Aphelenchoides bicaudatus from Ornamental Nurseries in Taiwan and Its Relationship with Some Agricultural Crops

Fan-Yu Jen, Tung-Tsuan Tsay, and Peichen Chen, Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan, 40223

Abstract

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Aphelenchoides bicaudatus nematodes were identified in planting materials and water samples from 35 floral ornamental nurseries in Taiwan. A pure line of the nematode was established from the bulk samples collected, and the nematode was identified as *A. bicaudatus* on the basis of morphometric data and the 18S gene sequence (GU984233). Host range tests were conducted by inoculating *A. bicaudatus* onto *Phalaenopsis* spp. strawberry, rice, bird's-nest fern, cyclamen, dendrobium, African violet, and saxifrage (all reported hosts

of *A. besseyi*). Symptoms did not develop during the 4 weeks following inoculation. However, after staining, both eggs and nematodes inside plant tissue of *Phalaenopsis* roots, strawberry leaf buds, and rice sheath indicated that *A. bicaudatus* had established in these hosts. These results revealed a potential new host-parasite relationship of *A. bicaudatus*, and suggest that this nematode may infect these economically important hosts.

The *Aphelenchoides* genus is divided into plant-parasitic, mycophagous and predatory groups of nematodes, according to their type of feeding (5). The habitat of *Aphelenchoides* spp. includes fresh water, soil, insects, plants, and marine water (4), and species are distributed worldwide. In spite of the number of species in the genus, only a few economically important species have been described in detail (9). Within the plant-parasitic group, four species are well studied and recognized: *Aphelenchoides fragariae* Christie 1932, *A. rizemabosi* Steiner and Buhner 1932, *A. besseyi* Christie 1942, and *A. arachidis* Bos 1977. Three of these have very wide plant host ranges: *A. fragariae* colonizes hosts in 47 families and more than 250 species, *A. rizemabosi* colonizes plants in 40 families and nearly 200 species, and *A. besseyi* colonizes more than 200 plant species (14).

The most common and economically important *Aphelenchoides* spp. in Taiwan is *A. besseyi* (15,16,18,21). *A. besseyi* parasitizes rice (*Oryza sativa* L.), strawberry (*Fragaria glandigloria* Ehrh.), bird's nest fern (*Asplenium nidus* L.), and many other ornamental plants (17,18,21). A rice field infested with the nematode incurred yield losses of 35 to 45% (15). In Taiwan, *Aphelenchoides besseyi* is a common pest of the bird's-nest fern, a cultivated perennial vegetable crop (21). Other *Aphelenchoides* spp. reported in Taiwan include *A. composticola* on the button mushroom (*Agaricus bisporus* (Lange) Imbach) and an unidentified species found on *Hydrilla verticillata* (L. f.) Royle (20).

Aphelenchoides spp. are also commonly found in the ornamental plants and nurseries, causing significant plant damage (6,8). *Aphelenchoides fragariae* is able to overwinter in soil, dry infected leaves, and dormant plant crowns (6). In order to establish a standard procedure for producing healthy seedlings, 48 floral ornamen-

tal plant nurseries in Taiwan were surveyed for *Aphelenchoides* spp. in 2007. The objectives of this study were to (i) investigate the prevalence of soil-inhabiting *Aphelenchoides* spp. in floral plant nurseries, (ii) identify species of *Aphelenchoides* found in nurseries based on their morphological characteristics and 18S rDNA sequences, and (iii) evaluate the disease potential of the predominant nematode species on several economically important crops.

Materials and Methods

Nematode samples from nurseries. A total of 48 nurseries was surveyed: 17, 30, and 1 in central, southern, and eastern Taiwan, respectively. From each nursery, 500 g of planting medium from 10 to 12 pots was collected. The planting medium, herein referred to as "used" planting medium, was mixed well in a bucket. One liter of irrigation water was also collected from each nursery. Nematodes were isolated from (i) three 100-g subsamples of used potting medium, (ii) two 100-g subsamples of potting medium that had not been used, and (iii) all irrigation water samples using a modified Baermann funnel nematode-extraction method (19). Each sample was slowly poured through a double layer of Kleenex tissue placed over a mesh on top of a funnel containing water, and left for 24 h to allow nematodes to descend into a vial attached to the bottom of the funnel. Samples were observed under a dissection microscope, and those with *Aphelenchoides* nematodes were recorded.

Establishing a pure-line nematode. Because the number of nematodes in each sample was very small, all *Aphelenchoides* nematodes in samples obtained from the same region were pooled and transferred onto slant cultures of *Alternaria citri* on potato dextrose agar to increase their population for further identification, as previously described (18). After 2 weeks, the increased population was collected and observed through a dissection microscope. Based on body size and the position of the vulva, a single female was picked to establish a pure-culture line. The morphological data and the rDNA information included here were from one of the pure lines established.

Morphological identification. The female nematodes were heat-killed and fixed in 2% triethanolamine (TAF) (7). Specimens were photographed under $\times 40$, $\times 200$, $\times 400$, and $\times 1,000$ magnification, and the photographs were calibrated and measured using SPOT software (Diagnostic Instruments). Fifty female nematodes were measured according to de Man's formula, and the data are presented as mean $\pm$ standard deviation. Fine structures were photographed using a cryo-field emission scanning electron micro-

Corresponding author: P. Chen, E-mail: janetchen@nchu.edu.tw

Current address of F.-Y. Jen: Taiwan Nihon Nohyaku Co., LTD. Room 902, No. 22, Nanking W. Rd. Taipei, Taiwan, 10352.

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scope (SEM; JEOL6330 Cryo-FESEM), including the lateral incisures and the tail morphology (20). In preparation for the SEM, nematodes were washed from the culture slants and the volume of water was reduced. The nematode counts were adjusted to approximately 350 to 100 μ l. Delicate Task Wipers (Kimberly-Clark) were cut into 5 mm² and two sheets were stacked together. The nematode suspension (20 μ l) was dripped onto the stacked sheets and the wiper was transferred to the metal carrier of the SEM, adhered to the carrier by double-sided tape. The sample was fixed in liquid N₂ for 90 s and sent into the vacuum chamber for observation. The images obtained were saved with 1,280 by 1,240 pixel resolution as a BMP file.

18S rDNA sequences. Nematodes from the culture slants were collected via the modified Baermann's funnel method to eliminate most fungal hyphae and spores (20). Approximately 1,500 nematodes were placed into an Eppendorf tube, and the nematode DNA was extracted using the Tissue & Cell Genomic DNA Purification Kit (GeneMark), following the procedures provided by the manufacturer. The 18S rDNA region was amplified using the Nem_18S_F (5'-CGCGAATRGCTCATTACAACAGC-3') and Nem_18S_R (5'-GGGCGGTATCTGATCGCC-3') primers (4), and the polymerase chain reaction (PCR) mix was completed with 0.5 U of Taq polymerase (GeneMark), and 2.5 μ l of 10 $\times$ Taq buffer in a 20 μ l of reaction mixture (3). The PCR conditions were 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and then 72°C for 1 min, and the reaction ended with a final extension at 72°C for 5 min. An aliquot of 2 μ l of the PCR products were used for ligation using the pOSI-T PCR Cloning Kit (GeneMark). The successfully transformed bacteria clones were sent to Mission Biotech Company for sequencing. A consensus sequence of the 18S gene was compared with the 18S rDNA sequences from other

Aphelenchoides spp. in National Center for Biotechnology Information (NCBI), including *Aphelenchoides saprophilus* (FJ040408), *A. xylocopae* (AB252221), *A. ritzmabosi* (DQ901554), *A. stammeri* (AB368535), *A. blastophtorus* (AY284644), *A. fragariae* (AJ966475), and *A. bicaudatus* (AY284643). The sequences were paired using DNASTAR software (DNASTAR Inc.) and the percentages of similarities were obtained.

Potential hosts. Economically important plants and crops used in a host range assay included strawberry (*F. glandigloria* Ehrh.), bird's nest fern (*Asplenium nidus* L.), rice (*O. sativa* L. 'Tainung No. 67'), dendrobium (*Dendrobium miyakei* Schltr.), cyclamen (*Cyclamen persicum* Mill.), saxifraga (*Saxifraga stolonifera* Meer b.), and African violet (*Saintpaulia ionantha* H.). A phalaenopsis orchid (*Phalaenopsis Sogo Yukidian* 'V3') was also included in this test, because these plants are often raised in moss, which was also sampled from several nurseries in this study. Nematodes for inoculation were propagated on fungal culture slants as previously described (1). Twenty-five rice seeds were soaked in the distilled water with approximately 1,000 *Aphelenchoides bicaudatus* nematodes until germination and transferred to pots. The nematode suspension was adjusted to 1,000 nematodes per 300 to 500 μ l for all inoculations. All the available buds on five plants of strawberry or saxifraga were inoculated by dripping the nematode suspension into the buds. For bird's nest fern, dendrobium, cyclamen, and African violet, three leaves per plant were randomly chosen, and the nematode suspension was injected into each leaf. A piece of cotton was used to cover each injection site to retain moisture. Nematodes were inoculated into wounds created by a needle puncture in leaves, buds, or roots of on each of 15 *Phalaenopsis* orchids (7). Tap water included as the control was mock inoculated in the same manner. All the inoculated plants were observed for symptom

Table 1. Survey results of *Aphelenchoides* spp. from irrigation water, planting medium, and used planting medium in Taiwan

Region in Taiwan	Number of nurseries with <i>Aphelenchoides</i> spp. per nurseries surveyed in the region		
	Irrigation water	Planting medium	Used planting medium
Central	1/17	1/17	13/17
Southern	0/30	2/30	21/30
Eastern	0/1	0/1	1/1

Fig. 1. Photomicrographs of female *Aphelenchoides bicaudatus*. **A**, Fixed specimen and **B**, anterior region; S indicates stylet and Mb indicates middle bulb. **C**, Posterior region; PUS = post-uterine sac. **D**, Tail, with typical bifurcation.

development for 4 weeks, and nematodes from at least one of the replicates in each treatment were reisolated by immersing the inoculation sites in water. The remaining replicates were stained with phenol-cotton blue following the method of Tsay et al. (17). The strawberry, saxifrage, and Phalaenopsis orchid buds were placed in the refrigerator for 1 min before processing for staining. The inoculated plant tissue was placed in the staining solution and heated in a microwave oven (National, NE-305A) for 30 s at full power, cooled down for 1 min, and heated again for 30 s in the microwave oven under the same conditions. To destain the background, the samples were transferred to glycerol and heated for 30 s before the examination. The host range trials were repeated twice.

Results

In the 48 nurseries surveyed, no plants exhibited typical symptoms of infection by foliar nematodes. One nursery located in the central region had *Aphelenchoides* spp. in the irrigation water samples, and three were found to contain *Aphelenchoides* spp. in planting medium samples (Table 1). Used planting media collected from 35 nurseries were infested with *Aphelenchoides* spp. (Table 1).

Morphometric data. No male individuals were observed in the pure line cultures. The female of *A. bicaudatus* is long and slender (Fig. 1A), the head is distinctly set off from the body, appearing smooth, while the lip region is slightly off-set, and the stylet is weak, with stylet knobs (Fig. 1B). The metacarpus is wider than 75% of the body width (Fig. 1B). The female is prodelphic and the ovary is arranged in a single row; vulva is a transverse slit, two-fifths of the body width long and located approximately two-thirds of the body length from the anterior end; the position of vulva from the anterior end, expressed as percentage of body length V%, was 68.53 ± 1.2 with an obvious post-uterine sac (Fig. 1C). The tail tapers gradually beyond the anus, and terminates in a bifurcate tip, of which the ventral prong is acute and always longer than the dorsal prong (Fig. 1D). Under SEM observation, the body has three lateral incisures (Fig. 2).

Fifty females were measured for de Man's formula. Morphometric data, including linear measurements, calculated ratios, and percentage values, were in the range of the paratype of *A. bicaudatus* published by Siddiqui and Taylor (12) (Table 2). Other morphometric data that are important traits for the *Aphelenchoides* genus are also included, such as the length of the post-uterine sac (18.98 ± 4.54), presented as the percentage of the length from vulva to anus.

rDNA sequences of 18S and internal transcribed spacer regions. A consensus 891 bp of the 18S sequence was obtained and submitted to NCBI (GU984233). A BLAST search of the NCBI database exhibited a 99% similarity with the sequence AY284643, which belonged to *A. bicaudatus*. The 18S sequence in our study

Fig. 2. Three lateral lines of *Aphelenchoides bicaudatus* observed under cryo-field emission scanning electron microscope ($\times 3,000$).

only had an 81 to 88% similarity with the other five *Aphelenchoides* spp. sequences deposited in NCBI. The results confirmed the morphometric data that the specific nematode population obtained from the field samples was *A. bicaudatus* (Table 3).

Potential hosts. Of the eight hosts inoculated with *A. bicaudatus*, three were apparent hosts of the nematode. After the sheaths of inoculated rice plants were carefully dissected in distilled water, *A. bicaudatus* was observed inside the rice tissue and the nematode was recovered in both trials. Inoculated strawberry plants grew normally but nematodes were reisolated after 4 weeks from 3 of 10 replicates, and were detected inside the buds by staining. The leaves, buds, and roots of Phalaenopsis orchids were inoculated but only the inoculation sites on the roots harbored *A. bicaudatus*. Staining also revealed eggs inside the orchid root tissue. No nematodes were reisolated from or observed in bird's nest fern, cyclamen, dendrobium, Phalaenopsis leaves and buds, African violet, and saxifrage.

Discussion

A. fragariae, *A. ritzemabosi*, and *A. besseyi* are often reported in ornamental nurseries, and usually cause symptoms in the aerial parts of plants (8). In the current survey, there were no symptoms of infection by foliar nematodes but *A. bicaudatus* was not uncommon. This is the first report of growth and survival of *A. bicaudatus* inside both rice and Phalaenopsis root tissue, and possibly on strawberry buds. The eggs found inside the Phalaenopsis root tissue suggested that *A. bicaudatus* was able to colonize and reproduce inside Phalaenopsis roots, although the infection was nonsymptomatic. Many *Aphelenchoides* spp. are on quarantine lists, and millions of dollars of revenue are lost due to returned

Table 2. Female morphometrics of 50 *Aphelenchoides bicaudatus* in this study and *A. bicaudatus* females recorded in 1967

Variable ^z	<i>A. bicaudatus</i> morphometrics (mean $\pm$ standard deviation and range)	
	This study	Siddiqui and Taylor (12)
n	50	50
L (μ m)	499.12 ± 67.95 (376–637)	460 (410–550)
K (μ m)	15.24 ± 2.69 (11–22)	...
a	33.03 ± 2.42 (27.00–38.64)	28 (25–31)
b	9.0 ± 0.7 (7.5–10.0)	8.2 (7.3–9.6)
b'	5.13 ± 0.76 (3.61–7.94)	...
c	11.94 ± 0.93 (10.16–14.80)	11.4 (9.8–13.7)
c'	5.41 ± 0.56 (4.13–7.14)	...
V (%)	68.53 ± 1.20 (64.90–71.83)	67.5 (65–70)
Stylet (μ m)	10.38 ± 0.63 (9–12)	11.2 (10–12)
PUS/V-A (%)	18.98 ± 4.54 (9.23–33.80)	...

^z Symbols: n = number of nematodes observed, L = total body length, K = maximum body width, a = total body length divided by maximum body width, b = total body length divided by esophageal length, b' = total body length divided by distance from anterior end of body to posterior end of esophageal glands, c = total body length divided by tail length, c' = tail length divided tail width, V(%) = position of vulva from anterior end expressed as percentage of body length, Stylet = length of stylet, and PUS/V-A(%) = length of post-uterine sac expressed as percentage of length from vulva to anus.

Table 3. Similarities of the 890-bp 18S rDNA sequence from *Aphelenchoides bicaudatus* isolated in this study compared with six *Aphelenchoides* spp. sequences obtained from National Center for Biotechnology Information

Nematode species	Accession number	Coverage	Similarities (%)	Gap (%)
<i>A. bicaudatus</i>	AY284643	883/890	99	0
<i>A. fragariae</i>	AJ966475	794/897	88	0
<i>A. blastophorus</i>	AY284644	787/901	87	1
<i>A. saprophilus</i>	FJ040408	785/904	86	2
<i>A. ritzemabosi</i>	DQ901554	746/919	81	4
<i>A. besseyi</i>	AY508035	747/919	81	3

shipments of nematode-infected plants (6). Further studies need to be done to clarify the etiology of *A. bicaudatus* on the Phalaenopsis orchid. *A. bicaudatus* has been found mostly in soil associated with corn, cotton, potato, sugarcane, rice, and tomato, and is considered primarily a mycophagous nematode (11,12). The nematode is known to reproduce on a number of fungi, algae, yeasts, and Lucerne sprouts (11,13), and has not been reported to parasitize on any economically important hosts.

In the survey, *Aphelenchoides* spp. were commonly found in used planting media collected from nurseries and, surprisingly, from three batches of planting media that had not been used. It is still not known how these nematodes were introduced into the nurseries. The superfamily Aphelenchoidea has adapted to a wide range of ecological relationships including phytoparasitism, predation, and fungus feeding (2), and many members are able to survive in low-moisture conditions for extensive periods of time (10). The majority of planting media in this survey were moss. Nurseries in Taiwan usually treat the dry commercial moss in a warm water bath for disinfection before use. The treatment apparently is not effective, probably because, when treating large quantities of material, the center of the mass may not reach the desired temperature. Because *A. bicaudatus* found in this survey might survive in rice, strawberry, and Phalaenopsis orchids, alternative methods of eliminating nematodes in the planting medium are currently under investigation.

When resampling the nursery in which *Aphelenchoides* nematodes were found in the irrigation water, the predominant nematode was also *A. bicaudatus*. Ground water is widely used for agricultural irrigation in areas where rainfall is abundant but is not evenly distributed. Therefore, ground water is often stored in uncovered reservoirs without further treatment. To avoid introducing nematodes, simple filtration may remove unwanted nematodes and their eggs.

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