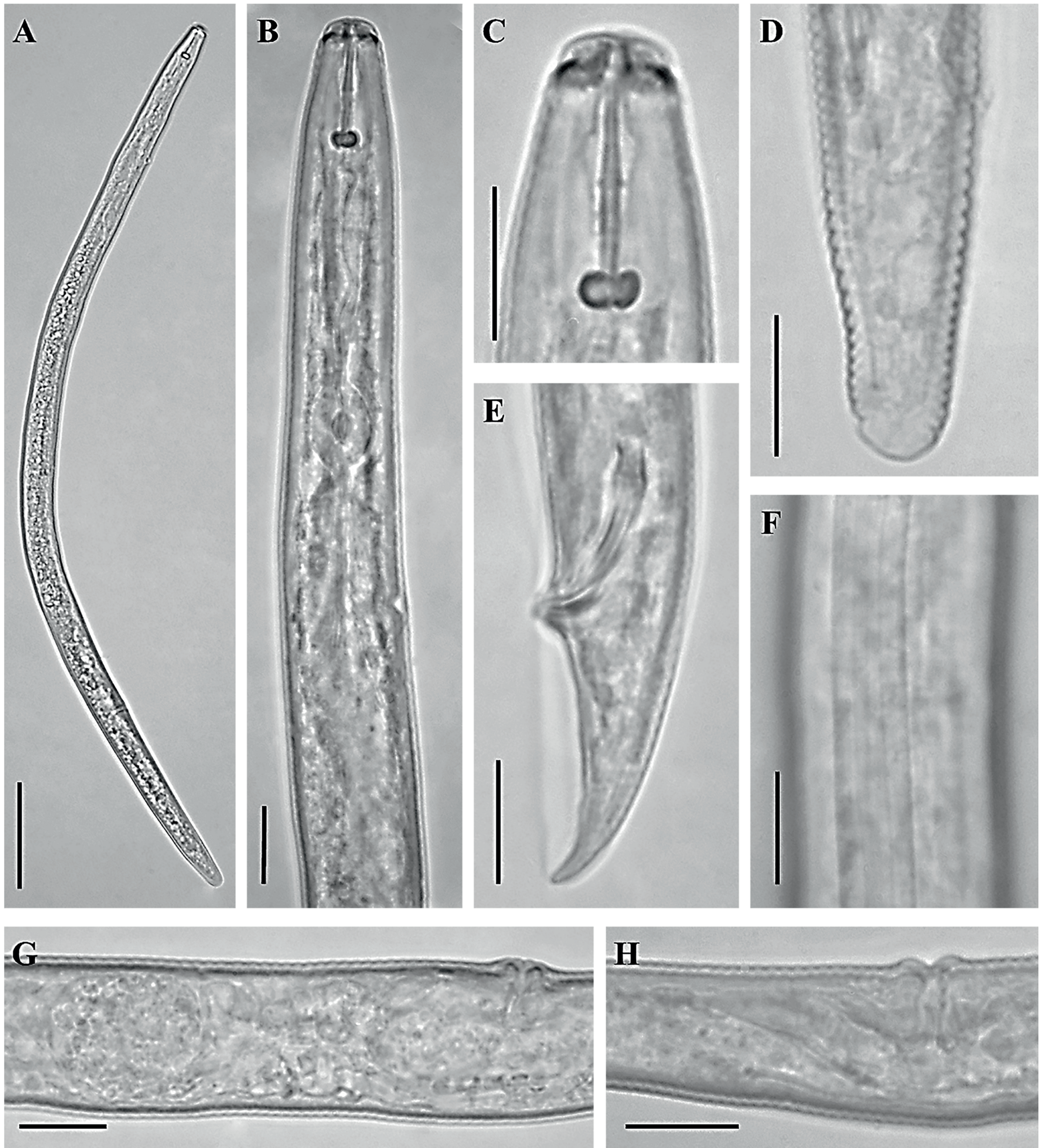

Fig. 1. Light microscopy pictures of *Pratylenchus coffeae* from this study. A-D, F-H: Female. A: Entire body, B: Anterior part of the body showing lip and pharynx region; C: Anterior end region; D: Tail region; F: Lateral field at mid-body; G: Vulva region showing spermatheca; H: vulva and post-uterine sac. E: Tail region of the male. (Scale bar: A: 50 µm; B-H: 10 µm).

sequences from GenBank. Multiple sequence alignments were performed using MUSCLE, and a Bayesian phylogenetic analysis was conducted using the MrBayes 3.2.6 add-in in Geneious R11. Mega 7 was employed to determine the best-fit models for the Bayesian phylogenetic analysis (Le *et al.*, 2022).

Cluster and Multidimensional scaling (MDS) analyses

To examine the relationships between various populations of *P. coffeae* based on measurement variables, the Primer version 6.1.12 was employed for Cluster Analysis (Anderson *et al.*, 2008). The Cluster and MDS analyses incorporated six morphometric measurements, i.e., L, a, b, c, V%, and Stylet length (abbreviations according to Castillo and Vovlas (2007)), from 17 female populations of *P. coffeae*, including the populations from Vietnam, Indonesia, India, Russia, Japan, and Brazil (Bajaj & Bhatti, 1984; Inserra *et al.*, 2001; Loof, 1960; Mizukubo, 1992; Nguyen *et al.*, 2012; Ryss, 1988). Prior to conducting the Cluster and MDS analyses, all the data were normalized due to the disparity in units. A resemblance matrix was created using the Euclidean distance measure. The MDS analysis was performed using the non-metric method with 1000 restarts. The parameters for the MDS analysis were set as follows: the minimal stress was set to 0.01 and the Kruskal stress formula was set to 1.

Ethical Approval and/or Informed Consent

The result of this work has not been published previously and is not under consideration elsewhere.

Results

Morphological characterization

In general, morphological characters of *P. coffeae* recovered from Ming aralia are highly similar to those from coffee, except for small variations in some measurements, such as body length (485 – 550 vs 440 – 531 μm), max body diam. (22 – 26 vs 16.2 – 19.3 μm), Post – uterine sac (PUS) (16.4 – 26 vs 24 – 30 μm), tail length (25 – 30 vs 21 – 27) in the females (Table 1). The morphological features observed in our *P. coffeae* populations (Table 1, Fig. 1) exhibit significant concordance with other populations of *P. coffeae* worldwide, as reported by Castillo and Vovlas (2007). The distinctive characteristics that delineate our nematode populations are as follows:

Female. The body of the nematode is slightly ventrally curved. The lateral fields typically exhibit four lines, although occasional occurrences of five or six lines are observed. The labial region is slightly separated from the body contour and comprises two distinct annuli, with instances of three annuli on one side of the labial region occasionally observed. The stylet is robust and possesses rounded to oblong basal knobs. The post-vulval uterine sac measures 1.5 – 2.0 times the diameter of the body at the vulva. The spermatheca is broadly oval to nearly rounded and typically contains sperm. The tail terminates in a flattened shape (truncate), occasionally appearing as smooth, rounded, or irregularly crenate.

Male. The males closely resemble the females except for their sexual characteristics. They possess slender and paired spicules, which are accompanied by a small gubernaculum. The tail exhibits a ventrally bent and conical shape, enveloped by a peloderan bursa.

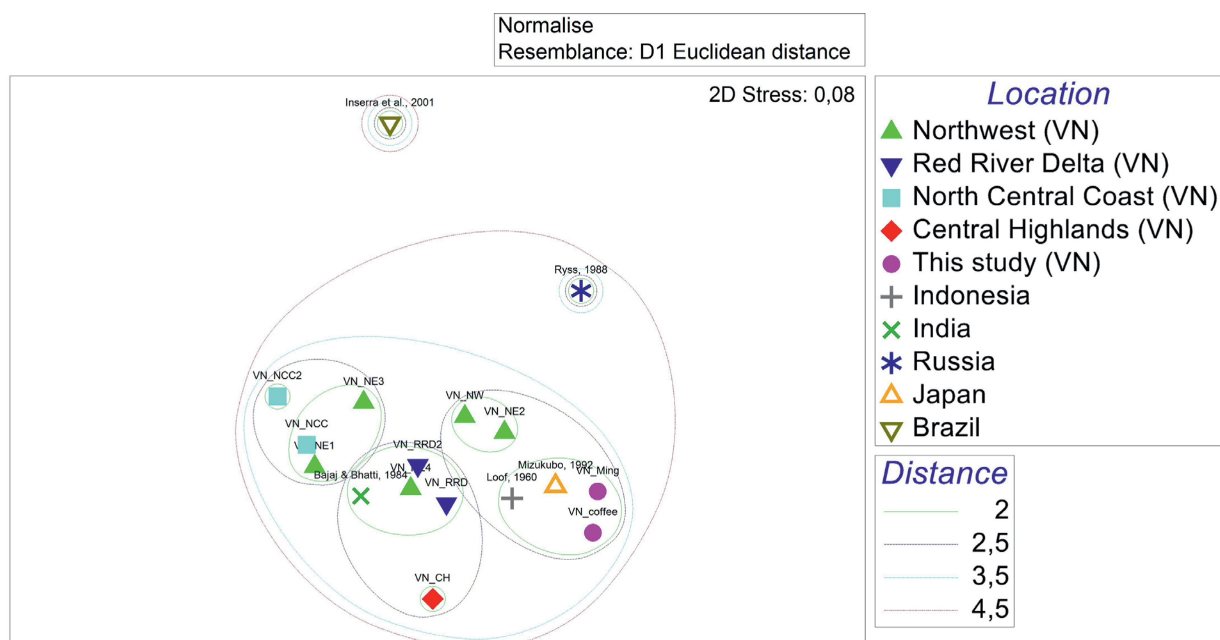

Fig. 2. Cluster and MDS analyses of *Pratylenchus coffeae* from 17 populations in the world.

Cluster and MDS analyses

Figure 2 shows that two populations of *P. coffeae* from this study are most closely related to the populations from Japan and Indonesia (Euclidean distance <2). The group of 4 aforementioned population is placed in a bigger group together with two other population of *P. coffeae* recovered from the Northwest of Vietnam (Euclidean distance <2.5). Among all studied populations, the population provided by Inserra et al., 2001 is the most distantly related population compared to all other populations of *P. coffeae*. The best 2-dimensional configuration resulted in a stress value of 0.08, indicating this is a reliable result.

Molecular characterization

In our molecular analysis, a range of 2 to 5 sequences for each gene and population was obtained, with the exact number varying based on the success of molecular analyses. Despite the availability of multiple sequences from different hosts and locations, a decision was made to analyze a single representative sequence for each gene and population, driven by the remarkable conservation observed.

<u>Characterization of D2-D3 of 28S rRNA region</u>

In this study, two sequences of D2-D3 of 28S rRNA region from two different hosts in this study (accession number: OR196106, OR196107) were presented with 100 % similarity to each other (960 bp long). These sequences are also 100 % identical to five other sequences of *P. coffeae* from GenBank (accession number: MT160082, KY424280, MW487595, HM469435, MK346210). The D2-D3 sequences of *P. coffeae* from this study differ by 0 – 3 nucleotides (99.4 – 100 % similar) to all other sequences of *P. coffeae* from GenBank. The Bayesian interference phylogenetic tree based on the D2-D3 sequences showed that all the sequences of *P. coffeae* were placed in a maximally supported clade (1 PP) with a sister relationship to a clade containing the sequences of *P. speijeri* (Fig. 3).

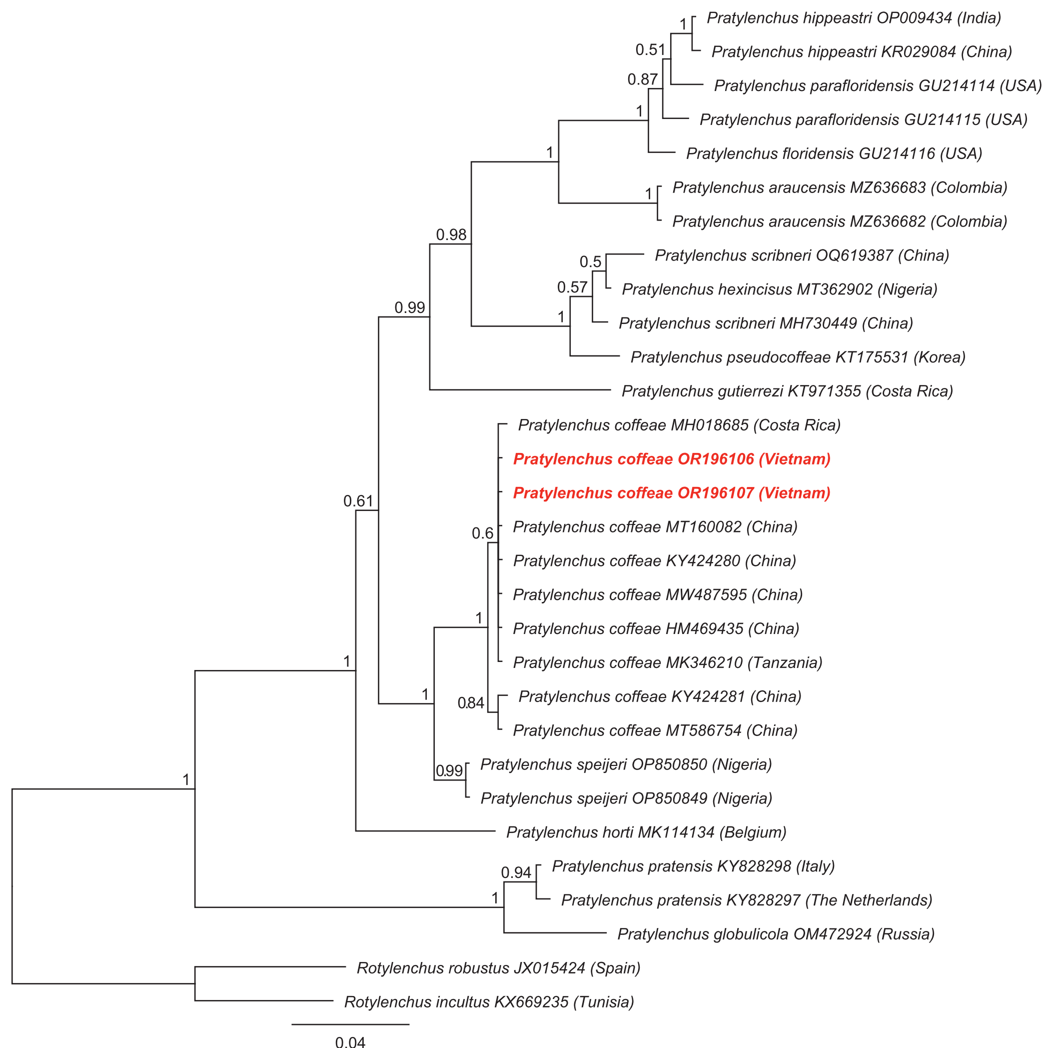

Fig. 3. Bayesian phylogenetic tree of *Pratylenchus* species generated from D2D3 of 28S rRNA genes using the HKY+G model. Posterior Probability support was given next to each node. The sequences from this study were marked by red and bold.

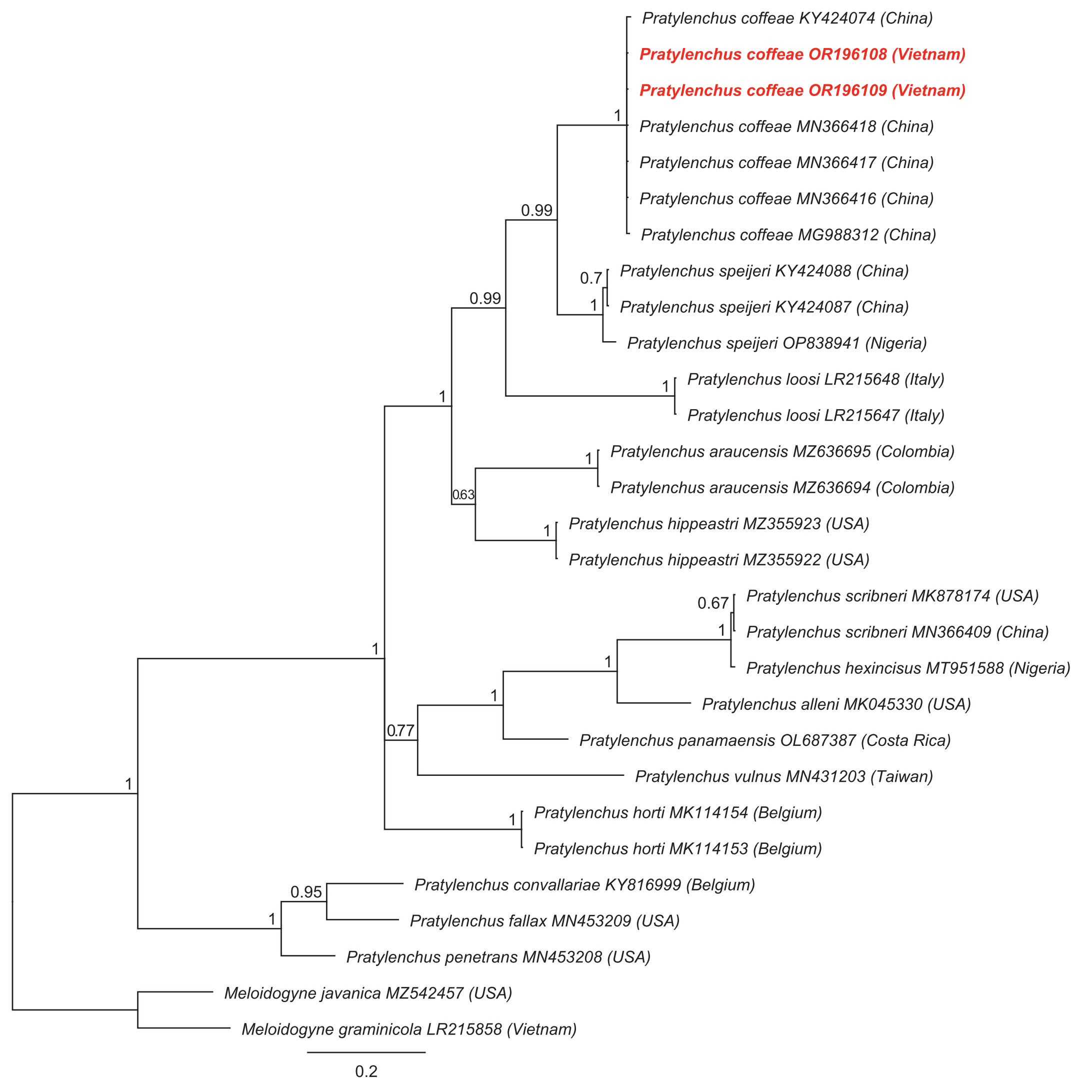

Fig. 4. Bayesian phylogenetic tree of *Pratylenchus* species generated from *COI* mtDNA genes using the HKY+G model. Posterior Probability support was given next to each node. The sequences from this study were marked by red and bold.

Characterization of COI mtDNA region

Two *COI* sequences of *P. coffeae* from two different hosts in this study (413 bp long; accession number: OR196108, OR196109) were selected for analyzing without variation. These sequences differ by 0 – 2 nucleotides (99.5 – 100 % similar) to all other sequences of *P. coffeae* from GenBank. The phylogenetic tree constructed from *COI* sequences using Bayesian interference indicated that all the *COI* sequences of *P. coffeae* were grouped together in a highest supported clade (1 PP), and this clade was most closely related to the clade of *P. speijeri* (Fig. 4).

Discussion

Although the morphological characterizations of our nematode populations are highly in agreement with the type and other descriptions of *P. coffeae* (Castillo & Vovlas, 2007), De Luca *et al.* (2012) reported that there exist cryptic species in *P. coffeae* species complex. To confirm the identification of *P. coffeae* populations in this study, a comprehensive analysis was conducted, which included morphological examination, morphometric measurements, Cluster and MDS analyses, and molecular characterizations of the *COI* mtDNA and D2-D3 regions of the 28S rRNA gene. These combined approaches allowed us to confidently distinguish *P. coffeae* from other nematode species. This comprehensive analysis not only contributes to our understanding of the morphological and genetic diversity of *P. coffeae* but also provides new insights into its host preference in Vietnam. Additionally, the observed genetic stability, between different populations of *P. coffeae* from different hosts and locations in this study, suggests a well-adapted species that maintains its genetic integrity across diverse environmental

conditions and host plants. Understanding the factors contributing to such genetic conservation could provide valuable insights into the biology and ecology of *P. coffeae*, with potential implications for pest management strategies and the sustainable cultivation of affected crops.

In the Cluster and MDS analyses, populations that exhibit higher similarity will be plotted closer to each other. Our Cluster and MDS analyses revealed that the two populations of *P. coffeae* in this study are more similar to the populations from Indonesia and Japan, despite the initial prediction that populations from the same region or country would exhibit greater morphological similarity. Furthermore, the twelve populations of *P. coffeae* from various regions in Vietnam were divided into three distinct groups, with an Euclidean distance of less than 2.5. This suggests that the geographical distribution of this species does not significantly influence its measurements.

On the other hand, our study revealed that *P. coffeae* was associated with specific symptoms in both Ming aralia and coffee hosts. These symptoms included chlorotic leaves, stunted growth, and root lesions, indicating the damaging effects of *P. coffeae* on the host plants. Given the substantial economic significance of both Ming aralia and coffee crops in Vietnam, along with the extensively documented destructive nature of *P. coffeae* on a global scale, the outcomes of this study underscore the compelling and immediate imperative for implementing proactive nematode management strategies to control this pest in Ming aralia and coffee.

Acknowledgments

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Conflict of interest

Authors state no conflict of interest.

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