CLEMEN JUNIOR DE OLIVEIRA

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF TWO FLORIDA POPULATIONS OF FOLIAR NEMATODES (*Aphelenchoides* spp.) ISOLATED FROM STRAWBERRY WITH NOTES ON THEIR PHYTOPARASITIC STATUS

Dissertation submitted to the Plant Pathology Program of the Universidade Federal de Viçosa, as part of the requirements to obtain the tittle of *Magister Scientiae*.

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APPROVED: February 27, 2019.

Robert Weingart Barreto

Renato Nunzio Inserra

Johan Desaeger
(Co-advisor)

Leandro Grassi De Freitas
(Advisor)

To my gramfather,

That passed away the day before I moved to Florida,

Manuel De Oliveira

I DEDICATE!

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To God, for being always presente in my journey.

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To all people that has helped me to conclude sucessuflly this step of my life

BIOGRAPHY

CLEMEN JUNIOR DE OLIVEIRA, son of Joaquim Rodrigues de Oliveira and Maria Zelia Pires Guimares de Oliveira, borned at Caldas Novas- Goias, on 17th of August of 1993.

Concluded his kindergardent at a rural school did his 5^{th} and 6^{th} grade at Rio Quente City and finished his middle and high school at Agua Limpa City on December, 2009.

In 2010, he moved to the capital of the state (Goiania) to prepare himself in order to be accept in the Agronomy Departament of the Federal university of Goias. He has been accepted one year later.

During his undergrade, from 2011 to 2016. Clemen has successful done up to 3,500.00 hours in Workshop, Courses, Conferences and Intership in 4 differents countries (Brazil, USA, Canada and Puerto Rico). He has study 18 moths at the University of Wisconsin through the Brazilian scientific mobility program.

In January of 2017, He has started his master's studies in Plant Pathology under supervise of Dr. Leandro Grassi De Freitas. In January of 2018, Clemen has move to Florida to sucessufully concluded his master's experiment under co-supervise of Dr. Johan Desaeger.

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ABSTRACT

DEOLIVEIRA, Clemen Junior, M.Sc., Universidade Federal de Viçosa, February, 2019. **Morphological and Molecular Identification of Two Florida Populations of Foliar Nematodes (*Aphelenchoides* spp.) Isolated from Strawberry with Notes on Their Phytoparasitic Habits.** Advisor: Leandro Grassi de Freitas. Co-advisor: Johan Deseager.

Morphological and molecular analyses were conducted to determine the taxonomic status of two populations of foliar nematodes from a Florida strawberry field tentatively identified as putative *Aphelenchoides besseyi* and *A. fujianensis*. Both nematode species were reared in plates containing cultures of the fungus *Monilinia fructicola*. The morphological characters of the Florida population of putative *A. besseyi* fit those of the original description and other re-descriptions of this species. The population of putative *A. fujianensis* did not fit the morphology described in the original description from China because it was male-less and with females lacking a functional spermatheca, whereas *A. fujianensis* was described as an amphimictic species and with females having a large spermatheca. Phylogenetic relationships among the Florida populations and other reference populations of Aphelenchida are given as inferred from the analyses of the near full-length small subunit ribosomal RNA (SSU), partial large subunit ribosomal RNA (LSU) and partial mitochondrial cytochrome oxidase gene subunit I (COI) gene sequences. These phylogenetic analyses were conducted using reference sequences of other geographical distant populations of *A. besseyi,* a species described without molecular analysis, and *A. fujanensis*, including that from the original description of this species. The results of the molecular study suggest that the sequences of the population of putative *A. besseyi* from Florida strawberry and those of other reference populations of this species are not congruous. The lack of congruity among them indicates that *A. besseyi* is a species complex. The Florida strawberry population was originating from strawberry stolons imported from North Carolina (USA), the type locality of *A. besseyi*. The new sequences obtained for this population are the most representative of this species. Furthermore, the results of the phylogenetic analyses indicated that the sequences of the Florida population of the putative *A. fujianensis* do not match those of the type population of *A. fujianensis* validating the results of the morphological analysis. The sequences of the Florida population were congruous with those of some morphological similar populations from Brazil identified as *A. fujianensis* suggesting that the Florida

and Brazilian populations are conspecific and are not *A. fujanensis*. Additional comparative studies of the Florida population with described *Aphelenchoides* species having the tail terminus with a mucro bearing hair-like processes will clarify its taxonomic status as a representative of another already described or an undescribed taxon in the group of *Aphelenchoides* species with stellate tail. In the phytoparasitic studies, the Florida population of putative *A. besseyi* from *M. fructicola* cultures infected strawberry and other selected plant species. Strawberry and gerbera daisy were the most suitable hosts compared to soybean. The population levels in strawberry were lower than those observed in gerbera daisy. The nematode behaved as an ectoparasite on strawberry without any evidence of endoparasitism. Ectoparasitism was observed also in gerbera daisy. Gerbera daisy is reported as a new host of *A. besseyi*. Very low population levels were observed in soybean indicating that this legume is not a good host of the Florida population of *A. besseyi* under the conditions of this experiment. Alfalfa was not infected by the nematode confirming the selective host preference of this Florida population of *A. besseyi.* The results of this study using the population of putative *A. fujianensis* at initial inoculum levels of 1000 specimens per plant clearly indicated that this species has mycetophagous habits. The population levels observed in the four crops mentioned above were low and did not exceed 34 specimens per plant (less than 2 specimens per gram of fresh tissues). On the contrary, inoculated and senescent soybean seedlings, kept in a greenhouse for 130 days, harbored high population levels to up 553 specimens per gram of desiccated tissues, when fungi infecting the decaying stem tissues became available as a food source. Although, this nematode population is mainly a mycetophagous species, it can behave as a phytoparasite under certain conditions as it was shown by the detection of nematode specimens inside the trichomes of soybean stem. Additionally, the localized inoculation of 300 nematodes applied with pieces of filter paper adhering to the blade of the soybean leaves resulted in nematode penetration and colonization of the mesophyll with subsequent development of foliar symptoms like those reported for other foliar nematodes.

RESUMO

DEOLIVEIRA, Clemen Junior, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Identificação morfológica e molecular de duas populações de nematoides foliares (*Aphelenchoides* spp.) isolados de morango em campos da Flórida com notas sobre seus hábitos fitoparasitários**. Orientador: Leandro Grassi de Freitas. Coorientador: Johan Deseager.

Análises morfológicas e moleculares foram realizadas para determinar o status taxonômico de duas populações de nematóides foliares coletados de um campo de morangos da Flórida, tentativamente identificados como putativos *Aphelenchoides besseyi* e *A. fujianensis*. Ambas as espécies de nematoides foram multiplicadas em placas contendo culturas do fungo *Monilinia fructicola*. Os caracteres morfológicos da população do suposto *A. besseyi* isolado da Flórida se ajustam aos da descrição original e outras descrições dessa espécie. A população do suposto *A. fujianensis* não se ajuta com a descrição original relatado na China. A população da Florida não foi encontrado macho e as fêmeas possuem espermateca não funcionais, enquanto que a espécie tipo do *A. fujianensis* foi descrita como uma espécie com machos e as fêmeas com uma grande espermateca. As relações filogenéticas entre as populações da Flórida e outras populações de referência de Aphelenchida foram analisadas utilizado a pequena subunidade ribossomal do RNA (SSU), grande subunidade parcial do RNA ribossômico (LSU) e da subunidade I do gene parcial do citocromo oxidase mitocondrial (COI). Estas análises filogenéticas foram conduzidas usando sequências de referência de outras populações geográficas distantes de *A. besseyi*, que é uma espécie descrita sem análise molecular, e *A. fujanensis*, incluindo aquela da descrição original desta espécie. Os resultados do estudo molecular sugerem que as seqüências da população de supostos *A. besseyi* coletado do morango em campos da Flórida e de outras populações de referência desta mesma espécie não são congruentes. A falta de congruência entre eles indica que *A. besseyi* é um complexo de espécies. A população de morangos da Flórida originou-se de estolões de morango importados da Carolina do Norte (EUA), a localidade-tipo do *A. besseyi*. As seqüências geradas neste estudo, para essa população, são as mais representativas desta espécie. Além disso, os resultados das análises filogenéticas indicaram que as seqüências da população da Florida do suposto *A. fujianensis* não coincidem com as da população tipo de *A. fujianensis* descrito na China, validando os resultados da análise morfológica. As sequências da população da Flórida foram congruentes com as de algumas populações

do Brasil identificadas como *A. fujianensis*, sugerindo que as populações da Flórida e do Brasil são coespecíficas e não são *A. fujanensis*. Estudos comparativos adicionais da população da Flórida com espécies descritas de Aphelenchoides tendo o terminal da cauda com mucro semelhantes a cabelos, clarificarão o seu status taxonômico como um representante de outro táxon já descrito ou não descrito no grupo de espécies de Aphelenchoides com cauda estrelada. Nos estudos fitoparasitários, a população do *A. besseyi* isolado do morando na Flórida e multiplicado em culturas de *M. fructicola* re-infectou morango e outras espécies de plantas selecionadas. Morango e gerbera foram os hospedeiros mais sucestíveis quando se comparado com à soja. Os níveis populacionais em morango foram menores que os observados na gerbera. O nematóide comportou-se como ectoparasita em morango sem evidência de endoparasitismo. Ectoparasitismo foi observado também em gerbera. Gerbera é relatado como um novo hospedeiro de *A. besseyi*. Níveis populacionais muito baixos foram observados em soja, indicando que esta leguminosa não é um bom hospedeiro para a população de *A. besseyi* da Flórida, nas condições deste experimento. A alfafa não foi infectada pela população de *A. besseyi* da Flórida confirmando a preferência seletiva do hospedeiro para com essa população. Os resultados deste estudo utilizando a população do suposto *A. fujianensis* com níveis iniciais de inóculo de 1000 espécimes por planta indicaram claramente que esta espécie possui hábitos micófagos. Os níveis populacionais observados nas quatro culturas mencionadas acima foram baixos e não excederam 34 espécimes por planta (menos de 2 espécimes por grama de tecidos frescos). Ao contrário, plântulas de soja inoculadas e mantidas em casa de vegetação por 130 dias, abrigaram altos níveis populacionais, de até 553 espécimes por grama de tecidos dessecados, quando fungos que infectam os tecidos caulinares em decomposição tornaram-se disponíveis como fonte de alimento. Embora esta população de nematoides seja principalmente uma espécie micofago, ela pode se comportar como fitoparasita sob certas condições, como foi demonstrado pela detecção de espécimes de nematoides dentro dos tricomas do caule da soja. Além disso, a inoculação localizada de 300 nematoides aplicados com pedaços de papel de filtro aderido à as folhas de soja resultou em penetração do nematoide e colonização do mesofilo com posterior desenvolvimento de sintomas foliares como os relatados para outros nematoides foliares.

GENERAL INTRODUCTION

Foliar nematodes are members of the genus *Aphelenchoides* Fisher, 1894, which contains 180 species (and 19 of uncertain status) (Sánchez-Monge et al., 2015; Cheng et al., 2013). They are mostly fungal-feeders (mycetophagous), but some species are known to also feed on aboveground plant parts (Fortuner and Williams, 1975; CABI, n.d.; Rybarczk-Mydlowska, 2012). The taxonomy of *Aphelenchoides* species is complicated. Despite the fact that the plant parasitic species are well described and defined taxons, other species have not been described sufficiently and there appears to be large intra-specific variation with minimal inter-specific relationships. Most taxa are not yet associated with discriminating molecular data, muddling the taxonomic work on this genus (Zhao, 2006.; Hunt, 1993).

The most important plant-feeding species, reported so far, are *A. besseyi* (Christie, 1942), *A. fragariae* (Ritzema Bos, 1891) Christie, 1932 and *A. ritzemabosi* (Schwartz, 1911) Steiner & Buher, 1932 with 200, 620 and 321 associated plant species, respectively. *A. besseyi*, *A. fragariae* and *A. ritzemabosi* are able to parasitize strawberry in many diverse geographical areas and the symptoms include leaf crinkling, distortion and dwarfing of the plant with reduced flowering.

Aphelenchoides besseyi is well known as the causal agent of rice white tip disease, which is found in rice-growing regions all over the world and has been reported to cause yield losses of 71% in China paddies (Cheng et al., 2013). It parasitizes strawberry, many ornamental plants and has also been found in bean fields in Costa Rica (Chaves et al., 2013; Morales et al., 1999), and recently also on soybean in Brazil, causing yield losses of up to 100% (Meyer et al., 2017).

Aphelenchoides fragariae is a damaging parasite of strawberry and many ornamental plants. This nematode has been reported mainly from Europe and North America. There are reports also from Australia, China, Estern Russia, India, Israel and Turkey (EPPO Global Database, 2016).

Aphelenchoides ritzemabosi causes foliar damage to *Chrysanthemum* spp., strawberry and ornamental plants in many countries around the world (CABI/EPPO, 2000; EPPO, n.d.).

In Florida, the three species have been reported on many ornamental plants, however only *A. besseyi* has been detecetd on strawberry. The symptoms that *A. besseyi* causes on strawberry are commmonly known as "summer crimp disease' (Christie, 1959). This disease was at first investigated in North Carolina and Florida by E. A. Bessey in 1901 and 1906, respectively. Bessey associated this disease with a nematode. Subsequent studies by Brooks (1931) and Christie (1938) demonstrated and confirmed that the agent of this disease was a nematode that was erroneously identified as *Aphelenchus fragariae* Ritzema Bos 1891, a nematode classified by Christie (1932) as junior synonym of the spring dwarf nematode, *Aphelenchoides fragariae,* the spring dwarf nematode. The confusion in the identification was caused by the fact that *A. fragariae* occurs on strawberry in Europe and in norhern states of the United States. However, Christie (1942) clarified the identitiy of the species causing the summer crimp symptoms and described it as a new species called *A. besseyi*, which is morphologically and biologically different from the spring dwarf nematode, *A. fragariae*. *Aphelenchoides besseyi* has been a prevalent damaging nematode on Florida strawberry from 1930 to early 1950's. Afterwards, the nematode infestations became were uncommon until 2016 when they reappeared again from a few fields in central Florida (Desaeger and Noling, 2017). Preliminary observations conducted in these fields infested with *A. besseyi* indicate that other *Aphelenchoides* species are present on declining strawberry plants. The identity of these aphelenchoidids from strawberry is not known in Florida. For ecological and agronomic purposes its important to obtain data in their biology and taxonomic status.

To establish an effective nematode management strategy, the most important and first step is to identify the species responsible for the yield losses and also of those that are associated with them. As the taxonomy of this genus *Aphelenchoides* is complex and not well understood, the aim of this project is to identify, by using morphological and molecular tools, which species of foliar nematodes are present in Florida strawberry fields and are causing disease symptoms. In addition, we will also study the host range of the different species found.

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Article**- Morphological and Molecular Identification of Two Florida Populations of Foliar Nematodes (Aphelenchoides spp.) Isolated from Strawberry with notes on their Phytoparasitic Status.** Manuscript written under the rules of journal Plant Disease.

Morphological and Molecular Identification of Two Florida Populations of Foliar Nematodes (Aphelenchoides spp.) Isolated from Strawberry with notes on their Phytoparasitic Status.

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Abstract

Morphological and molecular analyses were conducted to determine the taxonomic status of two populations of foliar nematodes from a Florida strawberry field tentatively identified as putative *Aphelenchoides besseyi* and *A. fujianensis*. Both nematode species were reared in plates containing cultures of the fungus *Monilinia fructicola*. The morphological characters of the Florida population of putative *A. besseyi* fit those of the original description and other re-descriptions of this species. The population of putative *A. fujianensis* did not fit the morphology described in the original description from China because it was male-less and with females lacking a functional spermatheca, whereas *A. fujianensis* was described as an amphimictic species and with females having a large spermatheca. Phylogenetic relationships among the Florida populations and other reference populations of Aphelenchida are given as inferred from the analyses of the near full-length small subunit ribosomal RNA (SSU), partial large subunit ribosomal RNA (LSU) and partial mitochondrial cytochrome oxidase gene subunit I (COI) gene sequences. These phylogenetic analyses were conducted using reference sequences of other geographical distant populations of *A. besseyi,* a species described without molecular analysis, and *A. fujanensis*, including that from the original description of this species. The results of the molecular study suggest that the sequences of the population of putative *A. besseyi* from Florida strawberry and those of other reference populations of this species are not congruous. The lack of congruity among them indicates that *A. besseyi* is a species complex. The Florida strawberry population was originating from strawberry stolons imported from North Carolina (USA), the type locality of *A. besseyi*. The new sequences obtained for this population are the most representative of this species. Furthermore, the results of the phylogenetic analyses indicated that the sequences of the Florida population of the putative *A. fujianensis* do not match those of the type population of *A. fujianensis* validating the results of the morphological analysis. The sequences of the Florida population were congruous with those of some morphological

similar populations from Brazil identified as *A. fujianensis* suggesting that the Florida and Brazilian populations are conspecific and are not *A. fujanensis*. Additional comparative studies of the Florida population with described *Aphelenchoides* species having the tail terminus with a mucro bearing hair-like processes will clarify its taxonomic status as a representative of another already described or an undescribed taxon in the group of *Aphelenchoides* species with stellate tail. In the phytoparasitic studies, the Florida population of putative *A. besseyi* from *M. fructicola* cultures infected strawberry and other selected plant species. Strawberry and gerbera daisy were the most suitable hosts compared to soybean. The population levels in strawberry were lower than those observed in gerbera daisy. The nematode behaved as an ectoparasite on strawberry without any evidence of endoparasitism. Ectoparasitism was observed also in gerbera daisy. Gerbera daisy is reported as a new host of *A. besseyi*. Very low population levels were observed in soybean indicating that this legume is not a good host of the Florida population of *A. besseyi* under the conditions of this experiment. Alfalfa was not infected by the nematode confirming the selective host preference of this Florida population of *A. besseyi.* The results of this study using the population of putative *A. fujianensis* at initial inoculum levels of 1000 specimens per plant clearly indicated that this species has mycetophagous habits rather than phytoparasite. The population levels observed in the four crops mentioned above were low and did not exceed 34 specimens per plant (less than 2 specimens per gram of fresh tissues). On the contrary, inoculated and senescent soybean seedlings, kept in a greenhouse for 130 days, harbored high population levels to up 553 specimens per gram of desiccated tissues, when fungi infecting the decaying stem tissues became available as a food source. Although, this nematode population is mainly a mycetophagous species, it can behave as a phytoparasite under certain conditions as it was shown by the detection of nematode specimens inside the trichomes of soybean stem. Additionally, the localized inoculation of 300 nematodes applied with pieces of filter paper adhering to the blade of the soybean leaves resulted in nematode penetration and colonization of the mesophyll with subsequent development of foliar symptoms like those reported for other foliar nematodes.

Key words: Foliar nematode, Summer crimp nematode, *A. besseyi*, *A.fujianensis*, Nematode Identification

Introduction

In Florida, infections of foliar nematodes (*Aphelenchoides besseyi* (Christie, 1942), *A. fragariae* (Ritzema Bos, 1891) Christie, 1932 and *A. ritzemabosi* (Schwartz, 1911) Steiner & Buher, 1932) are common on many ornamental plants (Lehman, 2002), but *A. besseyi* is the only species, in this group of nematodes, damaging to strawberries (*Fragaria* x *ananassa*) in the state. The symptoms that *A. besseyi* causes on strawberry are commmonly known as “summer crimp disease”. Christie (1959) proposed this epithet and reported that E. A. Bessey was the first to investigate and associate this

disease with a nematode during field observations he conducted in North Carolina and Florida, in 1901 and 1906, respectively. Subsequent studies by Brooks (1931) and Christie (1938) demonstrated and confirmed that the causal agent of this disease was a nematode, which was erroneously identified as *Aphelenchus fragariae* Ritzema Bos 1891, a nematode reclassified by Christie in 1932 as junior synonym of *Aphelenchoides fragariae,* the spring dwarf nematode (for information regarding these taxonomical revisions see Filipjev, 1934). The confusion in the identification arose because *A. fragariae* occurs on strawberry in Europe and in norhern states of the United States. However, Christie (1942) clarified the identity of the species causing the summer crimp symptoms in North Carolina and Florida, and described it as a new species called *A. besseyi*, which is morphologically and biologically different from the spring dwarf nematode, *A. fragariae*. *Aphelenchoides besseyi* was a prevalent damaging nematode on Florida strawberry from 1930 to the early 1950. Afterwards, the nematode infections were uncommon and not reported until 2016 when they reappeared from a few fields in Central Florida (Desaeger and Noling, 2017). Preliminary observations conducted in these fields indicate that different species of *Aphelenchoides* are present on declining strawberry plants. Three of these *Aphelenchoides* populations from strawberry in Florida were tentatively identified morphologically by Oliveira et al. (2018) as *A. besseyi*, *A. bicaudatus* (Imamura, 1931)

Filipjev & Schuurmans Stakhoven, 1941 and *A. fujianensis* Zhou,Cui,Ye,Luo,Wang,Hu & Liao, 2010. The identity of these aphelenchoidids from strawberry, in Florida, remains uncertain and needs to be validated by more extensive morphological and molecular analyses. Among the three species reported by Oliveira et al. (2018), *A. besseyi* is the most economically important; *A. fujanensis* is a mycetophogous described from dead pine (*Pinus massononiana*) in China (Zhou et al. 2010) and reported also associated with rice (*Oryza sativa*) seeds in Brazil without information on its phytoparasitic status (DeJesus et al. 2016); and *A. bicaudatus* is another mycetophagous species associated with several crops (Siddiqui, 1976). This study focuses only on the characterization of the Florida strawberry populations of putative *A. besseyi* and *A. fujianensis*. *Aphelenchoides besseyi* is a polyphagous facultative phytoparasite of rice and strawberry that can feed on both plant tissues and fungi (Christie, 1959; Franklin & Siddiqi 1972). It is not known whether the Florida populations of putative *A. besseyi* can parasitize other economic important plants such as the forage legume alfalfa (*Medicago sativa*) that is damaged by *A. rhitzemabosi* in

the Pacific Northwest of the United States (Gray et al., 1994), the flowering ornamental plant gerbera daisy (*Gerbera jamesonii*) that is infested by *A. fragariae* in Sri Lanka (Loos, 1941), or the agronomic crop soybean (*Glycine max*) that is damaged by local populations of *A. besseyi* in Brazil (Favoreto and Meyer, 2017; Favoreto et al. 2017). The objectives of this study were to: *i)* provide morphological chracterization of populations of putative *A. besseyi* and *A. fujianensis* from Florida strawberries that were cultured in plates containing the fungus *Monilinia fructicola* (G. Winter) Honey;
ii) provide molecular characterization of these populations of putative *A. besseyi* and *A. fujianensis* using full length small subunit ribosomal RNA (SSU), partial large subunit ribosomal RNA (LSU), and partial mitochondrial cytochrome oxidase subunit I (COI) gene sequences; *iii)* determine the ability these populations of putative *A. besseyi* and *A. fujianensis* to infect alfafa, gerbera daisy, soybean and strawberry.

Materials and Methods

Nematode Populations: The nematode populations used in this study were originally collected from strawberry plants originally imported from North Carolina and showing symptoms like those induced by foliar nematodes in a farm in Plant City, Hillsborough County, FL. These populations were tentatively identified as putative *Aphelenchoides besseyi* and *A. fujianensis*. Other species and populations of Aphelenchida from GenBank were included in our study and are listed in Table 1. The population of putative *A. besseyi* was obtained from photosynthetic leaves of nematode-infected strawberry plants from the same field and maintained in greenhouse. The population of putative *A. fujianensis* was obtained from senescent and desiccated strawberry cv. Florida Radiance leaves, collected from the same field during season 2017/2018. The nematodes were extracted by incubating leaf fragments in water per 24 hours (Young, 1954). Specimens were hand-picked and transferred into a Syracuse watch glass. Five to ten specimens having the same morphological characteristics were then pipetted in a 20 µl drop on two-weeks old *M. fructicola* cultures growing in PDA. This fungus was selected as a culturing medium because it has been reported as a good host for many aphelenchids (Giblin-Davis, 1987; Giblin-Davis et al., 1989). Plates were incubated in the dark at 23 ºC ± 3 ºC for 23 days. At the end of the incubation period, a large portion of the nematodes that reproduced on fungus has migrated to water drops

condensed on the lid of the plates. These specimens free of fungal hyphae were transferred in watch-glasses and used for morphological and molecular analyses and in experiments to determine their phytoparasitic habits. Additionally, *A. besseyi* specimens reared in vitro were used for comparison of their morphology with that of specimens extracted directly from strawberry.

Light microscopic study and morphological identification: Live adult specimens were hand-picked nematodes in water, immobilized by gentle heating and mounted in water agar (Esser, 1986) on a slide for measurements and photographs. Additional specimens were hand-picked and processed and mounted in glycerin in permanent slides (Seinhorst, 1959). Measurements of specimens were made using a Nikon (Optiphot) ocular micrometer. Photographs were taken with a Zeiss compound microscope, AXIO Scope A1 equipped with Nomarski interference contrast and an AxioCam ICc5 (Germany). Measurements taken included those reported by Fortuner (1970) and Franklin & Siddiqi (1972) for *Aphelenchoides* species and additional ones used in taxonomic studies (Siddiqi, 2000). The obtained characters of the Florida population of this putative *A. besseyi* and *A. fujianensis* were compared with those reported in the original description and re-descriptions of these species from distant geographical areas, including China and Brazil.

DNA extraction, PCR amplification: Three specimens for each putative *A. besseyi* and *A. fujianensis* were hand-picked and processed for DNA extraction (Floyd et al. 2002). DNA was used immediately for PCR. PCR amplification were carried out using a T100^{TM} Thermal Cycler (BIO RAD) and in a 50 µl reaction volume consisting of 39.75 µl of molecular water (HyClone™), 5 µl of 10 X ThermoPol® reaction buffer, 1 µl of deoxynucleotide (dNTPs) solution mix (10 mM), 1 µl each forward and reverse 10 µM primer (Genewiz, South Plainfield, NJ, USA), 0.25 µl of Taq DNA polymerase (5000 U/m) (New England Biolabs, Ipswich, MA, USA) and 2 µl of DNA extract. The primer sets and PCR conditions used in this study are listed in Table 2. Three different loci were used to elucidate relationships among the Aphelenchida populations: (i) Near full-length small subunit ribosomal RNA (SSU), (ii) partial large subunit ribosomal RNA (LSU) and (iii) partial mitochondrial cytochrome oxidase gene subunit I (COI)

sequence data. Amplified PCR products were sequenced directly at Genewiz Company (Genewiz, South Plainfield, NJ, (USA). The newly nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers: MK291493 and MK291494 (SSU); MK294342 and MK294343 (LSU); MK303401 and MK303402 (COI).

Sequencing and phylogenetic analyses: For SSU rRNA gene, the newly obtained sequences were aligned with their corresponding published gene sequences (Table 1) using ClustalX 1.83 (Thompson *et al*., 1997) with default parameter. Sequence alignments were analyzed with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The best fit model of DNA evolution was obtained using the program jModeltest (Posada, 2008) with the Akaike Information criterion. BI analysis was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples (10%), a 50% majority rule consensus tree was generated. Posterior probabilities (PP) in percentage are given on appropriate clades.

For the rRNA LSU and the mtCOI genes, the newly obtained DNA sequences were aligned and checked manually using Geneious Prime® (version 0.3, 2019), and subsequently subjected to a BLAST search (Altschul et al. 1990). Sequences of other Aphelenchida available from public databases (GenBank) were also included as reference (Table 1). Phylogenetic analyses were conducted using BI in MrBayes 3.2.2. (Ronquist et al., 2012) and maximum likelihood (ML) method using MEGA (version 7). For ML reconstruction, the best fitting model of sequence evolution was determined for each data set separately using MEGA. The model Kimura 2-parameter assuming a discrete gamma distribution (K2+G) was selected for LSU and the model Hasegawa-Kishino-Yano assuming a discrete gamma distribution with Invariant sites HKY+G+I for COI. Completed gaps were selected to run the trees. The support value for each node was determined by 1000 bootstrap replicates. For BI one cold and three heated Markov chains were used. Analyses were run for 30 million generations with sampling every 1000 generations. Each analysis consisted of four independent runs, utilizing four coupled Markov chains. The run convergence was assessed by finding

the plateau in the likelihood scores (standard deviation of split frequencies < 0.0015). In each of the runs, the first 25% trees were discarded as burn-in and 75% ML and 90% BL majority consensus trees were generated showing the posterior probability on each node. Similar tree topologies were obtained using BI and ML, therefore ML tree topologies only are shown. The branch values correspond to posterior probability based BI.

Phytoparasitic status: Pregerminated seeds of alfalfa, gerbera daisy and soybean cv. Patriot were sown in individual 15 cm-diam. plastic pots containing 1,700 cm^3 of a growing mix Metro-Mix® 380 (Fine bark, perlite, vermiculite, Canadian sphagnums and peat moss). Three-cm long stolons rather than seeds were used for obtaining strawberry cv. Florida Radiance plantlets. Prior the inoculation, seedlings of alfalfa and soybean grew for 15 days, whereas those of gerbera daisy and the strawberry stolons for 30 and 25 days, respectively. One inoculum level of 600 specimens per plant was used for putative *A. besseyi*, whereas two inoculum levels of 400 and 1000 specimens per plant were used for putative *A. fujianensis.* Plant species were set up in a complete randomized design in a greenhouse bench with five replications. All plants were harvested 60 days after inoculation (DAI), except those of gerbera daisy inoculated with putative *A. fujianensis* that were taken down after 84 days. Development of above ground symptoms was monitored 3-4 times per week. Additionally, just for soybean an extra set of five seedlings was inoculated each with 1000 specimens of putative *A. fujianensis* to assess nematode survival on desiccated stem tissues of senescent plants 130 DAI. A single inoculum level (600 specimens) was used for *A. besseyi* because this density is higher than the standard density (400 specimens) used, previously, in successful inoculations (Marlatt and Perry, 1971). This density was hypothesized sufficient to induce the infection of the tested plants. Smaller and greater levels of inocula (400 and 1000 specimens) were chosen for putative *A. fujianensis*, as that has not been reported as a phytoparasite and has not been used in other host test experiments. The inoculations were carried out by pipetting the aqueous suspension of the nematodes at each concentration in multiple 1-ml droplets delivered slowly on the crowns of the alfalfa and strawberry plantlets and on all the leaves of gerbera and soybean seedlings to contain loss of the inoculum leaking in the soil (Fig. 1 A). Localized and prolonged inoculations, described in the following section, were

conducted with putative *A. fujanensis* on soybean (Fig. 1 B) with the aim to verify the potential phytoparasitc status of this species. The inoculation of plant top rather than soil was used because the leaves of some of the selected plant species did not touch the soil and would have escaped the nematode infection from the inoculum in the soil. In fact, studies conducted by Marlatt (1970) and Marlatt and Perry (1971) have shown that *A. besseyi* is not able to climb the stem of *Ficus elastica* seedlings from the soil, but it is able to invade the leaves contacting the soil of the grass *Sporobolus poiretii* indicating that the nematodes should be in contact with the plant parts in order to start the infection. At harvest, above ground plant weights were recorded and nematode number per plant top and 200 cm^3 soil from the rizosphere were determined. Final populations in the entire plant top tissues were determined by incubating in water the entire macerated plant top tissues (Young, 1954) and are expressed as total number of nematodes found in the entire plant top. The final populations of the soybean set harvested 130 DAI were expressed as number of nematodes per gram of desiccated stem tissues. Soil final densities were obtained using the "salad bowl' incubation method (Rodríguez-Kábana and Pope, 1981). To avoid the interference of potential infestations of noxious arthropods such as mites and trips on the symptoms induced by *A. besseyi*, the experiments inoculated with the putative *A. besseyi* were enclosed in cages screened with a 4-μm-pore net and kept in a randomized design on greenhouse bench for 60 days. Once the experiments inoculated with the putative *A. fujianens* were carry out on bench in greenhouse for 60 days, except gerbera that was 84 days. Non-inoculated plants served as controls. Final nematode populations were assessed as in the previous experiment and shown as repeated treatments (Tables 5,6).

Temperature (T) and humidity (RH) in the greenhouse were recorded using Hobo® ProV2 onset. For tests conducted using un-caged plants and extra sets of soybean seedlings, temp and RH recorded were, respectively: Max 42.5 °C - Min 12.8 °C (Avg 23.8 °C) and Max 100 % - Min 35.1% (Avg 88.5%). The randomized plants on a greenhouse bench received nebulized water delivered for 3 minutes, at intervals of 6 hours from an automatic overhead irrigation system. For the test carried out using caged plants, temp and RH were respectively: Max 46.2 °C - Min 20.8 °C (Avg 29.5 °C) and Max 100 % - Min 28.6% (Avg 82.1%). The randomized plants on a bench received nebulized water delivered by hand with a sprayer twice per day.

Localized inoculation of putative *A. fujianensis* on soybean leaves: Potential penetration into leaf tissues by this nematode and development of symptoms of infection in soybean seedlings were determined by applying paper filter pieces containing 300 specimens of the nematode on a selected portion of the upper surface of all leaf blades as described by Riedel (1985) (Fig. 1B). Soybean seedlings were selected for this experiment because of the glabrous surface of their leaves that would have favored nematode penetration and development in the leaf tissues. After inoculation, the plants with attached pieces of filter paper on their leaves were enclosed in plastic bags and kept for 48 hours in a cabinet in the dark and at room temperature. After removal of the plastic bags, they were kept in a greenhouse for four weeks and irrigated with nebulized water delivered by hand with a sprayer twice per day. The inoculated leaves were examined for development of symptoms 3-4 times per week and at the end of the experiment using a stereomicroscope. Nematode penetration into the leaf tissues was verified by tearing with a needle the epidermis of the symptomatic leaves to observe and photograph nematode specimens in the mesophyll using a Zeiss compound microscope mentioned previously.

Results

Light microscopic study and morphological identification: Morphological examination of the putative *A. besseyi* populations obtained directly from photosynthetic strawberry leaves (*in vivo*) and *M. fructicola* cultures (*in vitro*) indicated that these populations belong to the species *A. besseyi*, as reported by Oliveira et al. (2018). Measurements are reported in Table 3. The morphometrics of the two populations were similar and did not differ from those reported in the original description by Christie (1942) nor from those of a population from rice (*Oryza sativa*) by Fortuner (1960). Many morphometrics were missed in the original description of Christie. Thus, the morphometrics obtained for the studied *A. besseyi* populations were also compared with those reported by Allen (1952) and Franklin & Siddiqi (1972) for other populations of *A. besseyi*. The morphological features of females of the two populations from leaves and fungal plates matched those of *A. besseyi*. The studied specimens from photosynthetic strawberry leaves and fungal plates had a stylet 12 (11.5-12.5 μm) and 11(10.9-11.2 μm) long, respectively, like that reported by Fortuner

(1960) (10-12.5 μm) from the rice population. Stylet knobs or swellings were 2 (1.9-2.2 μm) and 1.9 (1.8-2 μm) wide for the *in vivo* and *in vitro* specimens, respectively, and slightly larger than the average value of 1.75 μm reported by Franklin & Siddiqi (1972) for fixed specimens of *A. besseyi*; lateral field marked by four incisures; genital tracts with a conspicuous spermatheca packed with round sperm and a post uterine branch short and without sperm or with a few sperm in 5% of examined specimens. Tail terminus with a mucro having three or four processes as reported in the literature (Fig. 2). Only two male specimens were found in the population from photosynthetic leaves. These two specimens had a shorter body than that reported in the original description 525.3 (499.2-551.4 μm) *vs* 660-750 μm. Wider range of body length (440-720 μm) of *A. besseyi* males were reported by Allen (1952) for populations collected from Florida strawberry and Fortuner (1960) for a population from rice (530-610 μm) or reared on the fungus *Alternaria oleracea* Milb. (440-590 μm). Stylet and spicula were 12 and 17.5-18.8 μm long, respectively and in the range of the values 11.4 (10-12.5 μm) and 19.2 (18-21 μm), respectively reported for these characters by Fortuner (1960). The population reared on *M. fructicola* contained males that had body 541-592 μm long (Fig. 3). These values were smaller than those reported in the original description, but in the range of those reported for the populations measured by Allen (1952) and Fortuner (1960). In conclusion, the morphometrics of these populations from photosynthetic strawberry leaves and fungal cultures were not different and fit well those reported for *A. besseyi* both in the original description and later descriptions (Fortuner, 1960; Franklin & Siddiqi, 1972; Hunt, 1993).

The results of the morphological study of the nematode population isolated from Florida senescent and desiccated strawberry leaves, cultured in *M. fructicola*, and tentatively identified by Oliveira et al. (2018) as putative *A. fujianensis*, indicated that the morphological and biological characteristics of this population did not match those reported in the description of this nematode species from China (Zhuo et al., 2010). The analyzed Florida population was male-less, whereas the described type population of *A. fujianensis* is amphimictic (Zhuo et al., 2010).

Measurements of Florida putative *A. fujianensis* females are reported in Table 4. Some morphometrics of this population overlap those reported in the original description of type *A. fujianensis* and, also, those of a small number of characters published for a male-less *A. fujianensis* population from Brazil found in rice seeds and cultured on

Fusarium solani (De Jesus et al., 2016). The different reproductive habits of females of Florida putative *A. fujianensis* were reflected in morphological differences in their genital tract when compared to that of females from China. Females of the Florida population are different from those of the type species from China in that they lacked a functional and conspicuous spermatheca (Fig. 4). Characters that were shared between Florida females and those of the of the type population from China include a comparable stylet length (12.6 (12-13 μm) vs 13 (12.5-14 μm)) and a stellate tail (Shaina, 1996), having a terminal mucro consisting of a trunk bearing four hair-like and short processes (Fig. 4). However, females of putative *A. fujianensis* from Florida have oocytes disposed in several rows rather than a single row as described for the type *A. fujianensis*. They have also a shorter post-uterine branch (44.9 (28.7-74.2) vs 86 (68-110) and smaller values of PSU/VA (%) (22 (13.1-34.1) vs 37.6 (32.1-44.4)). These differences in the reproductive habits and morphology of the genital tract of females of Florida putative *A. fujianensis* compared to those of the type *A. fujianensis* from China indicate that these two aphelenchoidids are distinct species. Extensive morphological comparisons between the Florida population and other *Aphelenchoides* species with stellate tails are in progress for the correct identification of this Florida population.

Molecular characterization and phylogenetic relationships within selected *Aphelenchoides* species: Phylogenetic relationships among Florida strawberry populations of putative *A. besseyi*, putative *A. fujianensis* and reference aphelenchoidoids species were inferred from the analyses of nearly full length SSU rRNA (1600-1305 bp), partial large subunit LSU rRNA (709-710 bp), and partial nitochondrial cytochrome oxidase I COI mtDNA (679-689) gene sequence data.

SSU rRNA gene. The SSU alignment consisted of 51 sequences of species and populations of *Aphelenchoides*. The consensus tree inferred from the analysis of sequences of this gene is shown in Figure 5. The Bayesian consensus tree revealed: *i)* two highly supported sister clades containing populations of putative *A. besseyi* and *A. ritzemabosi. ii)* the clade with putative *A. besseyi* populations divided in a group with populations from rice, another group containing populations from fern and leguminous plants, and a monospecific clade with the new DNA sequence of the population of

the putative *A. besseyi* from Florida strawberry , which remained separated from the other two groups suggesting that *A. besseyi* should be considered a "species complex"; and *iii)* thirteen sequences of populations identified as *A. besseyi*, *A. fujianensis,* and the newly obtained sequence of the Florida population of putative *A. fujianensis* from strawberry clustering together and assembling with one small group of four Brazilian populations of *A. fujianensis* and one population of *Aphelenchoides* from USA; *iv)* all these populations in these groups, including the putative *A. fujianensis* from Florida, clearly separated from the type population of *A. fujiananesis* collected originally from *Pinus massoniana* in China, indicating the they are not representatives of the species *A. fujianensis*. These results corroborate those of the morphological analysis and support the classification of the Florida population of putative *A. fujianensis* as a distinct *Aphelenchoides* species belonging to the group of species having tail terminus with a stellate mucro (Shahina, 1996).

LSU rRNA gene. The LSU alignment consisted of 59 sequences of selected species and populations of Aphelenchoidoidea (53 *Aphelenchoides*, three of *Bursaphelenchus* and one each for *Ektaphelenchus*, *Laimaphelenchus* and *Sheraphelenchus*). Two additional sequences of *Paraphelenchus orientalis* and *Aphelenchus avenae* (Aphelenchoidea) were used as outgroup taxons. The maximum likelihood consensus tree inferred from the analysis of the sequences of this gene is shown in Figure 6. The tree revealed: i) nine moderately supported clades with those of *Aphelenchoides* species and *Laimaphelenchus heidelbergi* separated from the small clades with species of *Bursaphelenchus*, *Ektaphelenchus*, and *Spheraphelenchus*; ii) monophyly of the studied isolates and reference populations of *A. besseyi*, *A. fragariae*, putative *A.fujianensis*, *A. ritzemabosi*, *A. subtenuis*, *A. varicaudatus*, *A. xylocopae* and *Aphelenchoides* sp. and *L. heidelbergi* with a posterior probability of 96%; *iii)* sister taxa status between *A. besseyi* and *A. ritzemabosi*; *iv)* highly supported clades of putative *A. besseyi* and putative *A. fujianensis* (99%); *v)* the *A. besseyi* clade divided into a homogeneous group containing populations from rice and *Brachiaria* species; another group with populations from leguminous species and a monospecific clade with the population from Florida strawberry, sequenced in this study, which did not match the sequences of the other populations indicating that it was not conspecific, but was a component of *A. besseyi* species complex; *vi)* all reference of *A. fujianensis* populations and the strawberry population from Florida, sequenced in this study,

grouping in an undivided clade that contained sequences of populations of *A. fujianensis* from Brazil. The results of the phylogenetic analysis using the LSU consensus tree are not useful in validating those of the morphological analysis because no LSU sequences have been published for the type population of *A. fujianensis*. Three additional populations of *A. besseyi* identified by Chang et al. (2010) from Taiwan and two from the USA, one identified by Ye et al. (2007) on strawberry and the other by Zhao et al. (2008) on *Hosta* sp. clustered in this clade. The fact that these populations grouped with putative *A. fujianensis* populations rather than with those of *A. besseyi* cast doubt on the reliability of these identifications as *A. besseyi*.

COI mtDNA gene. The COI alignment consisted of 51 sequences of selected species and populations of Aphelenchoidoidea (46 of *Aphelenchoides*, three of *Bursaphelenchus* and one each for *Ektaphelenchus* and *Laimaphelenchus*). The consensus tree inferred from the analysis of this gene sequences is shown in Figure 7. The tree revealed: i) 13 moderately supported clades with those of *Aphelenchoides* species and *Laimaphelenchus heidelbergi* separated from the small clades with species of *Bursaphelenchus* and *Ektaphelenchus*; ii) monophyly of the studied populations and reference populations of *A. besseyi*, *A. fragariae*, *A. fujianensis*, *A. ritzemabosi*, *A. subtenuis*, *A. xylocopae* and *L. heidelbergi*; *iii)* highly (96%) supported sister taxa status between *A. besseyi* and *A. ritzemabosi* and also between *A. fragariae* and *A. subtenuis*; *iv)* clades of *A. besseyi* and *A. fujianensis* divided in groups; *v)* the *A. besseyi* clade divided into four moderately supported groups that did not reflect the botanical families of the plants where they were collected and nor showed that the grouped populations were conspecific; *vi)* the Florida population of putative *A. besseyi* matched one from *Brachiaria brizantha* from Brazil and did not match any of the sequences of the other populations of putative *A. besseyi* confirming that it was not conspecific with them; vii) all reference populations of *A. fujianensis* including that of putative *A. fujianensis* from Florida strawberry, sequenced in this study, grouped in a clade which was separated from that of the type population of *A. fujianensis* from China at the bottom of the clade indicating that these populations from other geographical areas are not *A. fujianensis*. These results agree with those obtained using the SSU gene sequences. The sequences (EU325686 and AY598072) of two *A. besseyi* populations from the USA, identified by Zhao et al. (2008) on *Hosta* sp. and

Ye et al. (2007) on strawberry, respectively, clustered in this clade confirming the doubt on the reliability of the identification of these two populations as *A. besseyi.*

Phytoparasitc status: Florida populations of *A. besseyi* re-infected strawberry plants and attained their highest reproduction rates in gerbera daisy seedlings. There was variability in the final nematode populations among strawberry plants that resulted in a mean value lower than that in gerbera (342 *vs.*1205). A smaller mean value (122) was also observed in strawberry from the repeated experiment. The symptoms observed in the strawberry infected plants were more accentuated in the inner than the outer leaves and consisted of crinkling, distortion and spider-like appearance (Fig. 8
A) as reported in the literature (Desaeger and Noling, 2017). The symptoms of nematode infection on gerbera daisy were like those seen in the nematode-infested strawberries (Fig. 8 B). A very low population (27 specimens) was recorded from soybean. Alfalfa seedlings were not infected or damaged by the nematode. No nematodes were found in the soil of the pots regardless of the plants (Table 5). Pots with alfalfa were an exception, because a residual population of 27 specimens persisted in their soil at the end of the experiment. No above ground plant weight suppression was observed in all the treatments at the inoculation levels used.

Low final densities of the Florida population of putative *A. fujianensis* were observed in all the treatments at the two inoculation levels (Table 6). The greatest population level was observed on alfalfa at the highest inoculum level of 1000 and did not exceed 35 specimens per plant top. No aboveground symptoms were observed regardless of the inoculum levels used. However, this nematode can be a phytophagous. Evidence of this behavior was obtained in live soybean stems where the nematode was found inside the trichomes that cover the stem of this legume (Fig. 9). The main nematode densities in live soybean plants, at the highest inoculum level (1000) and 60 DAI, were low, 19 specimens per plant top. These population levels/plant were equivalent to 2 specimens/gram of fresh plant tissues, and much smaller than those extracted from desiccated stem tissues (2 *vs.* 553) in an extra set of seedlings inoculated with 1000 specimens but kept in the greenhouse for 130 DAI until they died (data not reported in the tables).

Localized inoculation of putative *A. fujianensis* on soybean leaves: Definitive proof of phytoparasitism by the putative *A. fujianensis* population was obtained from the results of the inoculation test of soybean leaves with pieces of filter paper containing specimens of this nematode. The portion of the leaves in contact with the nematode infested filter paper became discolored and reddish after 9 ADI. days after the paper filter application. These symptomatic discolored areas were 7 mm long and 6 mm wide. Necrosis of the mesophyll was also observed in these areas (Fig. 10 A, B). Examination of the leaf discolored areas using a stereomicroscope allowed the observation of the nematodes inside the palisade and spongy parenchyma tissues of the mesophyll after tearing the leaf epidermis with a needle (Fig. 11). The number of nematodes found inside these discolored areas of the leaves varied from two to five (data not reported in table). The discolored spots of the leaves, however, did not expand after leaving the plants in the greenhouse for two additional weeks or 38 ADI. These results show that this nematode can behave as an endoparasite in the conditions of our experiments.

Discussion

The results of the molecular study suggest that the sequence of the population of putative *A. besseyi* from Florida strawberry and those of other reference populations of this species reported in the literature are not congruous. The lack of congruity among groups of these sequences indicates that *A. besseyi* is a species complex. The reference populations that were used for comparison in this study originate from distant geographical areas. Our Florida strawberry population was originating from strawberry stolons imported from North Carolina, the type locality of *A. besseyi*. We consider this population the morphological closest to that of *A. besseyi* described by Christie in 1942. Unfortunately no DNA sequences is available for the type population of *A. besseyi.* Other sequences for the three loci SSU, LSU and COI (AY508035, 508109 and 508072) of another population from Florida were identified as *A. besseyi* by Ye et al. (2007), but they do not match those of our population. In our study, they are congruous with those of *A. fujianensis* rather than *A. besseyi*. Therefore, the new sequences from this study are the only representative of *A. besseyi* in Florida. Our study confirms the reoccurrence of *A. besseyi* in Florida strawberry fields. Our findings validate the identification of this nematode on strawberries reported in previous studies

by Desaeger and Noling (2017) and Oliveira et al. (2018). This population behaved as a facultative phytoparasite by reproducing on both strawberries and the fungus *M. fructicola*. The morphology of these two isoltes from the two different food sources did not differ, although an adverse effect of the fungus on the body size of the males was observed. The males from our cultures were smaller than those reported in the literature. A similar effect from the fungus in inducing small body size was reported by Fortuner (1960) on males cultured on the fungus *A. oleracea*. Unfortunately, we found only two males, both with small bodies from the strawberry field population and could not confirm the fungal effect on male size. In our *M. fructicola* cultures, males remained active for 23 days and died soon after, while females remained alive for almost two months. These surviving females were active and showed the spermatheca packed with sperm. More biological, morphological and phylogenetic studies of putative *A. besseyi* are needed to better understand the genetical incongruity of the populations of *A. besseyi*. In our greenhouse host study, the populations from *M. fructicola* cultures maintained their phytoparasitic behavior and re-infested strawberry and colonized other selected plant species. Strawberry and gerbera daisy were the most suitable hosts compared to soybean. The mean values of the population levels observed on strawberry at the end of the two experiments matched those (235) reported in Florida infected strawberry by Brooks (1931). The population levels in strawberry were lower than those observed in gerbera daisy. These smaller levels may be due to due the different morphology of the leaves of the two hosts. The large leaves of gerbera daisy retained more initial inoculum than the small leaves of strawberries resulting in greater final population levels in the tissues of this flowering ornamental plant than in those of the strawberry plants. The nematode behaved as an ectoparasite on strawberry without any evidence of endoparasitism as reported by Christie, 1959. Ectoparasitism was observed also in gerbera daisy. Gerbera daisy is reported as new host of *A. besseyi.* Very low population levels were observed in soybean indicating that this legume is not a good host of the Florida population of *A. besseyi* under the conditions of this experiment. Alfalfa was not infested by the nematode confirming the selective host preference of this Florida population of *A. besseyi.* Our study shows that the initial inoculation levels that we used in the greenhouse experiments were sufficient to produce plant infection when delivered directly on the plant top. These inculum levels were as effective as those of 400 specimens applied in the soil in the inoculation tests conducted by Marlatt and Perry (1971). Our results suggest that the *A. besseyi*

population that we studied has the potential to become an emerging problem for the Florida strawberry growers. However, it is not certain that this nematode will become a serious problem as it was 50 years ago, because the infections of the nematode observed in the field in 2018 were less serious than those in 2017. Re-occurrence of nematode infections has been observed again in the season 2018/2019.The epidemiology of this foliar nematode on strawberries should be investigated for more years in Florida fields.

The population of the Florida putative *A. fujianensis* from *M. fructicola* plates shared only a few morphological characters with those of the type population of *A. fujianensis*, but it was biologically and morphologically different from this type population. The putative *A. fujianensis* from Florida was male-less and with the female's genital tract showing different arrangement of the oocytes than that of the type population of *A. fujiannesis* (multiple rows vs. single row) and, also without a functional spermatheca that is present in type *A. fujianensis*. These differences in the morphology and reproductive habits of our population compared to those in the original description indicate that these populations are not conspecific. These finding were confirmed by the results of the phylogenetic analyses using SSU, LSU and COI gene sequences, which provided definitive evidence that the Florida population is not *A. fujianensis*. The population of *A. fujianensis* described in Brazil by DeJesus et al. (2016) provides limited information of its morphological characters. Many of these characters, such as the lack of males and a functional spermatheca and the oocytes arranged in multiple rows in the females are shared by the Florida population indicating that Brazilian and Florida populations may belong morphologically to the same species. This morphological congruity between the Brazilian *A. fuijanensis* and the putative *A. fujjianensis* from Florida were confirmed by the results of the phylogenetic analyses, which indicate that these Brazilian and Florida populations are conspecific. However, the results of this study indicate that they both are not *A. fujianensis*. Additional comparisons of the Florida population with described *Aphelenchoides* species having the tail terminus with a mucro bearing hair-like processes will clarify the taxonomic status of the Florida population, which could be a representative of another already described or an undescribed taxon in the group of *Aphelenchoides* species with stellate tail (Shaina, 1996).

Under the field conditions of Florida, this unidentified species (indicated as putative *A. fujianensis*) associated with *A. besseyi* in senescent strawberry plants. These two species have similar morphological characters, but can be separated by the body width that is greater in this unidentified *Aphelenchoides* species than in *A. besseyi* (24.1 ± 2.4 (19.3-27.2) vs 15.7 ± 1.3 (13.3-17.8)) and the shape of tail's mucro that has shorter processes than that in *A. besseyi* (< 1 μm long vs > 1 μm long). The Florida population of this unidentified *Aphelenchoides* is male-less with female lacking a functional spermatheca whereas *A. besseyi* is amphimictic with numerus males and females having a large functional spermatheca filled with sperm.

These two species are also biologically different: *A. besseyi* is facultative phytoparasite, whereas this unidentified *Aphelenchoides* is mainly mycetophagous. This species, however, can become phytophagous under stressful environmental conditions. The localized inoculation of the nematodes applied with pieces of filter paper adhering to the blade of the soybean leaves resulted in nematode penetration and colonization of the mesophyll with subsequent development of symptoms like those reported for other foliar nematodes. There was evidence of phytoparasitism of this species in the host test, but the population level observed were low and less than 2 specimens per gram of fresh tissues. On the contrary, populations densities of the nematode in soybean senescent tissues were 20 folds greater because the nematode mostly likely developed and reproduced on its preferred food sources consisting of *Fusarium* spp. *Trichoderma* spp. and C*olletotrichum* spp. fungi that were isolated from the inside and outside of the dead soybean stem. Our work showed that this species does not have the aggressiveness and phytoparasitic abilities of other foliar nematodes such as *A. besseyi*, *A. fragariae* and *A. ritzemabosi*. It is not known whether this nematode is native to Florida or arrived with the trade of plant parts from abroad.

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Tables legends

Table 3. Morphometrics of live females and males of a Florida population of putative *Aphelenchoides besseyi* from strawberry and from *Monilinia fructicola* cultures compared to those in the original description by Christie (1942) and a re-description by Fortuner (1970). All measurements are in μm and in the form: mean ± SD (range).

Character	**Strawberry (N17-00341)**		***Monilinia fructicola* (N18-01001-2)**		**Strawberry Christie (1942)**		**Rice Fortuner (1970)**	
	♀	♂	♀	♂	♀	♂	♀	♂
n	15	2	10	10	-	-	-	-
L	657.0 ± 75.9 (516.8-810.8)	525.3 ± 36.9 (499.2-551.4)	710.3 ± 23.6 (669.3-747.3)	572.3 ± 19.4 (538.5-592.0)	(660-750)	(660-750)	681 (570-840)	618 (570-840)
a	41.7 ± 3.8 (34.0-49.7)	35.2 ± 0.5 (34.8-35.6)	50.3 ± 2.4 (45.3-53.3)	36.1 ± 1.6 (33.2-38.7)	(32-42)	(32-42)	47.7 (39.3-53.5)	47.7 (39.3-53.4)
b	9.8 ± 0.6 (8.6-11.2)	8.7 ± 0.6 (8.3-9.1)	10.3 ± 0.2 (10.0-10.6)	9.0 ± 0.3 (8.5-9.5)	(11.2-11.4)	-	11.4 (9.2-13.1)	11.5 (9.2-13.3)
b'	4.4 ± 0.2 (4.0-4.6)	4.2 ± 0.6 (3.8-4.7)	4.3 ± 0.1 (4.1-4.5)	4.3 ± 0.3 (3.9-4.8)	-	-	4.8 (4.0-5.7)	4.8 (4-5)
c	16.6 ± 1.2 (14.8-19.1)	17.6 ± 0.6 (17.2-18.0)	17.6 ± 0.8 (16.1-18.9)	17.7 ± 0.8 (16.0-19.1)	(17-20)	(17-20)	17.7 (13.8-20.4)	17.9 (16.6-20.0)

c'	3.9 ± 0.3 (3.6-4.7)	2.7	4.0 ± 0.2 (3.8-4.4)	2.7 ± 0.1 (2.5-2.8)	-	-	-	-
Max. body diameter	15.7 ± 1.3 (13.3-17.8)	14.9 ± 1.3 (14.0-15.8)	14.1 ± 0.6 (13.4-15.2)	15.9 ± 0.4 (14.9-16.3)	-	-	-	-
Body diameter at anus or cloacal opening	9.9 ± 0.7 (8.9-11.4)	10.7 ± 0.3 (10.5-11.0)	9.8 ± 0.5 (9-11)	12.0 ± 0.3 (11.5-12.5)	-	-	-	-
V	70.5 ± 0.5 (69.7-71.6)	-	69.8 ± 0.6 (68.8-70.8)	-	(68-70)	-	71.2 (68.7-73.6)	-
OV or Testis/L%	26.8 ± 2.5 (23.2-31.1)	52.5 ± 16.2 (41-64)	25.9 ± 1.1 (24.3-27.6)	44.2 ± 4.2 (38.0-51.4)	-	-	27.9 (19.9-39.3)	40.6 (28.2-52.3)
Anterior genital tract length	181.3 ± 19.2 (155.4-216.8)	279.2 ± 104.9 (205.0-353.4)	184.1 ± 9.7 (163-196)	-	-	-	-	-
Lip region width	6.9 ± 0.3 (6.0-7.5)	6.5	6.9 ± 0.1 (6.7-7.0)	6.6 ± 0.1 (6.4-6.8)	-	-	-	-
Lip region height	3.1 ± 0.2 (2.8-3.4)	2.8 ± 0.1 (2.7-2.9)	3	3	-	-	-	-
Stylet length	12.0 ± 0.2	12	11.0 ± 0.1	10.7 ± 0.2	-	-	11.9	11.4

	(11.5-12.5)		(10.9-11.2)	(10.4-11.0)			(10.0-12.5)	(10.0-12.5)
Stylet cone	6.7 ± 0.3 (6.2-7.0)	6.8	5.0 ± 0.1 (5.0-5.2)	5.0 ± 0.1 (4.8-5.0)	-	-	-	-
Stylet knob height	1.7 ± 0.1 (1.5-1.9)	1.7	1.6 ± 0.1 (1.5-1.7)	1.5 ± 0.1 (1.4-1.6)	-	-	-	-
Stylet knob width	2.0 ± 0.1 (1.9-2.2)	2.3 (-)	1.9 ± 0.1 (1.8-2.0)	1.9 (1.8-1.9)	-	-	-	-
Metacorpus length	14.3 ± 0.6 (13.3-15.5)	13.2 ± 0.5 (13-13.5)	14.5 ± 0.5 (14.0-15.2)	14.3 ± 0.4 (14-15)	-	-	-	-
Metacorpus width	10.0 ± 0.5 (9.2-11.0)	9.5 ± 0.7 (9-10)	9.9 ± 0.4 (9.0-10.5)	10.0 ± 0.3 (9.5-10.6)	-	-	-	-
Metacorpus valve length	3.0 ± 0.1 (2.9-3.3)	3.3 ± 0.3 (3.1-3.5)	3.3 ± 0.3 (3.0-3.8)	3.2 ± 0.2 (3.0-3.5)	-	-	-	-
Metacorpus valve width	2.0 ± 0.2 (1.9-2.5)	2.1	2.6 ± 0.1 (2.5-2.8)	2.4 ± 0.1 (2.3-2.5)	-	-	-	-
Pharynx length	66.9 ± 4.8 (59.0-73.2)	66.9 ± 4.8 (59.0-73.2)	68.4 ± 1.4 (66.3-70.3)	63.3 ± 1.5 (61.4-66.8)	-	-	-	-

Pharyngeal overlap	85.7 ± 1.0 (69.3-103.9)	60	95.0 ± 8.7 (85-105)	69.2 ± 8.1 (60.0-84.1)	-	-	-	-
Ant. end to pharyngeal gland lobe	151.8 ± 13.9 (129.0-176.2)	122.8 ± 10.5 (115.4-130.3)	162.9 ± 7.4 (152.0-172.3)	133.1 ± 9.5 (121.4-150.4)	-	-	-	-
Anterior end to excretory pore	80.1 ± 6.8 (68-93)	68	79.4 ± 3.7 (75.0-86.1)	76.2 ± 3.0 (70.3-81.2)	-	-	-	-
Post uterine sac	44.6 ± 6.6 (32.6-56.4)	-	45.6 ± 5.7 (37.0-56.4)	-	-	-	-	-
Vulva anus distance	152.2 ± 16.9 (123.8-182.2)	-	174.0 ± 5.7 (161.3-182.2)	-	-	-	-	-
Ant. end to vulva	464.1 ± 54.4 (360.4-574.2)	-	496 ± 19 (468.3-529.6)	-	-	-	-	-
Post end to vulva	193.0 ± 21.7 (156.4-236.6)	-	214.2 ± 6.1 (201.0-223.3)	-	-	-	-	-
Tail length	39.4 ± 3.9 (33.6-46.5)	29.7 ± 1.0 (29.0-30.5)	40.2 ± 1.8 (38.0-42.6)	32.4 ± 1.1 (30.2-33.6)	-	-	-	19.2 (18-21)

Spermatheca length	38.4 ± 9.5 (33.6-46.5)	-	58.0 ± 5.4 (49.5-69.3)	-	-	-	-	-
Spermatheca width	8.1 ± 0.6 (7.0-9.5)	-	8.2 ± 1.1 (6-10)	-	-	-	-	-
Spicule length	-	18.1 ± 0.9 (17.5-18.8)	-	18.3 ± 0.7 (17.0-19.3)	-	-	-	-
Gubernaculum length	-	-	-	-	-	-	-	-
PUS/VA %	29.3 ± 3.6 (21.9-33.7)	-	26.4 ± 3.7 (20.7-31.8)	-	-	-	-	-
Lateral field width	3.8 ± 0.4 (3.5-4.5)	-	3.2 ± 0.1 (3.0-3.5)	-	-	-	-	-
Spikes	-	2	-	-	-	-	-	(2-3)
Testis	-	-	-	253.1 ± 27.8 (213.8-300.1)	-	-	-	-

Post uterine sac	6.7 ± 0.8	-	6.4 ± 0.9	-	-	-	4.9	-
length/Body length	(5.0-7.6)		(5.0-7.8)				(4.1-6.2)	

Table 4. Morphometrics of live females of a Florida population of putative *A. fujianensis* from strawberry and reared on *Monilinia fructicola* compared to those in the original description of *A. fujianensis* by Zhou et al. (2010) in China and a re-description by De Jesus et al. (2016) in Brazil. All measurements are in μm and in the form: mean ± SD (range).

Character [a]	***Monilinia fructicola*** **(N18-01001-3)**	**Pine trees** **Zhou et al. (2010)**	**Rice** **De Jesus et al. (2016)**
n	10	20	-
L	821.8 ± 61.0(698.8-893.9)	866 ± 45 (803-941)	(603-868)
a	34.3 ± 2.4(30.0-38.0)	35.2 ± 1.4 (31.5-36.3)	(30.5-38.4)
b	10.4 ± 0.8 (8.5-11.5)	10.6± 0.7 (9.8-13.0)	-
b'	4.9 ± 0.3 (4.2-5.2)	-	-
c	17.4 ± 0.7(16.1-19.0)	16.9 ± 0.9 (15.1-18.2)	(15.5-19.2)
c'	3.4 ± 0.2 (3.0-3.8)	4.0± 0.3 (3.5-4.4)	(2.9-3.7)
Max. body diameter	24.1 ± 2.4(19.3-27.2)	25.0 ± 1.5 (23-28)	-
Body diameter at anus	14 ± 1 (11.4-15.3)	-	-

V	69.6 ± 0.7(68.1-70.8)	-	-
OV	25.3 ± 3.5(17.9-32.1)	-	-
Anterior genital tract length	208.6 ± 36.0(124.9-267.3)	-	-
Lip region width	7.9 ± 0.2 (7.5-8.2)	7.0 ± 0.6 (6.0-7.5)	-
Lip region height	3.2 ± 0.2 (3.0-3.7)	2.5 ± 0.2 (2-3)	-
Stylet length	12.6 ± 0.4 (12-13)	13.0 ± 0.3 (12.5-14.0)	(12.3-12.8)
Stylet cone	5.2 ± 0.3 (4.9-5.8)	-	-
Stylet knob height	1.6 ± 0.2 (1.2-2.0)	-	-
Stylet knob width	2.5 ± 0.4 (2.1-3.0)	-	-
Metacorpus length	18.6 ± 0.7 (18.0-19.8)	14 ± 1 (12.5-16.0)	-
Metacorpus width	13.9 ± 0.8 (12-15)	17.5 ± 1.1 (16-20)	-

Metacorpus valve length	5.4 ± 0.4 (5.0-5.9)	-	-
Metacorpus valve width	4.0 ± 0.2 (3.6-4.4)	-	-
Pharynx length	79.1 ± 3.3 (73.2-84.1)	-	-
Pharyngeal overlap	88.6 ± 7.8 (80.2-107.9)	-	-
Ant. end to pharyngeal gland lobe	157.8 ± 30.1 (71.2-185.1)	73.0 ± 2.7 (64-75)	-
Anterior end to excretory pore	94.7 ± 7.4 (85.6-108.9)	-	-
Post uterine sac	44.9 ± 13.4 (28.7-74.2)	86.0 ± 11.4 (68-110)	-
Vulva anus distance	202.3 ± 17.1 (165.3-219.0)	229.0 ± 15.4 (205-250)	-
Ant. end to vulva	572.2 ± 43.0 (490.0-625.7)	-	-
Post end to vulva	249.6 ± 19.4 (208.8-269.2)	-	-
Tail length	47.3 ± 2.8 (43.5-51.4)	51.0 ± 3.0 (46-58)	(36.1-50.7)

Body width at vulva	22.7 ± 2.1 (18.8-25.7)	-	-
PUS/BWV	2.0 ± 0.5 (4-5)	-	-
Mucro	2.5 ± 0.4 (2-3)	-	-
PUS/L	5.4 ± 1.5 (3.4-8.3)	-	-
PUS/VA %	22.3 ± 6.4 (13.1-34.1)	37.6 ± 4.5 (32.1-44.4)	(31.0-34.7)
Lateral field width	4.8 ± 0.3 (4-5)	-	-

Table 2. Primer sets, sequences and polymerase chain reaction conditions used to amplify each loci in this study.

Maker	Primer		Amplification (°C-sec)[a]						Reference
	Name	Sequence (5`-3')	In. Den (°C-min)	Den	Ann	Ext	Cyc	Final Ext (°C-min)	
COI	COI-F	CCTACTATGATTGGTGGTTTTGGTAATTG	94-5	94-30	51-30	68-120	42	68-10	Kanzaki and Futai 2002
	COI-R	GTAGCAGCAGTAAAATAAGCACG							
LSU	D2A- F	ACAAGTACCGTGAGGGAAAGT	95-5	94-30	55-45	68-120	35	68-10	Ye et al. 2007; DeJesus, et al. 2016
	D3B-R	TCGGAAGGAACCAGCTACTA							
SSU[b]	1813-F	CTGCGTGAGAGGTGAAAT	94-5	94-30	45-30	68-70	5		Holterman et at. 2009
	2646-R	GCTACCTTGTTACGACTTTT							
	988_F	CTCAAAGATTAAGCCATGC		94-30	54-30	68-70	35	68-5	
	1912_R	TTTACGGTCAGAACTAGGG							
	988_F	CTCAAAGATTAAGCCATGC	95-2	95-60	55-90	68-120	40	68-5	Holterman et at. 2009
	18SR-Burs	CTACGGCTACCTTGTTACGACTTTT							Ye et al. 2007

[a]PCR conditions for amplifications: Initial denaturation (In. Den), denaturation (Den) annealing (Ann), Extension (Ext) and cycle quantity (Cyc).

[b]DNA was amplified as two partially overlapping fragments.

Table 6. Final plant top and soil from the rhizosphere population densities of a population of putative *A. fujianensis* extracted from alfalfa, gerbera, soybean and strawberry, 60 and 84 (gerbera only) days after inoculation with various inoculum levels. Fresh plant top weight of each crop at plant harvest are shown in the last column†.

Crop	Inoculum levels[c]	Nematodes/plant top[a]	Nematodes/200 g of soil	Fresh plant weight[b]
Alfalfa	0	0b	0c	12 ± 1.4a
	400	10 ± 6.5a	245 ± 85.5a	16.3 ± 2.6a
	1000	6. ± 3a	96 ± 31.6b	17.3 ± 1.7a
Alfalfa Repeated treatment	0	0b	0b	35 ± 6.3a
	400	19 ± 9a	11 ± 4.6a	42.4 ± 10a
	1000	34 ± 7.3a	6.0 ± 1.6a	40.5 ± 6.3a
Gerbera	0	0b	0b	13.7 ± 5.7a
	400	3 ±0.8a	5 ± 0.4a	19.3 ± 2.7a
	1000	4 ± 1.2a	16.6 ± 9.8a	13 ± 5.7a
Soybean	0	0b	0c	34.8 ± 1.1a
	400	15 ± 10.7a	28 ± 9.7b	39 ± 4.5a
	1000	19 ± 11.6a	139 ± 34.3a	39.4 ± 1.6a
Soybean Repeated treatment	0	0b	0b	37.1 ± 2.3a
	400	15 ± 5.7a	4 ± 2.9ab	32.4 ± 1.5a
	1000	22 ± 5.1a	5.2 ± 1.8a	32.1± 2.3a
Strawberry	0	0b	0b	55.6 ± 3a
	400	11 ± 9.3a	30 ± 11.8a	45.9 ± 7.8a
	1000	16 ± 13a	19.± 14ab	46.3 ± 11.2a

†Values are mean of 5 replicates per treatment . [a]Nematode densities are expressed as total number of nematodes extracted from the entire plant top. [b]Values of plant top weights are expressed in grams. [c]Inoculum levels sharing the same letter within a column do not differ significantly, according to nonparametric test (P=0.05)(Kruskal Wallis)

Table 5. Final plant top and soil from the rhizosphere population densities of *A. besseyi* extracted from alfalfa, gerbera, soybean and strawberry, 60 days after inoculation. Fresh plant top weight of each tested crop at plant harvest are shown in the last column†.

Crop	Inoculum levels	Nematodes/Plant top[a]	Nematodes/200 g of soil	Fresh plant weight[b]
Alfalfa	0	0	0b	5.5 ± 0.3a
	600	0	27 ± 16.9a	5.3 ± 0.5a
Gerbera	0	0b	0	23.7 ± 2.3a
	600	1205 ± 1193a	0	23.5 ± 0.9a
Soybean	0	0b	0	15.2 ± 1.3a
	600	27 ± 16.9a	0	18.5 ± 0.6a
Strawberry	0	0b	0	15.6 ± 1.5a
	600	342 ± 153.2a	0	16.2 ± 1.1a
Strawberry Repeated treatment	0	0b	0	8.6 ± 0.9a
	600	122 ± 29.3a	0	6.4 ± 0.5a

†Values are mean of 5 replicates per treatment. Column means for each crop followed by common letters are not different according to Kruskall Wallinonparametric test (P=0.05). [a]Nematode densities are expressed as total number of nematodes extracted from the entire plant top. [b]Values of plant top weights are expressed in grams. [c]Inoculum levels sharing the same letter within a column do not differ significantly, according to nonparametric test (Kruskal Wallis)

Table 1. Species and populations of Aphelenchida used in this study.

Nematode species and code	Host	Localities	GenBank Accession number			References
			SSU	LSU	COI	
A. besseyi (N18-01001-2)	*Fragaria* x *ananassa*	Florida USA	MK291493	MK294342	MK303401	This study
A. besseyi	*Oryza sativa*	Japan	KT692671	KT692690	KT782798	Jesus et al. (2016)
A. besseyi	*Phaseolus vulgaris*	Costa Rica	-	KT692694	KT782801	Jesus et al. (2016)
A. besseyi	*Oryza sativa*	Taiwan	-	HQ540537	HQ540530	Chang et al. (2010)
A. besseyi	unknown	Russia	-	DQ328684	-	Subbotin et al. (2006)
A. besseyi	*Oryza sativa*	Taiwan	-	HQ540535	HQ540527	Chang et al. (2010)
A. besseyi	*Asplenium nidus*	Taiwan	-	HQ540536	HQ540528	Chang et al. (2010)
A. besseyi	*Oryza sativa*	Taiwan	-	HQ540534	-	Chang et al. (2010)
A. besseyi	*Fragaria x ananassa*	Taiwan	-	HQ540540	HQ540532	Chang et al. (2010)
A. besseyi	*Asplenium nidus*	Taiwan	-	HQ540541	HQ540533	Chang et al. (2010)
A. besseyi	*Hosta* sp.	USA	-	EU325682	EU325686	Zhao et al. (2008)
A. besseyi	*Oryza sativa*	Taiwan	-	-	EU983281	Lin et al. (2008)
A. besseyi	*Oryza sativa*	Taiwan	-	-	HQ540531	Chang et al. (2010)
A. besseyi	*Oryza sativa*	Taiwan	-	-	HQ540526	Chang. et al. (2010)

A. besseyi	unknown	China	-	-	NC025291	Sun, et al. (2014)
A. besseyi	soil	Europe	JQ957878	-	-	Rybarczyk-Mydlowska et al. (2012)
A. besseyi	*Oryza sativa*	China	-	KX356774	KX356862	Sanchez-Monge et al. (2016)
A. besseyi	*Oryza sativa*	Italy	-	KX356775	KX356863	Sanchez-Monge et al. (2016)
A. besseyi	*Oryza sativa*	Turkey	-	KX356776	KX356864	Sanchez-Monge et al. (2016)
A. besseyi	*Phaseolus vulgaris*	Costa Rica	-	KX356755	KX356845	Sanchez-Monge et al. (2016)
A. besseyi	*Phaseolus vulgaris*	Costa Rica	-	KX356753	-	Sanchez-Monge et al. (2016)
A. besseyi	*Oryza sativa*	Costa Rica	-	KX356760	KX356848	Sanchez-Monge et al. (20160
A. besseyi	*Oryza sativa*	Costa Rica	-	KX356761	KX356849	Sanchez-Monge. et al. (2016)
A. besseyi	*Oryza sativa*	Taiwan	KT454963	-	-	Wu et al. (2015)
A. besseyi	soil	Europe	JQ957877	-	-	Rybarczyk-Mydlowska et al. (2012)
A. besseyi	*Glycine max*	Brazil	KY510835	KY510839	-	Favoreto et al. (2017)
A. besseyi	*Glycine max*	Brazil	KY510837	KY510841	-	Favoreto et al. (2017)
A. besseyi	*Glycine max*	Brazil	KY510838	KY510842	-	Favoreto et al. (2017)
A. besseyi	*Glycine max*	Brazil	KY510836	KY510840	-	Favoreto et al. (2017)
A. besseyi	*Asplenium nidus*	Taiwan	KT943534	-	-	Wu et al. (2015)
A. besseyi	*Setaria italica*	China	-	KP757370	-	Wang et al. (2015)

A. besseyi	*Setaria italica*	China	-	KP757368	-	Wang et al. (2015)
A. besseyi	*Asplenium nidus*	Taiwan	-	-	MF669521	Yu and Yang et al. (2017)
A. besseyi	*Asplenium nidus*	Taiwan	-	-	MF669520	Yu and Yang et al. (2017)
A. besseyi	*Asplenium nidus*	Taiwan	-	-	MF669522	Yu and Yang et al. (2017)
A. besseyi	*Fragaria x ananassa*	Florida, USA	AY508035	AY508109	AY508072	Ye et al. (2007)
A. besseyi	*Oryza sativa*	Japan	KT692669	KT692689	KT782796	Jesus et al. (2016)
A. besseyi	*Oryza sativa*	Spain	KT692682	KT692703	KT782810	Jesus et al. (2016)
A. besseyi	*Brachiaria brizantha*	Brazil	-	KT692704	KT782811	Jesus et al. (2016)
A. besseyi	*Oryza sativa*	Brazil	KT692675	KT692696	KT782803	Jesus et al. (2016)
A. besseyi	*Brachiaria humidicola*	Brazil	-	KT692705	-	Jesus et al. (2016)
A. besseyi	*Brachiaria ruziziensis*	Brazil	-	KT692708	-	Jesus et al. (2016)
A. besseyi	*Oryza sativa*	Taiwan	KT943536	-	-	Wu et al. (2016)
A. besseyi	*Oryza sativa*	China	GU337995	-	-	Cui et al. (2009)
A. besseyi	*Oryza sativa*	Brazil	KT692679	-	-	Jesus et al. (2016)
A. besseyi	*Asplenium nidus*	Taiwan	KT943535	-	-	Wu et al. (2015)
A. besseyi	*Asplenium nidus*	Taiwan	KT454962	-	-	Wu et al. (2015)
A. composticola	unknown	Netherlands	KJ636363	-	-	Van Megen et al. (2014)

A. fragariae	*Woodwardia fimbriata*	UK	-	KT692710	-	Jesus et al. (2016)
A. fragariae	unknown	Japan	-	-	AB067761	Kanzaki and Futai (2002)
A. fragariae	soil	USA	-	AB368540	-	Kanzaki et al. (2008)
A. fragariae	*Anemone* sp.	Netherlands	-	KX356779	KX356857	Sanchez-Monge et al. (2016)
A. fragariae	soil	Europe	-	-	-	Rybarczyk-Mydlowska et al. (2012)
A. fragariae	soil	Belgium	-	-	-	Meldal et al. (2007)
A. fragariae	unknown	Europe	AY284645	-	-	Holterman et al. (2006)
Putative *A fujianensis*(N18-01001-3)	*Fragaria x ananassa*	Florida, USA	MK291494	MK294343	MK303402	This study
A. fujianensis	*Brachiaria brizantha*	Brazil	KT692672	-	-	Jesus et al. (2016)
A. fujianensis	Unidentified grass seed	Brazil	KT692676	-	-	Jesus et al. (2016)
A. fujianensis	*Brachiaria brizantha*	Brazil	KT692680	-	-	Jesus et al. (2016)
A. fujianensis	*Oryza sativa*	Brazil	KT692678	KT692699	KT782806	Jesus et al. (2016)
A. fujianensis	*Oryza sativa*	Brazil	KT692674	KT692695	KT782802	Jesus et al. (2016)
A. fujianensis	*Brachiaria brizantha*	Brazil	KT692663	KT692683	KT782790	Jesus et al. (2016)
A. fujianensis	*Oryza sativa*	Japan	KT692668	KT692688	KT782795	Jesus et al. (2065)
A. fujianensis	*Oryza sativa*	Brazil	KT692667	KT692687	KT782794	Jesus et al. (2016)

A. fujianensis	*Brachiaria decumbens*	Brazil	KT692665	KT692685	KT782792	Jesus et al. (2016)
A. fujianensis	*Brachiaria brizantha*	Brazil	KT692677	KT692698	KT782805	Jesus et al. (2016)
A. fujianensis	*Brachiaria decumbens*	Brazil	KT692664	KT692684	KT782791	Jesus. et al. (2016)
A. fujianensis	*Oryza sativa*	Japan	KT692670	KT692691	KT782797	Jesus et al. (2016)
A. fujianensis	*Panicum maximum*	Brazil	KT692666	KT692686	KT782793	Jesus et al. (2016)
A. fujianensis	*Brachiaria decumbens*	Brazil	KT692681	KT692702	KT782809	Jesus et al. (2016)
A. fujianensis	*Brachiaria brizantha*	Brazil	KT692673	KT692693	KT782800	Jesus. et al. (2016)
A. fujianensis	*Pinus massoniana*	China	FJ520227	-	FJ520226	Zhuo,K. et al. (2010)
A. paradalianensis	Packing wood	South Korea	GU337993	-	-	Cui et al. (2009)
A. ritzemabosi	unknown	UK	-	KT692713	KT782812	Jesus et al. (2016)
A. ritzemabosi	soil	Europe	JQ957882	-	-	Rybarczyk-Mydlowska et al. (2012)
A. ritzemabosi	Ornamental	Russia	DQ901554	-	-	Chizhov et al. (2006)
A. ritzemabosi	unknown	undetermined	MK301116	-	-	Halterman et al. (2018)
A. ritzemabosi	unknown	undetermined	JQ957881	-	-	Rybarczyk-Mydlowska et al. (2012)
A. saprophilus	unknown	undetermined	FJ040408	-	-	Halterman et al. (2008)
A. subtenuis	*Crocrus* sp.	Netherlands	-	-	KX356859	Sanchez-Monge et al. (2016)
A. subtenuis	*Allium cepa*	Netherlands	-	-	KX356860	Sanchez-Monge et al. (2016)

A. subtenuis	unknown	China	-	KY695135	-	Gu and He (2017)
A. subtenuis	unknown	China	-	KY695134	-	Gu. and He (2017)
A. subtenuis	unknown	undetermined	JQ957889	-	-	Rybarczyk-Mydlowska et al. (2012)
A. varicaudatus	*Pinus kesiva*	China	-	HQ283353	-	Huang. et al. (2012)
A. xylocopae	*Xylocopa appendiculata*	Japan	-	AB434933	-	Kanzaki et al. (2008)
A. xylocopae	*Xylocopa appendiculata*	Japan	-	-	AB252222	Kanzaki (2006)
Aphelenchoides sp.	Wood packing	Japan	-	KF638651	-	Fang et al. (2013)
Aphelenchoides sp.	uknown	undetermined	GU337994	-	-	Cui et al. (2009)
Aphelenchoides sp.	Packing wood	undetermined	KF032031	-	-	Ye and Giblin-Davis (2013)
Aphelenchoides sp.	*Medicago sativa*	USA	MH844706	-	-	Wang et al. (2018)
Aphelenchoides sp.	*Medicago sativa*	USA	MH844704	-	-	Wang et al. (2018)
Aphelenchoides sp.	*Medicago sativa*	USA	MH844705	-	-	Wang et al. (2018)
Aphelenchus avenae	*Brassica napus*	Czech Republic	-	Jq348400	-	Kumari (2012)
Bursaphelenchus cocophilus	*Cocos nucifera*	Honduras	-	AY508077	-	Ye et al. (2007)
B. xylophilus	*Pinus densiflora*	Japan	-	AY508106	AY508069	Ye et al. (2007)
B. mucronatus	unknown	Japan	-	-	AB067765	Kanzaki and Futai (2002)
B. rufipennis	*Dendroctonus rufipennis*	Alaska_USA	-	AB368530	AB368527	Kanzaki et al. (2008)

Ektaphelenchus obtusus	*Dendroctonus rufipennis*	Alaska_USA	-	AB368533	AB368531	Kanzaki et al. (2008)
Laimaphelenchus heidelbergi	*Quercus suber*	Portugal	-	KJ564293	KJ564292	Maleita et al. (2014)
Peraphelenchus orientalis	*Nicrophorus quadripnctatus*	Japan	-	AB786909	-	Kanzaki et al. (2013)
Schistonchus caprifici	*Ficus carica*	Turkey	-	-	EU287639	Gulcu et al. (2008)
Sheraphelenchus sucus	*Quercus serrata*	Japan	-	AB808721	-	Kanzaki and Tanaka (2013)

Figure legends

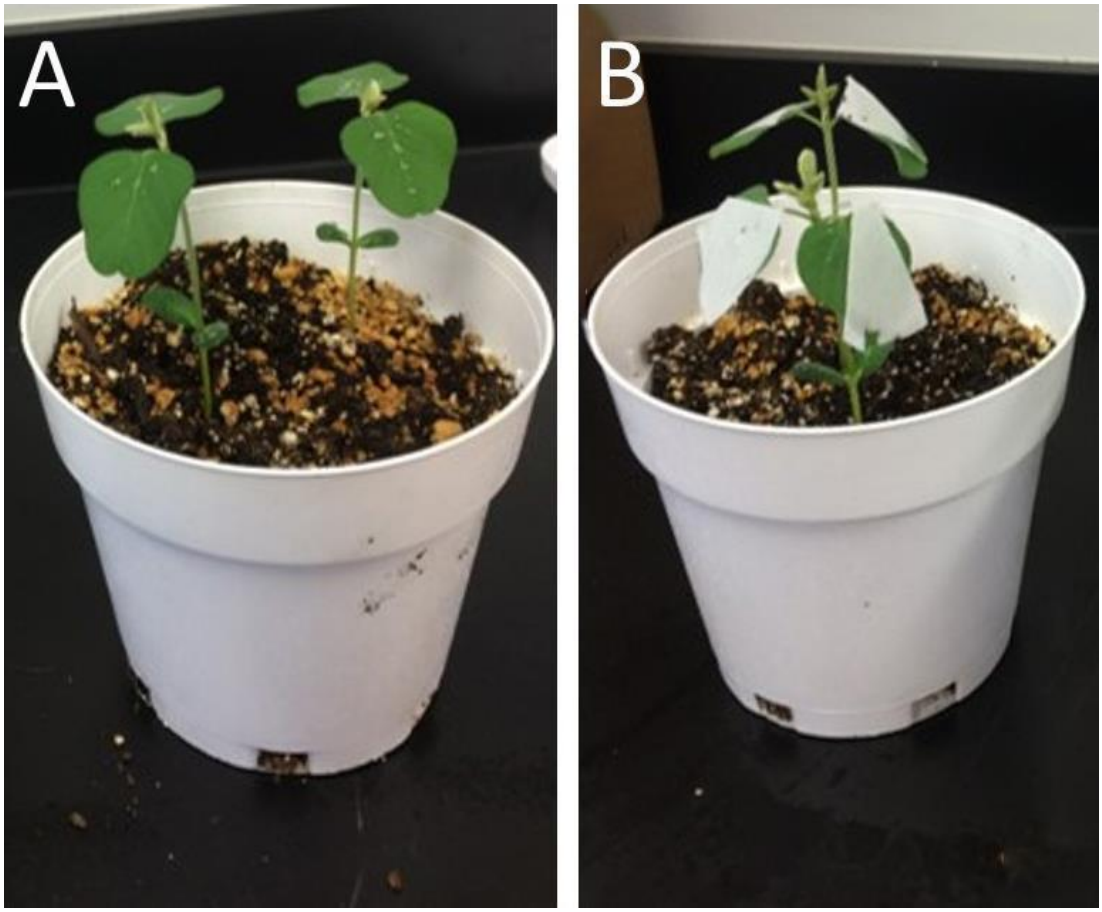

Fig. 1. Soybean seedlings inoculated with putative *Aphelenchoides fujianensis* using two techniques. A: Seedlings showing the upper surface of the leaf blade partially covered by droplets of the aqueous suspension of the nematodes delivered with a pipette; B: Seedlings showing pieces of filter paper containing the nematodes attached to the upper surface of the leaf blades.

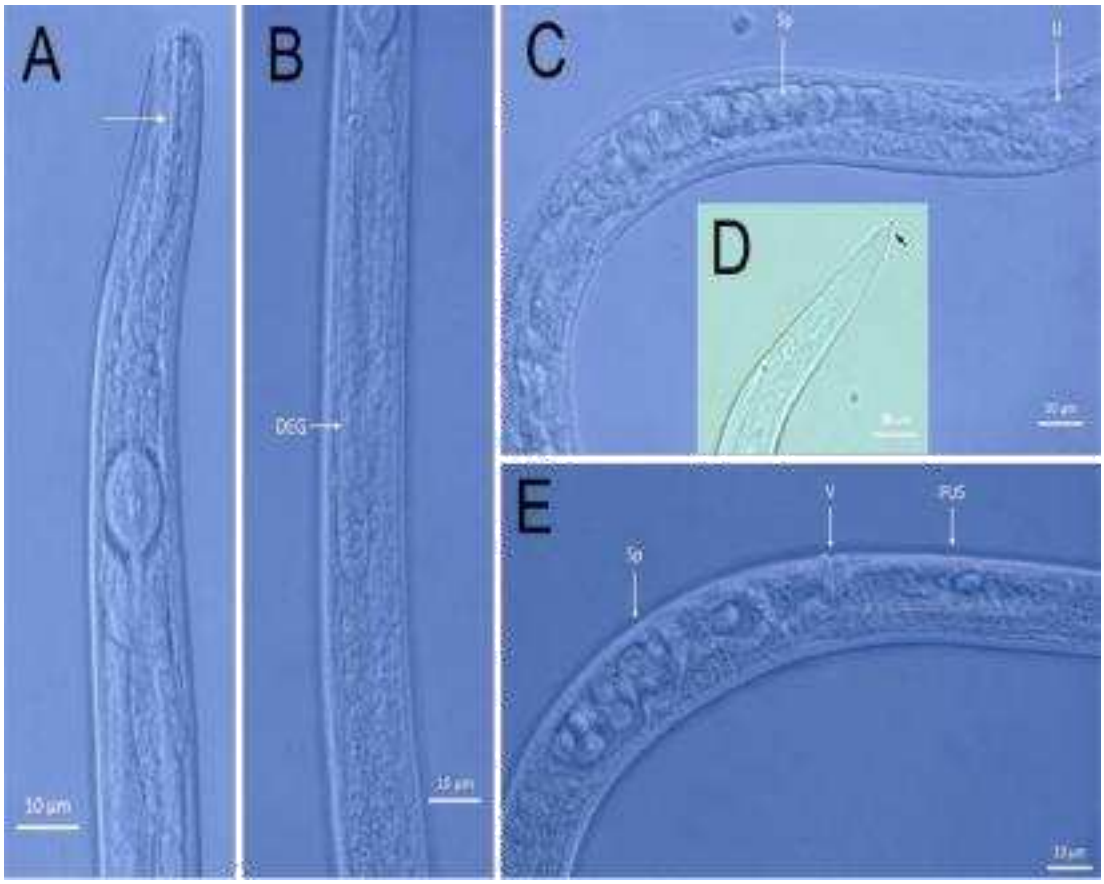

Fig. 2. Photomicrographs of putative *Aphelenchoides besseyi* female. A, B: Anterior portions of the body. Note, in A, the stylet (arrowed) and, in B, the dorsal esophageal gland (DEG) overlapping the intestine (B); C: Portion of the genital tract showing the long spermatheca filled with round sperm (Sp). Note the sperm packed in rectangular cases in the anterior portion of the spermatheca; D: Tail ending in a mucro with three processes (arrowed). E: Posterior portion of the genital tract showing an enlarged oval spermatheca (Sp) filled with round sperm, the vulva (V) and the postuterine sac (PUS) containing a few sperm.

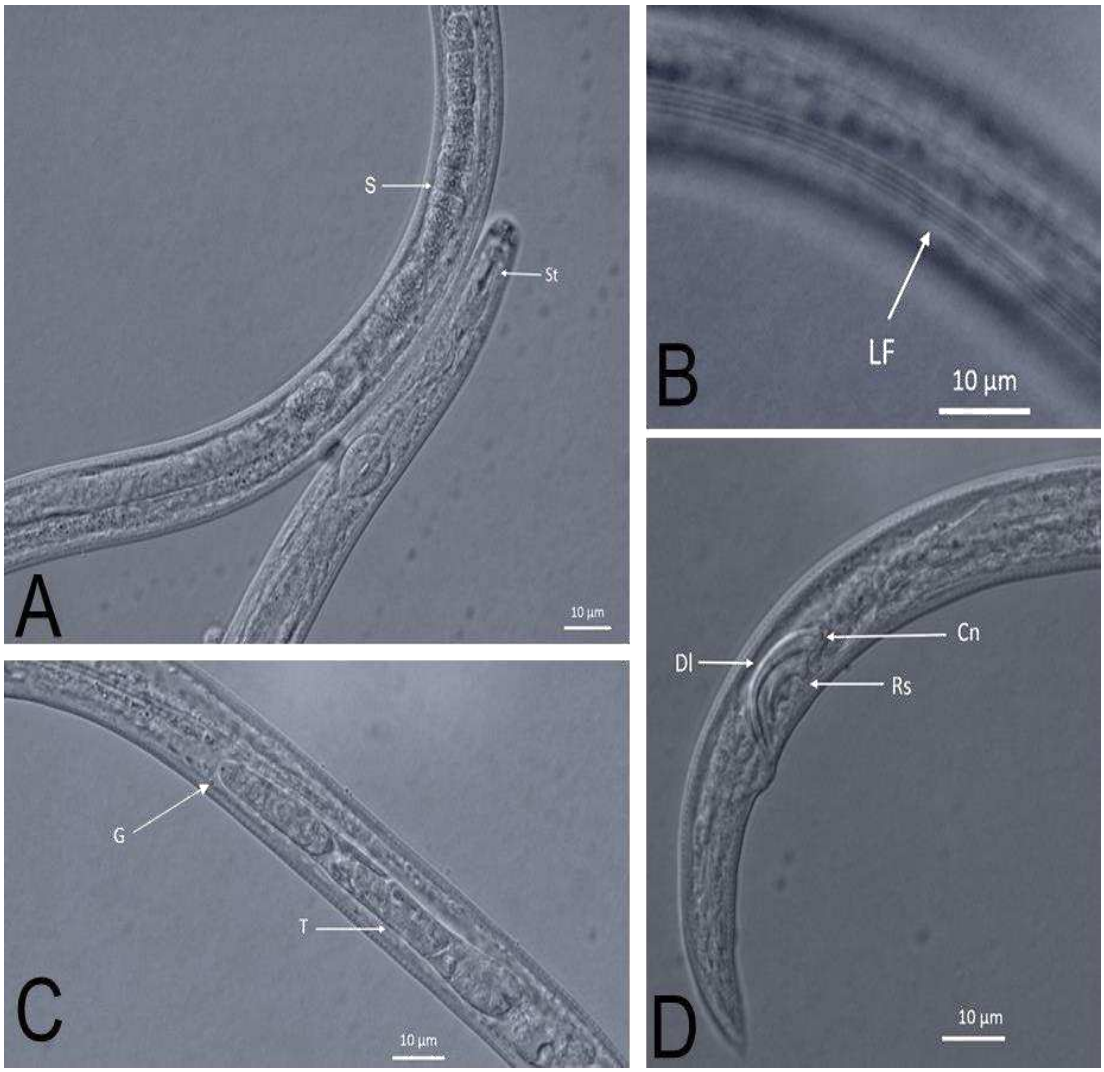

Fig. 3. Photomicrographs of putative *Aphelenchoides besseyi* male. A: Anterior and middle portions of the body showing the style (St) and the sperm packed in rectangular cases in the testis; B: Lateral field (LF) marked by four incisures; C: Anterior portion of the testis showing the germinal zone (G) at the tip; D: Posterior portion of the body showing the spicules. Their dorsal limb (DL) is well defined. The condilus (Cn) and rostrum (Rs) are not well developed.

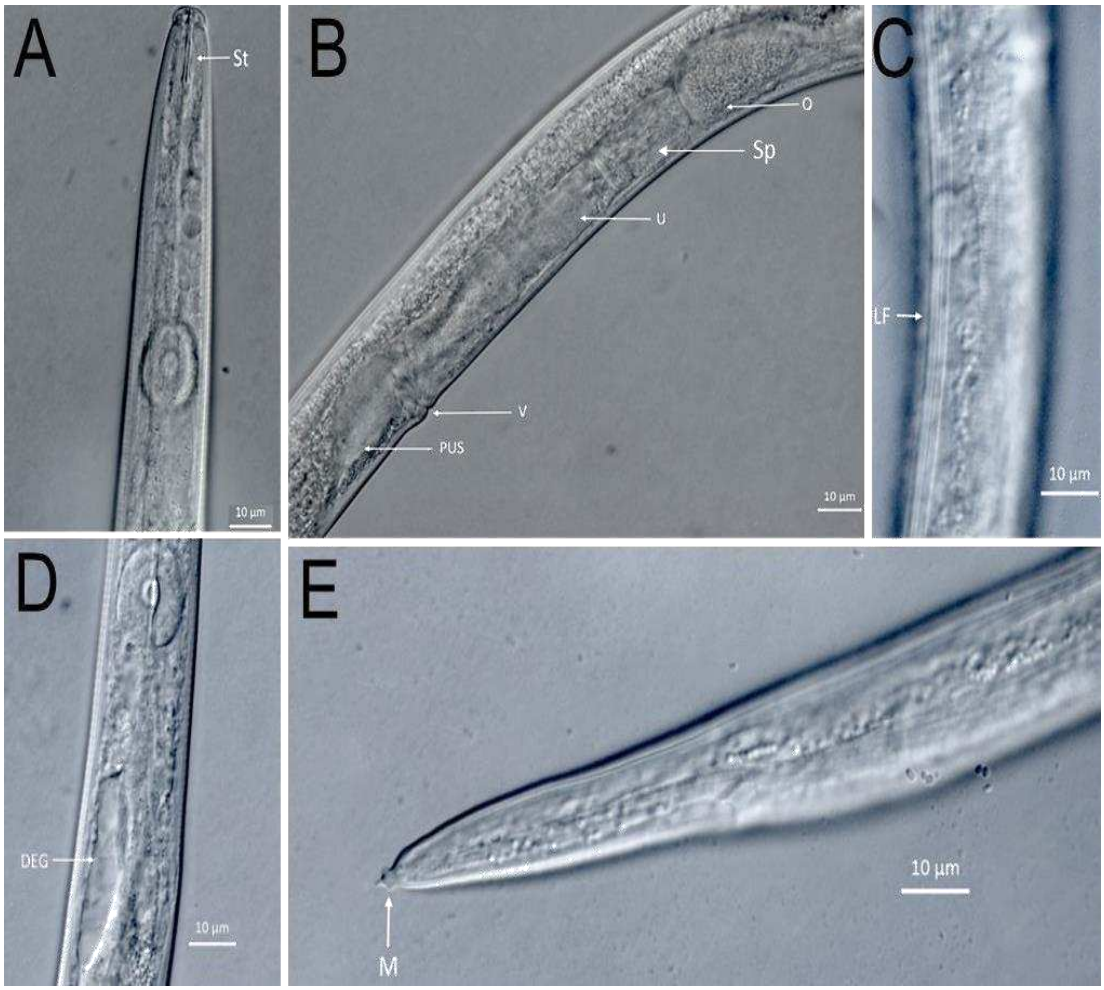

Fig. 4. Photomicrographs of putative *A. fujianensis* female. A: Anterior portion of the body showing the stylet (St); B: Posterior portion of the genital tract with a large oocyte (O) adjacent to a non-functional and rectangular spermatheca (Sp). Note the large uterus (U), the vulva (V) and the saccate postuterine branch (PUS); C: Lateral field marked by four incisures; D: Posterior portion of the body with the tail ending in a tricuspid mucro.

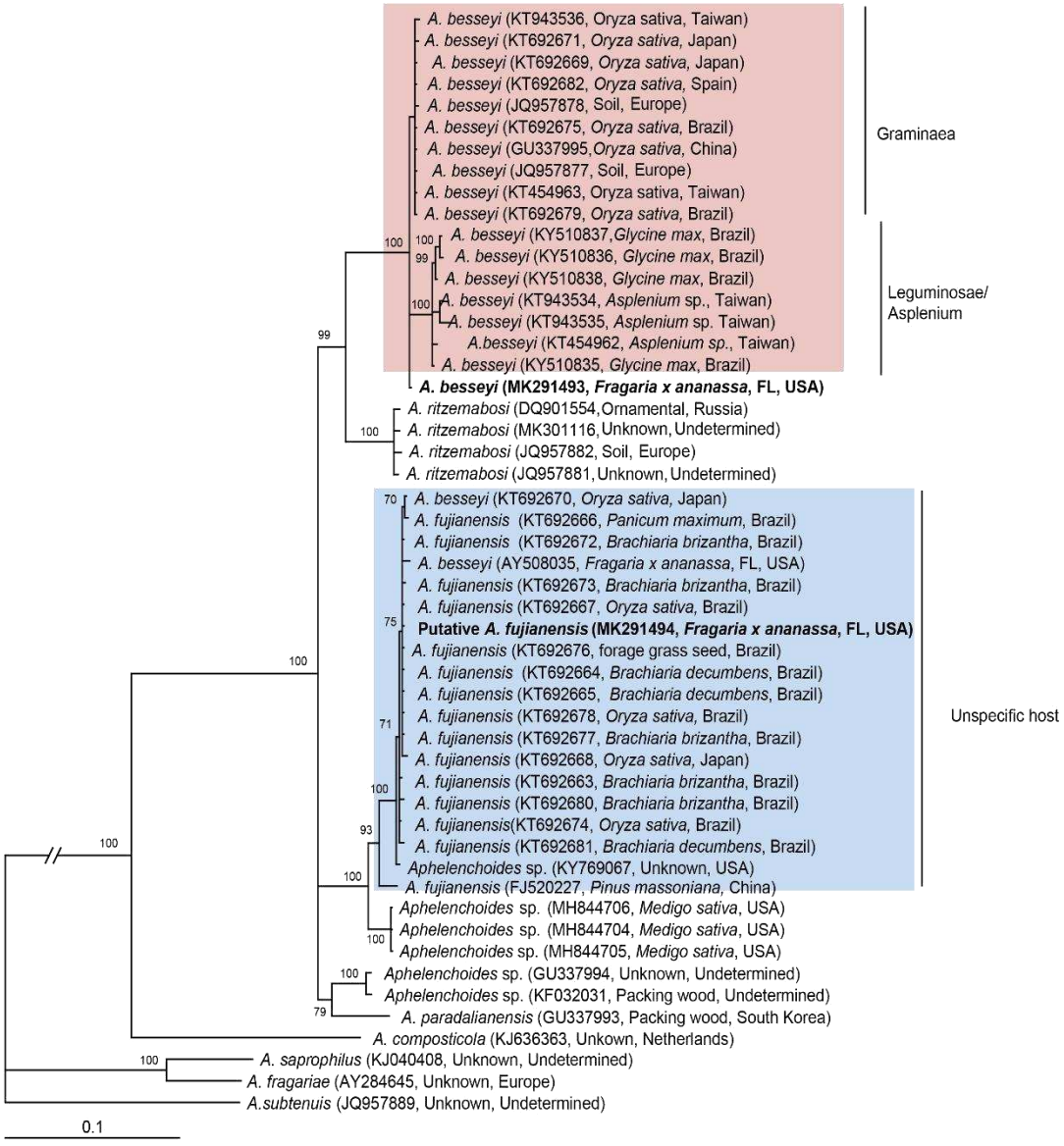

Fig 5. Phylogenetic relationships within selected species of *Aphelenchoides*: Bayesan 50% majority rule consensus tree from two runs as inferred from analysis of the near full-length small subunit ribosomal RNA (SSU) gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. Original sequences are indicated in bold font. *Aphelenchoides subtenuis* was used as an outgroup.

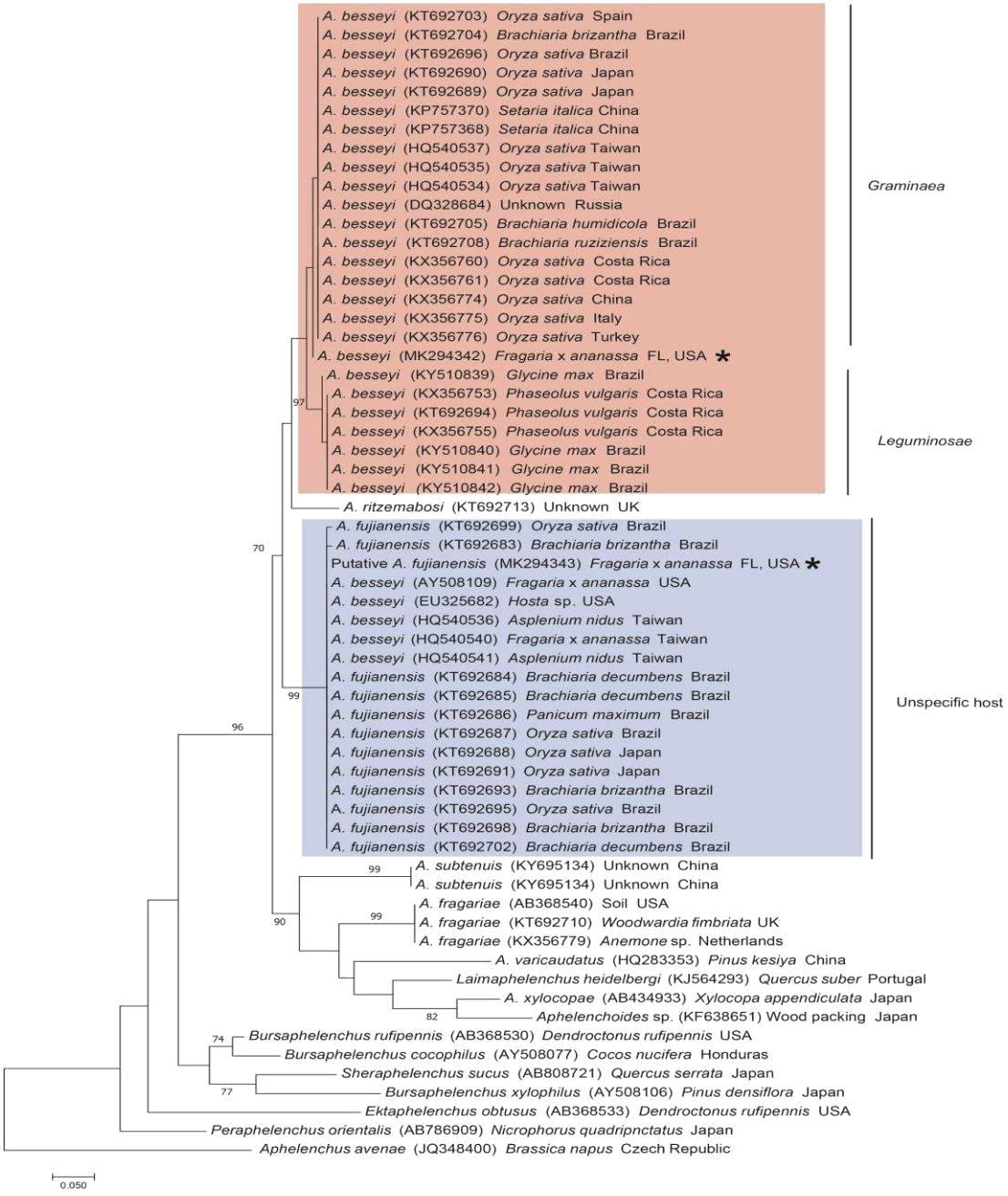

Fig. 6. Maximum likelihood tree of Aphelenchida spp. based on the alignments LSU loci. The tree Bayesian inference generated had a similar topology. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. Black vertical bars on the right delineate the clades based on host. Monilochaetes infuscans was used as outgroup. Orange box highlights the clades with populations of *A. besseyi* species complex and blue box highlights the populations of putative *A. fujianensis* and putative *A. besseyi. Aphelenchus avenae* was used as an outgroup.

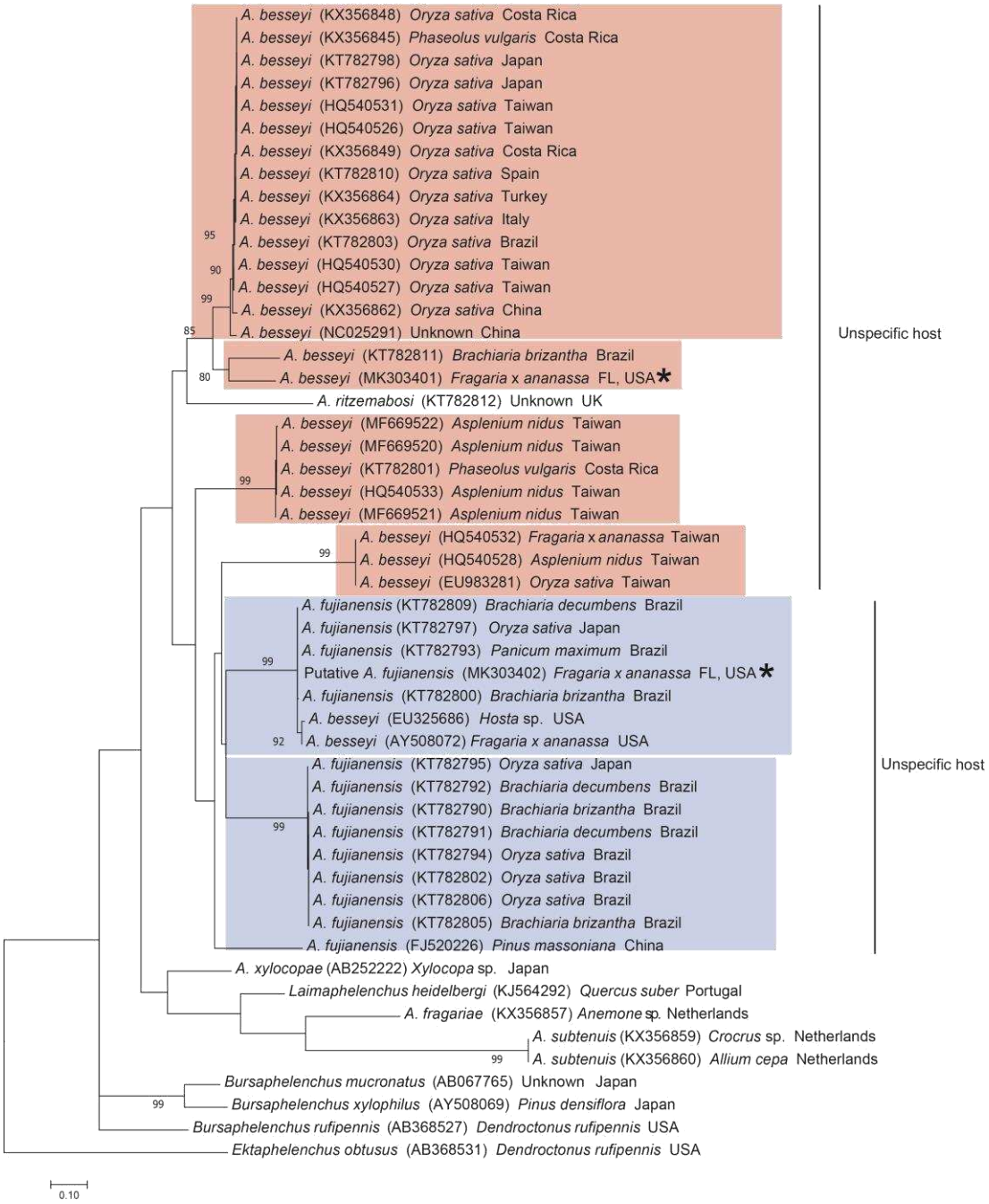

Fig. 7. Maximum likelihood tree of Aphelenchida spp. based on the alignments COI loci. The tree Bayesian inference generated had a similar topology. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. Black vertical bars on the right delineate the clades based on host. Monilochaetes infuscans was used as outgroup. Orange box highlights the species *A. besseyi* and blue box highlights the species *A. fujianensis.* The outgroup was *Ektaphelenchus obtusus.*

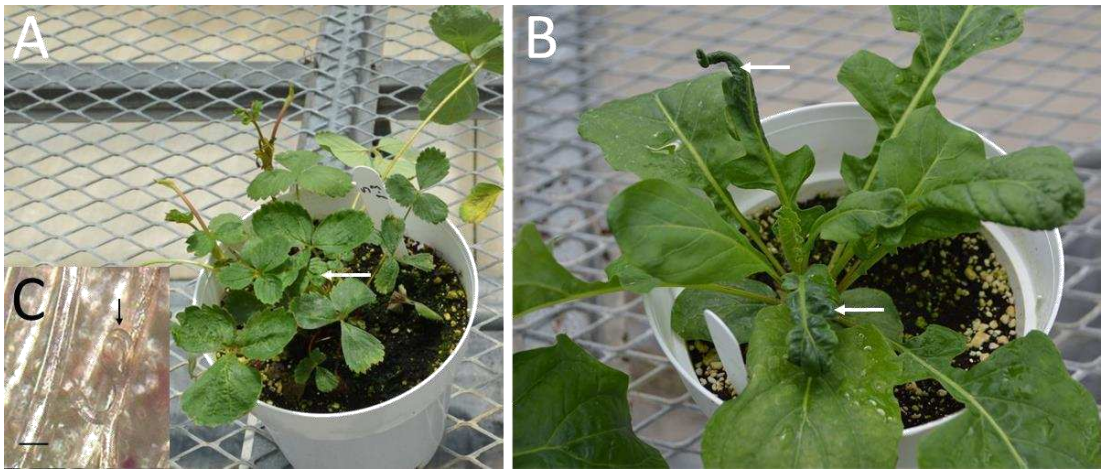

Fig. 8. Symptoms induced by putative *Aphelenchoides bessyei*'s infestations. A: Plantlet of strawberry cv. Florida Radiance showing crinkled and distorted leaves (arrowed) 60 days after the inoculation with 600 nematodes; B: Small plant of gerbera daisy showing distorted, deformed and dwarfish leaves (arrowed) 60 days after inoculation with 600 nematodes; C: A coiled nematode specimens on the surface of the blade of a strawberry leaflet where it feeds ectoparasitically. Scale bar = 30 μm.

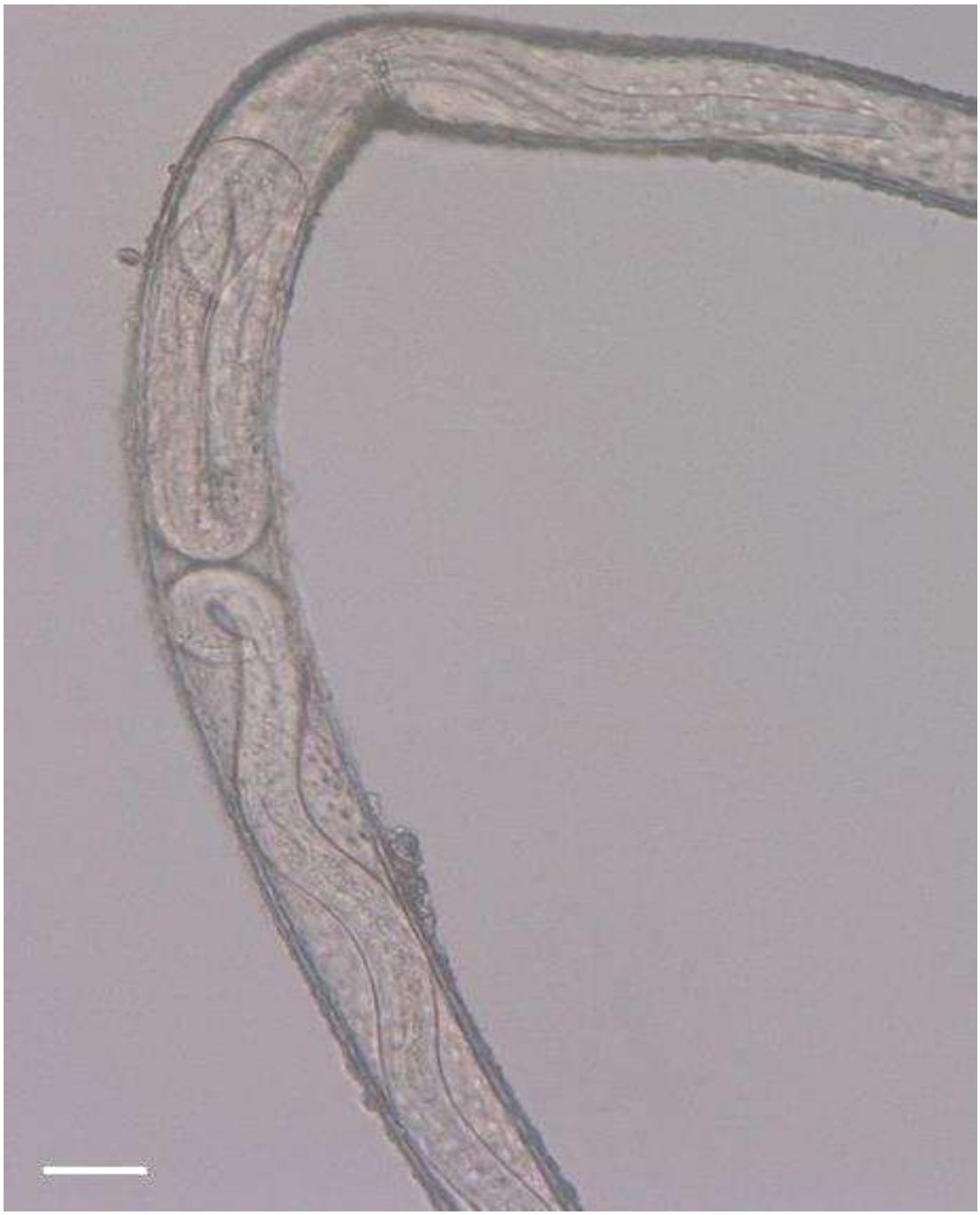

Fig. 9. Evidence of phytoparasitic habits of putative *A. fujianensis* in soybean. Nematode specimens inside a trichome of the stem of a soybean seedling 60 days after the inoculation of 1000 nematodes in an aqueous suspension delivered in droplets on the surface and petiole of its leaves. (Scale bar = 39 μm).

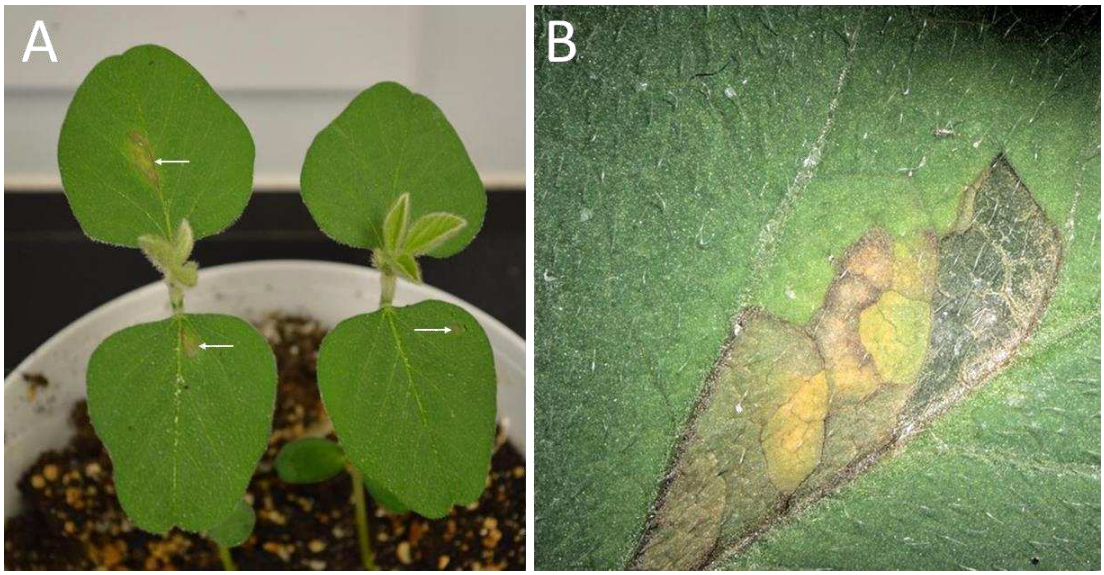

Fig. 10. Evidence of phytoparasitism induced by putative *A. fujianensis* in soybean seedlings. A: Seedlings with discolored spots (arrowed) on the upper surface of the blade 9 days after inoculation of the nematode that was applied with pieces of filter paper attached to the leaf blade. B: A magnified discolored section between the veins of the leaflet showing chlorotic and desiccated areas and dark tissues along the veins. Phytoparasitic habits of putative *A. fujianensis* in soybean.

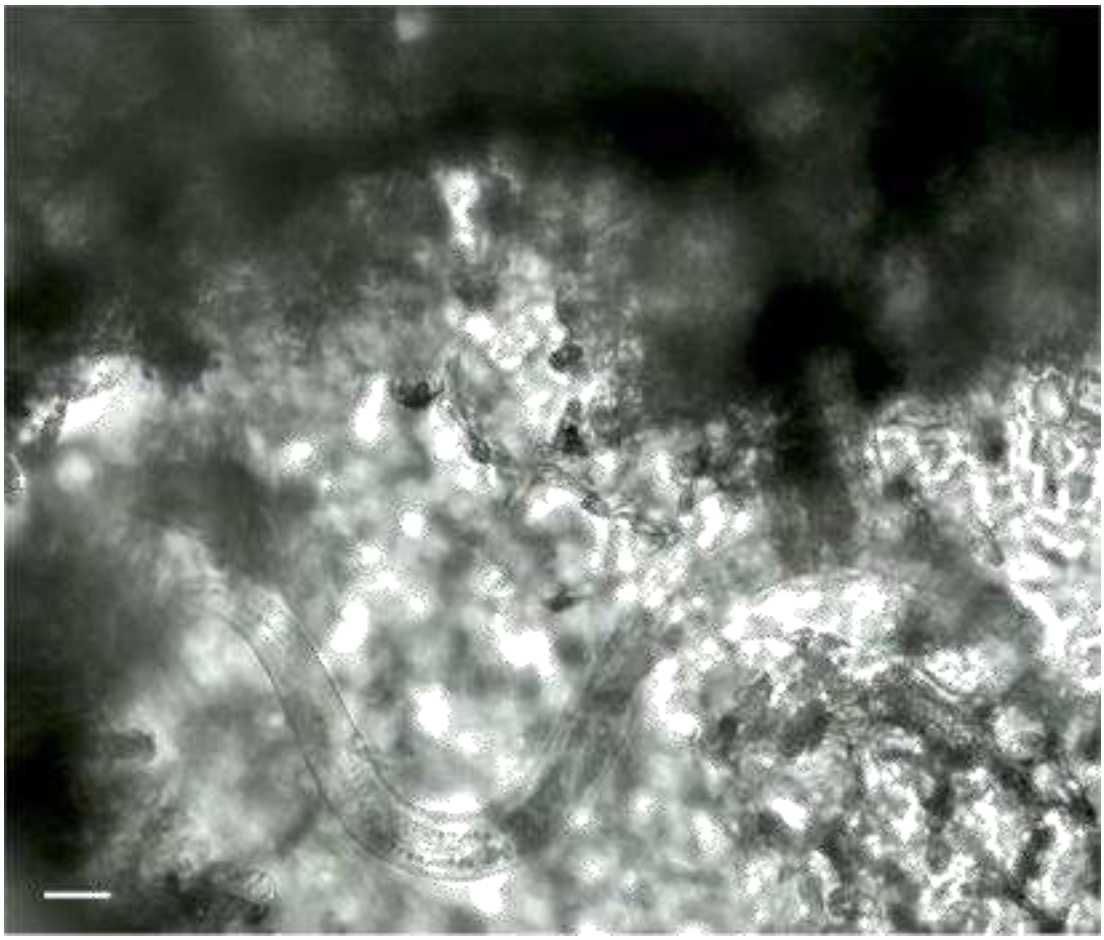

Fig. 11. A dissected symptomatic area of a soybean leaf showing a nematode specimen tunneling the mesophyll 24 days after the inoculation of 300 specimens delivered with a piece of filter paper attached to the leaf blade. (Scale bar = 24 μm).

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