Research

Detection of Nematophagous Fungi from *Heterodera schachtii* Females Using a Baiting Experiment with Soils Cropped to *Brassica* Species from California's Central Coast

Ying-Yu Chen¹ | Steven T. Koike² | Geoffrey D. Logan³ | Christopher Drozd³ | Juliana De Oliveira Silva¹ | Nicole B. Colindres³ | Beth B. Peacock³ | Jennifer Smith Becker¹ | Angelo Loffredo¹ | Haiyan Wu⁴ | Paul M. Ruegger³ | J. Ole Becker^{1,†} | James Borneman^{3,†} |

¹ Department of Nematology, University of California, Riverside, CA 92521, U.S.A.

² TriCal Diagnostics, Gilroy, CA 95020, U.S.A.

³ Department of Microbiology and Plant Pathology, University of California, Riverside, CA 92521, U.S.A.

⁴ Agricultural College of Guangxi University, Nanning 530004, China

[†] Corresponding authors: J. O. Becker; obecker@ucr.edu and J. Borneman; borneman@ucr.edu

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Abstract

Until the early 1990s, cyst nematodes were abundant pathogens in fields where hosts of *Heterodera* spp. were frequent members of crop rotations along California's Central Coast. To mitigate damage caused by *Heterodera schachtii* and *H. cruciferae*, the soil fumigant 1,3-dichloropropene (1,3-D) was used by more than 43% of surveyed broccoli growers. Over the last few decades, use of 1,3-D and other nematicides has dramatically diminished, suggesting a decline in nematode disease pressure. The goal of this study was to examine the hypothesis that increased population densities of nematophagous fungi contribute to the low populations of *Heterodera* spp. in fields frequently cropped to their hosts. In 2016, soil samples were collected from 152 *Brassica* fields with a broad geographical distribution, from Santa Barbara County to Santa Cruz County. The average number of *Heterodera* cysts per 250 cm³ of soil ranged from 0.5 to 27.5, with 62% of the soils harboring no detectable cyst nematodes and only a few samples reaching a potentially damaging threshold level. A baiting experiment with *H. schachtii* and cabbage was performed in a greenhouse to detect nematophagous fungi associated with nematode females as their posterior end emerged and became exposed to the soil's rhizosphere. An Illumina-based sequence analysis of these *H. schachtii* females identified several known nematophagous fungi, including members of the *Hyalorbilia oviparasitica* clade, *Pochonia chlamydosporia*, certain *Fusarium* spp., and others. These soils clearly harbor a diverse population of hyperparasitic fungi that could be biologically suppressing cyst nematodes below a damaging threshold.

Keywords: *Hyalorbilia oviparasitica*, *Dactylella oviparasitica*, *Brachyphoris oviparasitica*, *Hyalorbilia* aff. *multiguttulata*, sugarbeet cyst nematode, suppressive soils, biological control

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California is by far the largest U.S. producer of cole crops (USDA NASS 2020), with approximately two-thirds of them being grown in the fertile soils of its Central Coast, from Santa Barbara County to Santa Cruz County. Various cultivars of *Brassica oleracea* are grown, including broccoli, Brussels sprouts, cabbage, and cauliflower, among others (Johnson 1998). Many additional host crops of *Heterodera* spp. are also grown, such as spinach (*Spinacia oleracea*), table beets (*Beta vulgaris*), Swiss chard (*Beta vulgaris* subsp. *vulgaris*), and radish (*Raphanus sativus*). Also, mustards (*Brassica juncea*, *B. napus*, *Sinapis alba*) are often used as winter cover crops (Smith et al. 2011). All of these crops are hosts to either the sugarbeet (*Heterodera schachtii*) or the cabbage (*H. cruciferae*) cyst nematode, or both, and they are considered important vegetable pathogens. Indeed, *H. schachtii* has been reported to be among the most damaging plant-parasitic nematodes in the world (Jones et al. 2013; Sasser and Freckman 1987).

H. schachtii was most likely introduced to California from central Europe in the late 1800s via imported sugar beet seeds that were contaminated with nematode cysts (Cooke and Thomason 1978). For many decades, sugar beet production and processing were primarily concentrated along California's Central Coast, from the San Francisco Bay Area to Ventura County. Two sugar factories operated in this area, one in Salinas (Monterey County) and the other further south in Betteravia (Santa Barbara County). Over the years, it became less profitable to grow sugar beets compared to vegetables; a 9- to 10-month crop producing a relatively low-value commodity could not compete with two to three vegetable crops per year (S. Kaffka, *personal communication*).

When all sugarbeet production moved south to California's Imperial Valley, the coastal cyst nematode populations continued to thrive on susceptible host plants. Frequent plantings of these host crops resulted in a significant buildup of cyst nematode populations that caused considerable crop damage. Accordingly, use of the fumigant nematicide 1,3-dichloropropene (1,3-D) was quite common for the most profitable cole crops. Flint et al. (1992) reported that 43.2% of broccoli growers in Monterey County who were queried about their use of pesticides had used 1,3-D before its temporary withdrawal by the manufacturer in April 1990. This action was in response to the detection of high concentrations of this fumigant in the ambient air of a junior high school. This registration withdrawal led to predicted annual production losses of \$15.7 million for broccoli alone (Landels 1992). However, no overall adverse effect on cole crop yields was reported. For example, broccoli yield has steadily increased over the past 70 years by about 250 kg/ha each year (Geisseler and Horwath 2016). In addition, when 1,3-D once again became available 6 years after the registration withdrawal, its use with most cole crops never reached previous levels. More strikingly, 1,3-D applications declined to negligible levels in subsequent years (CA-DPR 2019).

The goal of this study was to examine the hypothesis that nematophagous fungi contribute to the low populations of *Heterodera* spp. in *Brassica* field soils from California's Central Coast. The population densities of *Heterodera* spp. were determined in 152 soils cropped to *Brassica* spp. from this region. In addition, an Illumina-based ribosomal RNA (rRNA) internal transcribed spacer (ITS) sequence analysis was used to determine whether nematophagous fungi were associated with *H. schachtii* females derived from those soils using a baiting experiment.

MATERIALS AND METHODS

Soil collection, processing, and initial nematode enumeration

During the spring of 2016, soils from 152 cole crop fields were sampled from California's Central Coast, approximately from Santa Cruz to Lompoc. A composite soil sample from each location was shipped overnight to the University of California, Riverside, CA. After air drying in paper bags, the samples were sieved (about 4 mm aperture) and thoroughly mixed. To enumerate cyst nematodes, two separate 250-cm³ subsamples were extracted by sieving and centrifugation (Dunn 1969). Cysts were counted using a dissecting microscope at 20× magnification. For each sample, five mature cysts were collected, placed into a 1.5-ml microcentrifuge tube, and 500 µl of a 2% commercial bleach solution was added to free the eggs from the gel matrix. The cysts were crushed with a mini plunger. The egg suspensions were well mixed, and 100 µl was placed in a counting dish for enumeration. The counting step was performed twice.

Nematode inoculum preparation

H. schachtii was initially isolated from a parasitized sugar beet root, obtained from the USDA-ARS Desert Research Station, Brawley, CA. Nematode inoculum was reared by growing 4-week-old cabbage (*Brassica oleracea* var. *capitata* 'Copenhagen Market Early') seedlings in steam-pasteurized sandy loam (approximately 75°C for 3 h, Sanyo Laboratory Autoclave, Scientific Model MLS-3781L, Sanyo Electric Biomedical Division, Tokyo, Japan) infested with freshly hatched *H. schachtii* second-stage juveniles (J2). The plants were maintained in greenhouse pot cultures for approximately 3 months. Cysts were extracted as described above and placed on modified Baermann funnels (Flegg and Hooper 1970) containing 4 mM ZnCl₂ to stimulate juvenile hatching. Hatched *H. schachtii* J2 were collected daily and stored in aerated water at 15 ± 1°C for not more than 48 h before soil infestation. The numbers of J2 in the suspensions were determined using a stereomicroscope at 40× magnification.

Greenhouse experiment for fungal baiting

Each of the 152 soil samples was mixed with autoclaved sand (1:1 v/v) to improve aeration and drainage. In previous research, the addition of 50% autoclaved sand did not significantly change the status of *H. schachtii*-suppressive soil (unpublished). Plastic cones (15 cm long × 2.5 cm diameter, Ray Leach Cone-tainers, Stuewe and Sons, Tangent, OR) were filled with about 250 cm³ of the soil mixes, leaving approximately 4 cm of headspace for watering. Three replicate cones were prepared. Cabbage (Copenhagen Market Early) seeds were incubated in a Petri dish containing moist tissue paper. After 1 week at 24 ± 2°C, each cone with soil was planted with one cabbage seedling. The cones were arranged in a hanging position in racks and incubated in a greenhouse at 26 ± 2°C. The seedlings were fertilized with liquid fertilizer (Miracle Gro 20-20-20, Scotts Company, Marysville, OH) every week. Four weeks after transplanting, approximately 1,000 *H. schachtii* J2 in 2 ml of water were added to each cone (~4 J2/cm³ of soil). The cones were incubated at 26 ± 2°C for 10 weeks with ambient light, corresponding to approximately 1,260 degree-days calculated using a base temperature of 8°C for *H. schachtii* (Curi and Zmoray 1966). From each replicate cone, 10 mature females were randomly collected from plant roots and stored in a 1.5-ml microcentrifuge tube at -80°C for DNA isolation.

TABLE 1

Soils used in this study by crop, county, and densities of *Heterodera* cysts and eggs

Soil no. ^v	Crop ^w	County locations of the fields ^x	No. of <i>Heterodera</i> spp. cysts/eggs per 250 cm ³ of soil ^y	Soil no. ^v	Crop ^w	County locations of the fields ^x	No. of <i>Heterodera</i> spp. cysts/eggs per 250 cm ³ of soil ^y
*1	Kale	San Benito	0	*61	Broccoli	Monterey	0
*2	Kale	San Benito	0	62	Broccoli	Monterey	0
*3	Broccoli	Santa Cruz	6.5/270.8	*63	Broccoli	Monterey	0
*4	Red cabb.	Santa Cruz	0	64	Cauliflower	Monterey	2.5/151.5
*5	Broccoli	Santa Cruz	0	*65	Cauliflower	Monterey	0.5/102.5
*6	Cauliflower	Santa Cruz	0	*66	Cauliflower	Monterey	0
*7	Broccoli	Monterey	0	67	Cauliflower	Monterey	4/224.4
8	Broccoli	Monterey	0	*68	Broccoli	Monterey	0
*9	Broccoli	Monterey	0	*69	Broccoli	Monterey	0
*10	Broccoli	Monterey	0	*70	Broccoli	Monterey	0
*11	Broccoli	Monterey	0	*71	Cauliflower	Monterey	1/148.1
*12	Broccoli	Monterey	0	72	Cauliflower	Monterey	0
*13	Cauliflower	Monterey	0	*73	Broccoli	Monterey	0
*14	Broccoli	Monterey	0	*74	Cauliflower	Monterey	0
*15	Broccoli	Monterey	0	*75	B. sprouts	Monterey	0
*16	Broccoli	Monterey	0	*76	B. sprouts	Monterey	2.5/281.7
*17	Cauliflower	Monterey	0	*77	B. sprouts	Monterey	0
18	Cauliflower	Monterey	0	*78	B. sprouts	Monterey	7.0/235.0
*19	Broccoli	Monterey	0	*79	B. sprouts	Monterey	11.0/351.8
*20	Broccoli	Monterey	0	*80	B. sprouts	Monterey	26.5/314.3
21	Broccoli	Monterey	0	*81	B. sprouts	Monterey	4.5/39.1
*22	Broccoli	Monterey	0	*82	B. sprouts	Monterey	0.5/65.0
23	Cauliflower	Monterey	0	*83	Broccoli	Monterey	4.5/95.6
*24	Broccoli	Monterey	1/149.4	*84	Broccoli	Monterey	0.5/65.0
*25	Cauliflower	Monterey	0	*85	Broccoli	Monterey	0
26	Cauliflower	Monterey	0	*86	Broccoli	Monterey	1/63.8
27	Broccoli	Monterey	0	87	Cabbage	Monterey	0
28	Broccoli	Monterey	0	88	Broccoli	Monterey	0
*29	Broccoli	Monterey	0	*89	Broccoli	Monterey	5/307.3
*30	Broccoli	Monterey	0	*90	Cabbage	Monterey	0
31	Cauliflower	Monterey	0	*91	Broccoli	Monterey	0
*32	Broccoli	Monterey	0	*92	Broccoli	Monterey	1/15.0
*33	Cauliflower	Monterey	0.5/88.8	93	Broccoli	Monterey	1.5/146.9
*34	B. sprouts	Monterey	0	94	Kale	San Benito	0
*35	Broccoli	Monterey	0	*95	Kale	Santa Clara	0
*36	Broccoli	Monterey	0	*96	Broccoli	Santa Clara	0
37	Cauliflower	Monterey	0	*97	Kale	Monterey	0
*38	Cauliflower	Monterey	0.5/91.3	*98	Broccoli	Monterey	0
*39	Cauliflower	Monterey	1.5/236.3	*99	Cabbage	Monterey	0
40	Broccoli	Monterey	0.5/58.8	*100	Broccoli	Monterey	0
*41	Broccoli	Monterey	1.5/158.1	*101	Broccoli	Monterey	0
*42	Cauliflower	Monterey	0	*102	B. sprouts	Monterey	0
*43	Bok Choy	Monterey	0	*103	B. sprouts	Monterey	0
44	Broccoli	Monterey	0	104	B. sprouts	Monterey	0
*45	Broccoli	Monterey	2/219.4	*105	Broccoli	S. Barbara	1/93.8
46	Broccoli	Monterey	0	106	Broccoli	S. Barbara	6.5/113.5
*47	Broccoli	Monterey	0.5/125.0	*107	B. sprouts	S. Barbara	2/248.1
*48	Broccoli	Monterey	0	*108	B. sprouts	S. Barbara	0
49	Broccoli	Monterey	0	*109	Cauliflower	S. Barbara	7.5/149.8
50	Broccoli	Monterey	0	*110	B. sprouts	S. Barbara	3/215.4
*51	Broccoli	Monterey	0	*111	Broccoli	S. Barbara	8/326.8
*52	Cauliflower	Monterey	0	112	Broccoli	S. Barbara	3/312.3
*53	B. sprouts	Monterey	0	*113	Cauliflower	S. Barbara	0
54	Cauliflower	Monterey	14.5/229.0	*114	Broccoli	S. Barbara	0.5/98.8
*55 ^z	Broccoli	Monterey	0	115	Cauliflower	Monterey	0.5/223.8
*56	Broccoli	Monterey	0	*116	Broccoli	Monterey	4.5/150.6
*57	Broccoli	Monterey	0	117	Broccoli	Monterey	1/98.8
*58 ^z	Broccoli	Monterey	0	*118	Broccoli	Monterey	1/98.8
59	Broccoli	Monterey	1/30.0	*119	Broccoli	Monterey	4/117.5
*60 ^z	Cauliflower	Monterey	0	*120	Broccoli	Monterey	0

(Continued on next page)

^v Asterisks (*) indicate soils used in the fungal analysis of *Heterodera schachtii* females.^w B. sprouts = Brussels sprouts; and Red cabb. = red cabbage.^x S. Barbara = Santa Barbara; and S.L. Obispo = San Luis Obispo.^y Average values.^z Soils not used for the fungal community analyses of the *H. schachtii* females because of low fungal rRNA ITS sequence counts (see Materials and Methods).

TABLE 1

(Continued from previous page)

Soil no. ^v	Crop ^w	County locations of the fields ^x	No. of <i>Heterodera</i> spp. cysts/eggs per 250 cm ³ of soil ^y	Soil no. ^v	Crop ^w	County locations of the fields ^x	No. of <i>Heterodera</i> spp. cysts/eggs per 250 cm ³ of soil ^y
*121	Broccoli	Monterey	0	*137	Cauliflower	S. Barbara	27.5/327.5
*122	Broccoli	Monterey	0	*138	B. sprouts	S. Barbara	18.5/276.3
*123	Broccoli	Monterey	0	139	Cauliflower	S. Barbara	0
*124	Broccoli	Monterey	0	*140	Cauliflower	S. Barbara	1/49.4
*125	Broccoli	Monterey	0	*141	Broccoli	S. Barbara	18.5/251.0
*126	Broccoli	Monterey	5.5/125.5	142	Broccoli	S. Barbara	7/115.0
*127	Broccoli	Monterey	7.0/140.0	*143	Red cabb.	S. Barbara	7/132.3
*128	Broccoli	S. Barbara	0	*144	Cauliflower	S. Barbara	0
*129	Broccoli	S. Barbara	9.0/307.8	*145	Broccoli	S. Barbara	0
*130	Cauliflower	S.L. Obispo	3.0/124.1	*146	Broccoli	S. Barbara	9.0/159.5
*131	Broccoli	S.L. Obispo	0	*147	Cauliflower	S. Barbara	1.5/126.3
*132	Broccoli	S.L. Obispo	2.0/96.3	*148	Broccoli	S. Barbara	0
*133	Broccoli	S.L. Obispo	0.5/155.0	*149	Broccoli	S. Barbara	10.5/246.5
*134	Broccoli	S.L. Obispo	7.5/87.8	150	Cauliflower	S. Barbara	10.5/329.3
*135	Broccoli	S.L. Obispo	0	151	Cabbage	S. Barbara	2.5/166.9
*136	Broccoli	S. Barbara	4.5/137.8	*152	Cabbage	S. Barbara	3.5/370.0

DNA isolation from *H. schachtii* females

DNA was isolated from *H. schachtii* females that were collected from plant roots at the end of the baiting experiment. Thirty-two of the 152 soils were not used in this analysis because of an insufficient number (<10) of *H. schachtii* females. This usually occurred because at least two plants in the three replicate cones grew poorly or died. For each of the remaining 119 soils, DNA was isolated from one of the replicate samples of 10 *H. schachtii* females. The nematode females were first washed three times with 500 µl of sterile water, and then DNA was isolated using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD) with a 150-s bead-beating step using a Mini-Beadbeater-16 (BioSpec), but using the bead tubes (Matrix A) from the FastDNA Soil Kit (MP Biomedicals, Irvine, CA).

Illumina fungal rRNA ITS library construction and sequencing

Illumina fungal rRNA ITS libraries were constructed as follows. Polymerase chain reactions (PCRs) were performed using a DNA Engine thermal cycler (Bio-Rad, Hercules, CA) and 25-µl reactions containing Phusion High-Fidelity DNA Polymerase Mix (New

England Biolabs, Ipswich, MA) supplemented with 500 µg/ml of bovine serum albumin, 1 mM MgCl₂, 250 µM of each deoxy-nucleotide triphosphate (dNTP), 400 nM of each primer, and 2.5 µl of DNA template. The PCR primers gITS7 (GTGARTCATC GARTCTTTG) and ITS4 (TCCTCCGCTTATTGATATGC) targeted the ITS2 region of the ribosomal rRNA gene operon (Ihrmark et al. 2012; White et al. 1990), with the reverse primers including 12-base barcodes, and both primers including the Illumina sequences needed for cluster formation. Thermal cycling parameters were 94°C for 5 min; 35 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s; followed by 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, and libraries were constructed using an equal amount (ng) of each sample. DNA sequencing (single-end 300 base) was performed using an Illumina MiSeq.

Illumina fungal rRNA ITS sequence data processing

The UPARSE pipeline was used for demultiplexing, length trimming, quality filtering, and operational taxonomic unit (OTU) picking using default parameters or recommended guidelines that were initially described in Edgar (2013) and that have been updated at https://www.drive5.com/usearch/manual/uparse_pipeline.html.

FIGURE 1

Fungal community analysis of *Heterodera schachtii* females derived from soils cropped to *Brassica* species and produced by a baiting experiment: **A**, county analyses; and **B**, crop analyses. Beta diversity distance values were generated from fungal rRNA ITS sequences and displayed using principal coordinates analysis. Values in parentheses after county and crop are the number of replicate samples.

Briefly, after demultiplexing and using the recommended 1.0 expected error threshold, sequences were trimmed to a uniform length of 297 bases. Sequences were then dereplicated and clustered into zero-radius OTUs using the UNOISE3 algorithm

(Edgar 2016), which detects and removes chimeric sequences. An amplicon sequence variant (ASV) table was then generated using the otutab command. Taxonomic assignments of the fungal ASVs were performed using the RDP Classifier version 2.12

FIGURE 2

Fungal species associated with *Heterodera schachtii* females derived from soils cropped to *Brassica* species and produced by a baiting experiment: **A**, county analyses; and **B**, crop analyses. Average relative abundances were determined by an examination of fungal rRNA ITS sequences. Asterisk (*) indicates this phylotype had more than one best match, and therefore its taxonomic assignment is not definitive. Other fungi are all of the taxa with less than 0.74% relative abundance combined. The number of replicate samples is shown in parentheses.

(Wang et al. 2007), trained on the ver8_99_s_02.02.2019 release of the UNITE database (Kõljalg et al. 2013), and ASVs having nonfungal assignments were removed. Because the fungal sequence counts from the nematodes derived from three of the 119 soils were low (<668), they were removed from further analysis.

The fungal rRNA ITS sequences have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under BioProject accession number PRJNA638436.

Illumina fungal rRNA ITS sequence analyses

QIIME (Caporaso et al. 2010) was used to calculate abundance Jaccard beta diversity distance matrices, which were depicted using principal coordinates analysis and were statistically assessed using Adonis PERMANOVA tests. Statistical differences among the taxa were determined using edgeR and the false discovery rate method (Benjamini and Hochberg 1995; McCarthy et al. 2012; Robinson et al. 2010). Prism was used to make the fungal species plots (GraphPad, La Jolla, CA).

Phylogenetic tree construction

The phylogenetic tree was constructed using fungal rRNA ITS sequences with high identity to members of the *H. oviparasitica* clade (Smith Becker et al. 2020) that were identified by this study (*Brassica* ASVs) and by a related study examining soils cropped to sugar beets (Sugar Beet ASVs) (Witte et al. 2020), as well as from nematophagous fungi belonging to the *H. oviparasitica* clade (Olatinwo et al. 2006b; Stirling and Mankau 1978; Timper and Riggs 1998; Yin et al. 2003) and related fungi described in Baral et al. (2018).

The phylogenetic tree was constructed by performing the following analyses using GenomeNet (<https://www.genome.jp/>). Sequences were aligned using the CLUSTAL W algorithm (Thompson et al. 1994). The final alignment had a length of 273 nucleotides, and the sequences ranged from 184 to 261 nucleotides. Alignment and phylogenetic reconstructions were performed using the function “build” of ETE3 v3.0.0b32 (Huerta-Cepas et al. 2016) as implemented on the GenomeNet (<https://www.genome.jp/tools/ete/>). Maximum likelihood trees were inferred using RAxML v8.1.20, which was run with model

TABLE 2

Statistical analysis of the relative abundances of the fungal species associated with the *Heterodera schachtii* females by county and obtained from a baiting experiment^z

Taxa	Monterey (79)	Santa Barbara (23)
<i>Hyalorhiza oviparasitica</i> clade	2.64 a	17.41 b
<i>Arthrobotrys conoides</i>	3.12 a	0.02 b
<i>Rhizopus oryzae</i>	2.94	1.33
<i>Mortierella</i> spp. ASV 6	2.98 a	0.00 b
<i>Mortierella</i> spp. ASV 9	2.09 a	0.00 b
* <i>Pochonia chlamydosporia</i>	0.03 a	4.30 b
<i>Fusarium</i> spp.	0.68 a	0.00 b
<i>Cyberlindnera fabianii</i>	0.80	0.31
<i>Mortierella elongata</i>	0.92	0.26
<i>Arthrobotrys scaphoides</i>	1.09 a	0.00 b

^z Numbers in the cells are mean relative abundance. For each row, means followed by a different letter are significantly different (edgeR analyses, false discovery rate-adjusted *P* values < 0.05). The number of replicate samples is shown in parentheses after county. Asterisk (*) indicates this phylotype had more than one best match, and thus its taxonomic assignment is not definitive.

GTRGAMMA and the default parameters (Stamatakis 2014). Branch supports were computed out of 100 bootstrapped trees. The tree figure was created using the Interactive Tree of Life (Letunic and Bork 2007) (<https://itol.embl.de/>).

The Illumina fungal rRNA ITS sequences from this study with high identity to those from the *H. oviparasitica* clade, and that were included in this phylogenetic analysis, have been deposited in the NCBI under the accession numbers MT248325, MT248326, MT248327, MT248328, MT248329, MT248330, MT248331, MT248332, and MT573527.

RESULTS AND DISCUSSION

Soils examined

Soils from 152 fields cropped to *Brassica* species along California’s Central Coast were examined. These soils came from fields primarily cropped to broccoli and that had a broad geographical distribution, from Santa Barbara County to Santa Cruz County (Table 1).

Population densities of *Heterodera* spp.

The average number of *Heterodera* spp. cysts per 250-cm³ of soil ranged from 0.5 to 27.5, with 62% of the soils harboring no detectable cysts and only a few samples reaching a potentially damaging threshold level of 12 eggs/g of soil (Abawi and Mai 1980) (Table 1). These low values support the hypothesis that many of the soils in this region suppress *Heterodera* spp. despite their originally widespread occurrence and frequent cropping to host plants of these nematodes.

Identifying fungi associated with *H. schachtii* females using a baiting experiment

The baiting experiment enabled the identification of fungi associated with *H. schachtii* females, an important stage of nematode development, increasing the likelihood of identifying nematophagous fungi. This experiment involved planting a

TABLE 3

Statistical analysis of the relative abundances of the fungal species associated with the *Heterodera schachtii* females by crop and obtained from a baiting experiment^z

Taxa	Broccoli (70)	B. sprouts (16)	Cauliflower (20)
<i>H. oviparasitica</i>	4.68	10.20	3.36
<i>A. conoides</i>	3.09	1.97	4.46
<i>Rhizopus oryzae</i>	3.15	1.02	2.95
<i>Mortierella</i> ASV 6	3.28 a	0.00 b	0.10
<i>Mortierella</i> ASV 9	0.00 a	10.28 bc	0.00 c
* <i>P. chlamydosporia</i>	0.89	0.27	2.75
<i>Fusarium</i> spp.	0.77 a	0.00 b	0.00 b
<i>C. fabianii</i>	0.85	0.41	0.63
<i>M. elongata</i>	1.17 a	0.02 b	0.00 b
<i>A. scaphoides</i>	1.23 a	0.00 b	0.01 b

^z Numbers in the cells are mean relative abundance. For each row, means followed by a different letter are significantly different (edgeR analyses, false discovery rate-adjusted *P* values < 0.05). The number of replicate samples is shown in parentheses after crop. Asterisk (*) indicates this phylotype had more than one best match, and thus its taxonomic assignment is not definitive. B. sprouts = Brussels sprouts; *H. oviparasitica* = *Hyalorhiza oviparasitica* clade; *Mortierella* ASV 6 = *Mortierella* spp. ASV 6; *Mortierella* ASV 9 = *Mortierella* spp. ASV 9; *P. chlamydosporia* = *Pochonia chlamydosporia*; *C. fabianii* = *Cyberlindnera fabianii*; *M. elongata* = *Mortierella elongata*; and *A. scaphoides* = *Arthrobotrys scaphoides*.

H. schachtii host crop (cabbage) in these soils, infesting the soils with *H. schachtii* J2, and then collecting nematode females from the plant roots after two nematode generations. Only 119 of these soils produced a sufficient number of *H. schachtii* females for further analysis, primarily because of poor plant growth or seedling diseases that were present in the other 33 soils.

Fungal community and species analysis of *H. schachtii* females derived from soils cropped to *Brassica* species

An Illumina-based sequence analysis of the rRNA ITS region was used to examine the fungal communities associated with *H. schachtii* females that were produced by the baiting experiment. Nematode samples from three of the 119 soils were removed from further analysis because of low numbers of fungal sequences. Statistical analyses were only performed on sample types with more than six replicate samples.

A beta diversity analysis showed that the fungal communities associated with the *H. schachtii* females were distinct in Monterey and Santa Barbara Counties (PERMANOVA test $P = 0.001$) (Fig. 1A). A similar beta diversity analysis determined that there were no differences in the fungal communities among the different crop types (Fig. 1B, PERMANOVA test $P > 0.05$).

To examine the relative abundance of the fungal species associated with the *H. schachtii* females from the baiting experiment, taxonomic plots were constructed using the fungal rRNA ITS sequence data. The most abundant fungi associated with the *H. schachtii* females included at least five nematophagous species or genera including members of the *H. oviparasitica* clade, *Arthrobotrys conoides*, *Pochonia chlamydosporia*, *Fusarium*

spp., *Mortierella* spp., and *Arthrobotrys scaphoides* (Fig. 2, all samples).

There were clear differences in the relative abundances of the nematode-associated fungal species between both the counties and the crops (Fig. 2). The statistical analyses (Tables 2 and 3) showed that there were higher relative abundances of the *H. oviparasitica* clade and *P. chlamydosporia* in *H. schachtii* females derived from soils in Santa Barbara County than from soils in Monterey County. Conversely, *A. conoides* was found in higher abundances in Monterey County than in Santa Barbara County. *Mortierella* spp. ASV 6 and 9 were most abundant in Monterey County and in fields cropped to broccoli and Brussels sprouts, respectively. *Fusarium* spp. and *A. scaphoides* were most abundant in Monterey County and in fields cropped to broccoli. *Mortierella elongata* was most abundant in fields cropped to broccoli.

These results are consistent with the hypothesis that increased population densities of nematophagous fungi contribute to low populations of *Heterodera* spp. in fields cropped to *Brassica* species along California's Central Coast. Fungi that are both known parasites of *H. schachtii* females and that were abundant enough to impact cyst nematode populations included members of the *H. oviparasitica* clade (Olatinwo et al. 2006a, 2006b; Smith Becker et al. 2020), *Pochonia chlamydosporia* (Kerry and Bourne 1996; Kerry et al. 1993), and certain *Fusarium* spp. (Chen and Chen 2002; Chen et al. 1994; Gao et al. 2008; Jorgenson 1970; Westphal and Becker 2001). Given that the relative abundances of these three nematode-associated fungi are different between the two counties, this suggests that this putative nematode population suppressiveness may be caused by different

FIGURE 3

Phylogenetic relationships of nematophagous fungi and related members of the Orbiliomycetes. The tree was constructed by performing a maximum-likelihood analysis of fungal rRNA ITS sequences obtained from four cultured nematophagous fungi, related phylotypes obtained by culture-independent analyses, and members of the Orbiliomycetes. Fungal phylotypes from culture-independent analyses of *Heterodera schachtii* females derived from soils cropped to (i) *Brassica* species (this study) and (ii) sugar beets (Witte et al. 2020) are indicated by *Brassica* followed by an ASV number and *Sugar Beet* followed by an ASV number, respectively. The outgroup is *Tuber borchii*. Bootstrap values (out of 100) are shown on the branches if ≥ 0.50 . Branch lengths are the expected number of substitutions per nucleotide. Additional information for *Hyalorbilia oviparasitica* StM is ATCC 38908, CBS 379.84, and MH861748, and for *H. oviparasitica* GloDo it is CBS 347.85. Additional information for *Hyalorbilia aff. multiguttulata* DoUCR50 is JQ638668.

organisms and/or combinations of organisms. However, because members of the *H. oviparasitica* clade had the highest relative abundance when all 116 samples were examined (Fig. 2), this suggests that this group of fungi might play the most important role. In addition, although the relative abundances of members of the *H. oviparasitica* clade and *P. chlamydosporia* did not vary among the crops, *Fusarium* spp. were highest in broccoli.

The other fungal species shown in Figure 2 and Tables 2 and 3 were likely associated with the females as spores or propagules but unable to attack the female stage of the nematode; trapping fungi in particular likely fall into this group. Their relatively high abundance in this analysis might be related to the fact that many of these species produce abundant conidia.

Phylogenetic analysis of the fungi identified in this study in relation to nematophagous fungi and other members of the *Orbiliomycetes*

Next, a phylogenetic analysis was performed to examine the relationships among the fungal sequences identified by this study that had high sequence identity to *H. oviparasitica* (formerly *Dactylella oviparasitica* and *Brachyphoris oviparasitica*, Smith Becker et al. 2020) and related fungi (Fig. 3). This analysis shows a coherent assemblage of organisms and phylotypes composed of four nematophagous fungi (green text), sequences from this study (red text), sequences from a similar study examining *H. schachtii* females derived from soils cropped to sugar beets (blue text) (Witte et al. 2020), and *H. brevistipitata*. This group was called the *H. oviparasitica* clade.

The fungal rRNA ITS sequences obtained from the two culture-independent studies, designated by either Sugar Beet or *Brassica* and an ASV number, are posited to come from nematophagous fungi because of their placement in the *H. oviparasitica* clade (Fig. 3) and because they were found in the analyses of *H. schachtii* females. In addition, given that the sequences from these two studies are intermingled in the *H. oviparasitica* clade, it appears that these fungi are not crop specific. This is consistent with prior research that showed that *H. aff. multiguttulata* DoUCR50 (Smith Becker et al. 2020) was attracted to emerging nematode females but not to plant roots (Smith Becker et al. 2013).

The presence of *H. brevistipitata* in the *H. oviparasitica* clade is intriguing because it suggests that this fungus might be nematophagous. However, because *H. brevistipitata* was originally isolated in its teleomorphic state from decaying plant material (Liu et al. 2005), its nematophagous status is unknown.

CONCLUSIONS

The results presented in this study are consistent with the hypothesis that indigenous members of the *H. oviparasitica* clade contribute to the low populations of *Heterodera* spp. in *Brassica* field soils from California's Central Coast. Other research supporting this hypothesis includes the discovery that a member of the *H. oviparasitica* clade was responsible for the *H. schachtii* suppressiveness in field 9E at the University of California Riverside's Agricultural Operations (Olatinwo et al. 2006b). In addition, several strains of fungi in the *H. oviparasitica* clade (ARF or Arkansas Fungus) (Yang et al. 2012), which were isolated from parasitized soybean cyst nematodes (*H. glycines*) from a suppressive soil, were able to reduce the population densities of this nematode when amended to a conducive soil (Kim and Riggs 1995; Timper and Riggs 1998). Finally, in greenhouse experiments, the amount of *H. schachtii* suppressiveness was positively associated with the initial population

densities of a member of the *H. oviparasitica* clade (Yang et al. 2012).

Taken together, the interpretation of these data leads to the conclusion that fungi belonging to the *H. oviparasitica* clade, which appear to be globally distributed (Yang et al. 2012), could be reducing the populations of several species of cyst nematodes that detrimentally impact a wide variety of economically important crops. In addition, similar to take-all decline in wheat, in which monoculture leads to the suppression of take-all disease (Cook and Baker 1983), the frequent cropping of *Heterodera* host crops in soils from California's Central Coast is speculated to lead to an increase in fungi that are suppressing these cyst nematodes. Because the results presented in this paper are only associations, they will need to be followed up by experiments that isolate these fungi and then test them by adding them to soil in nematode population suppression assays.

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