Spatial relationships between entomopathogenic nematodes and nematophagous fungi in Florida citrus orchards

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Abstract

Relationships between entomopathogenic nematodes (EPNs), nematophagous fungi (NF) and soil physical and chemical properties were studied in a survey of 53 citrus orchards in central ridge and flatwoods ecoregions of Florida. Seven species of NF associated with nematodes were quantified directly using a real time qPCR assay. All nematophagous fungi studied except *Arthrobotrys musiformis* and *Hirsutella rhossiliensis* were frequently detected (24-56%) in both regions. *Paecilomyces lilacinus* and *Gamsylella gephyropagum* were encountered more frequently in the flatwoods (P = 0.03) and on the ridge (P = 0.02), respectively. Redundancy analysis revealed seven abiotic and biotic factors as significantly related to the NF occurrence. Multiple regression of fungi on these variables explained 78%, 66%, 48%, 36%, 23% and 4% of the variation in *Catenaria* sp., *A. musiformis*, A. *dactyloides*, *P. lilacinus*, *A. oligospora* and *G. gepharopagum*, respectively. When the data from citrus were pooled with those reported previously from natural areas and subjected to principle component analysis, the first two principle components explained 43% of the variation in NF communities. The surveys (citrus vs natural areas) were discriminated by PC2 (P <0.001) and the ecoregion by PC1 (P <0.002), and all but one NF species were related (P < 0.01) to one or both components. NF communities tended to have more species and greater diversity in the flatwoods, where EPN richness and diversity were the least. However, the strength of associations between individual EPN and NF species as measured by SADIE reflected the associations between each species and ground water depth, suggesting that ecoregion preferences affected the species associations. Within each ecoregion, significant relationships between the individual NF and EPN species measured by stepwise regression tended to be positive. The results did not support the hypothesis that NF modulate the spatial patterns of EPN species between or within these two ecoregions.

Key words: Nematophagous fungi; quantitative real–time PCR; soil ecology; species–specific primers and probe; entomopathogenic nematodes; multivariate analysis

1. Introduction

Entomopathogenic nematodes (EPNs) have been studied for more than a quarter century in Florida citrus orchards for their potential as components of root weevil IPM programs. Following the deregistration of soil-applied organochlorine pesticides in the mid-1970s, the invasive pest *Diaprepes abbreviatus* and other root weevils such as *Pachnaeus litus* became the most economically damaging arthropods in Florida citrus due to a lack of adequate control measures (Dolinski et al., 2012). Disrupting the weevil's long life cycle, which comprises both arboreal and subterranean stages, is difficult using non-persistent chemical and biological insecticides. The introduction of the bacterial disease huanglongbing (HLB) into the citrus industry, first detected in 2005 (Halbert, 2005), intensified the need for root weevil management because HLB destroys more than half of the fibrous roots on trees before aboveground symptoms become evident (Johnson et al., 2014). As a consequence, orchards damaged by both weevils and HLB decline much more rapidly than those affected by just the bacterium or weevil (Duncan, 2014) .

The two primary genera of EPNs, *Steinernema* and *Heterorhabditis* (Dillman et al., 2012), are each represented by several native species in Florida. A survey of orchards (Campos-Herrera et al., 2013) and another of natural areas in the citrus growing regions of the state (Campos-Herrera et al., 2016), revealed similar associations between EPN communities and two ecoregions, the "central ridge" running north to south in the middle of the peninsula and the "flatwoods" regions surrounding the central ridge and extending to the coasts. Orchards on the higher elevation, well drained sandy soils of the central ridge were almost always inhabited by both *H. indica* and *S. diaprepesi* and frequently also by *H. zealandica*. By contrast, EPN communities in orchards on lower elevation, shallow, less well drained soils of the flatwoods were almost always composed of *H. indica*, but only occasionally in combination with *S. diaprepesi* and/or an undescribed *Steinernema* species in the *S. glaseri* group proposed by Spiridonov et al. (2004). The population density of *D. abbreviatus* is larger in flatwoods orchards than in those on the central ridge (Futch et al., 2005), perhaps due in part to lower rates of biological control by EPNs in the flatwoods (Duncan et al., 2003). These patterns and their relationships suggest that natural control of

69 weevils can be increased by discovering properties of habitats that can be modified in ways that conserve
70 EPNs and enhance their services.

71 Soil properties that affect soil water potential explained the greatest amount of EPN variation in
72 each of the two surveys by Campos Herrera et al. (2013, 2016) and results of subsequent experiments
73 supported the possibility that different EPN spatial patterns in Florida result partly from differential
74 species adaptation to soil moisture (Duncan et al., 2013; El-Borai et al., 2016). Additionally, natural
75 enemies such as nematophagous fungi (NF) and micro-arthropods regulate EPN numbers locally (Ekmen
76 et al., 2010; Hodson et al., 2012) and could also modulate EPN spatial patterns in the landscape if their
77 effects differ by region. NF comprise a guild of diverse fungi that incinclude saprophyte/facultative
78 predators (most notably the 'trapping fungi') and obligate predators of nematodes and other soil fauna
79 such as Chytridiomycete species that produce infective zoospores. Because many members of the guild
80 can function at different trophic levels, conventional methods of quantifying these fungi in soil samples
81 do not discriminate propagules derived from nematode prey from those derived from other nutrient
82 sources. The development of molecular tools that can quantify NF associations with nematodes in nature,
83 without the artifacts introduced by culturing methods, provides a means to study the importance of habitat
84 properties on those associations (Pathak et al., 2012). Campos-Herrera et al. (2016) detected primarily
85 positive associations between NF and either free-living bactivorous nematodes or EPNs from nematode
86 samples taken at 91 natural (non-agricultural) sites. Those relationships suggested that r-selected, rapidly
87 increasing nematode species provided local resources for NF populations. The relative lack of negative
88 associations might mean that NF predation of nematodes did not vary much across regions and did not
89 affect EPN spatial patterns at the landscape scale. Jaffee et al., (1996) similarly concluded that NF were
90 unrelated to the spatial pattern of *Heterorhabditus marelatus* that regulate subterranean ghost moth larvae
91 and the damage they do to the roots of bush lupines in coastal California. Relationships between EPNs
92 and NF in different habitats were also reported from field plots of citrus trees planted into holes filled
93 either with sand or the native loam soil, thus creating well-drained or poorly drained conditions (Duncan
94 et al., 2013). EPNs that were introduced into the plots persisted and killed sentinel weevil larvae at higher

rates in the sand than the loam during four years. However, real time PCR assays of DNA from nematodes isolated from soil samples showed that the NF species *Purpureocillium lilacinus* and *Catenaria* sp. consistently infected nematodes at higher rates in sand than loam, whereas no NF species appeared to be favored by conditions in the loam soil. Consequently, there is little evidence to date that the spatial patterns of EPN species at a broad, landscape scale are modulated by variation in susceptibility to NF across habitats.

The DNA of seven NF species recovered from samples of nematodes was identified and quantified, but not reported, in a survey of EPNs in Florida citrus orchards (Campos-Herrera et al., 2013). The agricultural inputs in those orchards created soil conditions very different than those of natural areas in the same region reported by Campos-Herrera et al. (2016). Here we report the relationships between NF and soil properties in the citrus orchards and compare them with those detected in the natural areas. Results from both surveys were also combined to explore spatial relationships between NF, member of the EPNs soil food web and abiotic factors. Our hypotheses were that 1) the occurrence frequency of some NF species would differ between orchards and natural areas due to differences in soil chemistry and management, 2) some spatial associations between species of EPNs and NF would be due to similar habitat adaptations and therefore, 3) associations between NF and EPNs within a habitat might provide better insights into the role played by NF in modulating EPN occurrence.

2. Materials and methods

2.1. General survey design, sampling and soil processing

The survey area extended nearly 200 km east to west (from the gulf coast to the Atlantic coast) and more than 300 km north to south (from Umatilla to Immokalee). The orchards were selected on the basis that they were known to be infested by *Diaprepes abbreviatus* and that representative numbers were located in each of the two ecoregions (23 sites in the central ridge and 30 in the flatwoods) in which

Florida's orchards occur. Sampling and sample processing methods are described in detail by Campos-Herrera et al. (2013). Briefly, two samples (30 cores, 2.5-cm diam. x 30 cm deep per sample) were obtained systematically at each 2-3 ha site during summer-early autumn 2009-2010. Samples were mixed and nematodes extracted (Jenkins, 1964) from 2, 500-cm^3 subsamples (i.e., 2 liters of soil per site). DNA was extracted using the UltraClean Soil DNA Extraction Kit (MoBio), quality was ensured (Nanodrop system 1000 v.3.3.0, Thermo Scientific, Wilmington, DE, USA), and the quantity adjusted for further molecular analysis. Following previous optimized protocols, seven species of NF were identified and quantified using real-time qPCR approaches (Atkins et al., 2005; Zhang et al., 2006; Pathak et al., 2012). Selected organisms previously reported in this survey by Campos-Herrera et al. (2013) were also combined in the evaluation, which included the species specific qPCR quantification of EPNs (13 species), and several other nematodes, bacteria and oomycete phytopathogens, all of which are known to interact with EPNs (Huang et al., 2010; Campos-Herrera et al., 2011a, 2011b, 2012; 2013) (Supplementary data 1). In all the cases, primers and probes were synthesized by Integrated DNA Technologies Inc. (IDT, San Diego, CA), with all TaqMan® PCR probes labelled with the fluorogenic reporter dye FAM, the quencher Iowa Black™ FG and the ZEN molecule in the middle of the probe. Real time qPCR runs were performed in ABI Prism 7000 (Applied Biosystem), in optical-well reaction plates (USA Scientific, Orlando, FL, USA) with a final volume of 20 µL, and employing those reactive materials and primers/probe concentrations, annealing temperature and number of cycles described previously (Supplementary data 1). For each of the species, the identification and quantification was performed using positive (standard curve), negative (sterile de-ionized water) controls and the corresponding unknowns, with two technical replicates in each run.

Soil characteristics were described for the citrus survey (Campos-Herrera et al., 2013) and the natural areas study (Campos-Herrera et al., 2016). Briefly, remaining soil was air dried and sand fractions were determined by wet sieving (53 mm) followed by re-drying and processing on stacked sieves on a mechanical shaker (W.S. Tyler Co., Mentor, OH). Soil pH, organic matter (OM), percentage sand, silt and clay, electric conductivity (EC), cation-exchange capacity (CEC) and soil nutrient elements (P, K, Mg,

147 Ca, and B) were measured in a commercial laboratory (Waters Agricultural Laboratories, Camilla, GA).
148 In addition to these studies, common for both surveys (citrus and natural areas), additional measurements
149 were performed in the citrus surveys as follow: soil field capacity (FC) at pF 2.7, wilting point (WP) at pF
150 4.2 and water holding capacity by Richards' method (Duchaufour, 1975); soil metal content (Cd, Cr, Cu,
151 Fe, Mn, Ni, Pb, Zn) and others (As and Se) by Optical Emission Spectrometry (IPC) with a Plasma
152 Spectrometer; and pesticide residues such as organochlorines, BHCs, Cyclodienes and DDT compounds
153 following ISO 10382 (2002), in the Chromatographic service of the National Museum of Natural
154 Sciences (Madrid, Spain) (Campos-Herrera et al., 2013).
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156 *2.2. Analysis of fungal distribution in the citrus survey*
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158 NF DNA quantity was divided by the total DNA in a sample to estimate the infection rate. The
159 infection rate of each species was then normalized (0-1) to permit comparison of the relative effects of
160 each species in an NF community (Pathak et al., 2012; Duncan et al., 2013). The occurrence and mean
161 infection rate of NF was calculated for each eco-region (central ridge and flatwoods). To compare species
162 abundance, the quantities of NF were square root transformed prior to statistical analyses. The ecological
163 indices (*i*) species richness (number of species, S), (*ii*) the Shannon Wiener diversity index $H' = -\sum p_i \ln$
164 p_i, where "p_i" is the proportion of individuals of the ith species and (*iii*) the evenness, a measure of the
165 equilibrium of species abundance ($J' = H'/H_{max}$,where H_{max} is lnS) were estimated for all samples and
166 within regions. Data are presented as mean ± SEM.
167 Regional differences in species occurrence, abundance, and ecological indices were evaluated by
168 T-test (20.0 IBM SPSS Inc., Chicago, IL, USA). Redundancy analysis of NF species and selected
169 organisms and soil properties were performed using the software CANOCO 5 (Ter Braak, 2009; Smilauer
170 and Lepš, 2014). The biotic and abiotic properties used as explanatory variables were reported by
171 Campos-Herrera et al. (2013). In particular, those derived from principal component analysis (PCA) as

contributing significantly to the total spatial variability (data not shown) together with GWD. The graphical results of the RDA were presented with bi-plot scaling (CANOCO 5). Stepwise multiple regression analysis (Minitab 15, State College, PA) of each NF species on the independent variables found to be significant by RDA was used to assess the relative explanatory contributions of those variables.

2.3. Analysis of fungal distribution and relationships in the combined citrus and natural area surveys

The biotic and abiotic data from natural areas and citrus orchards reported by Campos-Herrera et al. (2013, 2016) were combined with the NF data from citrus reported here for more comprehensive analyses. NF communities were characterized by principal component analysis (PCA) of the square root-transformed infection rates and subjected to ANOVA (GLM Minitab 15) to assess differences in the first two principal components between 1) surveys and 2) ecoregions. For comparison, Spatial Analysis by Distance Indices (SADIE; http://www.rothamsted.ac.uk/pie/sadie/SADIE-home_page_1.htm) was also used to assess the degree of association between each NF species and each EPN species as well as NF species and GWD (Spiridonov et al., 2007). The pooled data from the citrus and natural area surveys were also subjected to RDA as described previously. Pooled data from within each ecoregion were used to determine whether NF species occurrence differed (T-test, Minitab 15) in sites with or without a given EPN species. Stepwise multiple regression of the pooled data within each ecoregion was used to assess relationships between EPNs and NF.

3. Results

3.1. Analysis of fungal distribution in the citrus survey

197 Few NF species varied in abundance or detection frequency across the two regions. Two
198 exceptions were *P. lilacinus*, which was detected more frequently and at higher abundance in the
199 flatwoods and *M. gephyropagum*, which was more frequently detected on the central ridge (Table 1). The
200 number of species did not differ between the central ridge (2.3 ± 0.24) or the flatwoods (2.70 ± 0.21), but
201 the species diversity (H' = 0.30 ± 0.073 vs 0.56 ± 0.08; t = 2.32, P = 0.02) and evenness (J' = 0.34 ±
202 0.066 vs 0.55 ± 0.063; t = 2.25, P = 0.03) were highest in the flatwoods.
203 Seven environmental variables explained significant NF species variation in the redundancy
204 analysis (Fig. 1). Stepwise multiple regression of each NF on those seven factors as independent
205 variables revealed potassium and electrical conductivity to be positively and inversely, respectively,
206 associated with five of the six species, AWC to be directly related to three species, pH to be positively
207 related to two species and inversely related to one, boron to be inversely related to two species and
208 directly related to one and phosphorus to be positively related to two species (Table 2). These variables
209 explained 78% of the variation in *Catenaria* sp. and 66% of variation in *A. musiformis*, 48% in *A.*
210 *dactyloides*, 36% in *P. lilacinus*, 23% in *A. oligospora* and 4% in *G. gepharopagum*.
211
212 *3.2. Analysis of fungal distribution and relationships in the combined citrus and natural area surveys*
213
214 Two way analysis of variance of data from citrus orchards and those reported previously from
215 nearby natural areas (Campos-Herrera et al., 2016) revealed that *A. oligospora*, *A. dactyloides*, *M.*
216 *gephyropagum* and *Catenaria* sp. were detected more frequently (all species $P < 0.001$) in citrus orchards
217 than in natural areas (Fig. 2). The pooled data confirmed that *P. lilacinus* and *G. gephyropagum* were
218 detected more frequently in flatwoods and central ridge ecoregions, respectively. There was no significant
219 interaction between the botanical habitat (orchard *vs* natural area) and the ecoregion for detection
220 frequency of any NF species.
221 The first two principal components of the PCA accounted for 40% of the variation in the NF
222 species composition associated with nematodes in the two ecoregions of the combined surveys (Fig. 3).

223 The first principal component discriminated the two surveys (P <0.001) along the x-axis with *A.*
224 *oligospora*, *A. dactyloides*, *G. gephyrophagum*, and *Catenaria* sp., each significantly contributing (P <
225 0.001) to the difference. NF communities on the central ridge were distinguished from those in the
226 flatwoods (P = 0.002) by PC2 on the y-axis, with *P. lilacinus*, *A. dactyloides*, *A. musiformis*, and G.
227 *gephyrophagum* (each P < 0.001) and *A. oligospora* (P = 0.01) being related to the components. There
228 were no significant interactions between the survey and ecoregion effects on NF community structure.
229 The relative richness, diversity and evenness of the NF and EPN communities in the two
230 ecoregions differed from each other consistently in both surveys (Fig. 4). NF communities tended to have
231 more species and greater diversity in the flatwoods, where EPN richness and diversity were the least.
232 A redundancy analysis using the pooled data from both the orchard and natural area survey again
233 revealed pH, potassium, phosphorus and *S. diaprepesi* to be significant descriptors of the overall variation
234 in NF species (data not shown). The other significant predictive variables for citrus orchards (electrical
235 conductivity, soil water holding capacity and boron, Fig. 1) were not measured in the natural area survey
236 and therefore, stepwise regression using data from both surveys was not conducted.
237 SADIE association indices (non-parametric) between NF species and groundwater depth (GWD)
238 were positively related to association indices between NF species and *S. diaprepesi* (P = 0.01) and *H.*
239 *zealandica* (P < 0.05) and inversely related to those for *H. indica* (P = 0.02) and *Steinernema* sp. (P =
240 0.03) (Fig. 5). Because *S. diaprepesi* and *H. zealandica* were reported to be directly associated with GWD
241 and *H. indica* and *Steinernema* sp. inversely related to GWD (Campos-Herrera et al. 2013), the
242 associations between EPN and NF species may have resulted from varying responses by each species to
243 GWD which is greater on the central ridge than in the flatwoods. Therefore, we examined relationships
244 between NF and EPNs separately for each ecoregion. In the flatwoods sites, the detection frequencies of
245 six out of seven NF species in both the orchards and natural areas were greater in sites where *Steinernema*
246 spp. was not detected (Fig. 6). There were no apparent differences in NF and EPN occurrence for any
247 other EPN species in either region (not shown for central ridge). For central ridge sites, stepwise multiple
248 regression of each EPN species against NF and all other measured variables for those EPN species

commonly detected on the central ridge (*S. diaprepesi*, *H. indica* and *H. zealandica*) revealed significant positive relationships between EPNs and citrus sites, potassium, phosphorus, *Acrobeloides*-group, *H. rhossiliensis*, *P. lilacinus*, *Catenaria* sp. and *P. nicotianae*, and negative relationships between EPNs and sand content and *A. musiformis* (Table 4). Subsets of those independent variables explained 55%, 73% and 46% of the variation in *S. diaprepesi*, *H. indica* and *H. zealandica*, respectively. For flatwoods sites, regression of EPN species commonly encountered in the flatwoods (*H. indica*, *S. diaprepesi* and *Steinernema* sp.) showed positive relationships between EPNs and natural areas, GWD, calcium, boron, magnesium, copper, cation exchange capacity, *G. gephyropagum*, *P. lilacinus*, *Catenaria* sp., *P. nicotianae* and *Acrobeloides*-group, and inverse relationships between EPNs and silt and clay content, elevation, boron, manganese, iron and between *H. indica* and both of the steinernematid species (Table 3).

4. Discussion

As predicted, several NF species were detected at different frequency in nematode samples from the citrus orchards than were reported from natural areas nearby these orchards (Campos-Herrera et al., 2016). Each of those species (*A. oligospora*, *A. dactyloides*, *G. gephyropagum*, *Catenaria* sp.) tended to be detected in nearly half of the samples from orchards and fewer than 10% of natural sites. It is unknown whether these differences were due to the different survey times, sample processing (operator) variability or orchard *versus* natural area properties. A remarkably similar trend was reported from a Mediterranean region in which trapping fungi (primarily *A. oligospora*) and the endoparasitic *Catenaria anguliluae* were detected (by sprinkling soil on agar and baiting with nematodes) in nearly all samples from seven citrus orchards, but fewer than half of the samples from 19 nearby forests (Olivares-Bernabeu et al., 2003). If the occurrence of NF is generally greater in orchards, the potential to develop conservation biological control tactics for managing plant or animal parasitic nematodes with these fungi is evident. Several edaphic properties measured here that reflect the use of irrigation and fertilizers were significantly related to the abundance of each NF species both in the orchards and when orchard and natural area data were

pooled, and, therefore, merit controlled studies of their effects on these fungi (discussed below). Additionally, our orchard samples were gotten only where *Diaprepes* root weevil infestations were documented, whereas the natural sites were chosen without knowledge of soil arthropod host status. Campos-Herrera et al., (2012, 2016) reported close associations between EPNs, free-living nematodes and several species of NF, and noted that insect cadavers provide abundant resources to r-selected nematode species and their natural enemies. The lack of overall species diversity in monoculture orchards compared to natural areas may also have resulted in more EPN hosts in the orchards. Efron et al. (2001) reported a strong temporal correlation between seasonal abundance (host availability) of the grub *Maladera matrida* and steinernematids and heterorhabditids in citrus orchards in Israel.

We also confirmed that some spatial associations between EPNs and NF may have resulted from the ecoregion preferences of each species. SADIE association indices derived from georeferenced spatial patterns revealed consistently that the degree to which NF and EPN species were associated or disassociated was predictable by whether each tends to occupy habitats with deep or shallow water table. Nevertheless, in contrast to EPNs (Campos-Herrera et al., 2013, 2016), the regional differences between NF communities measured by either SADIE or PCA were very subtle. Only *P. lilacinus* and *G. gephyropagum* occurred significantly more frequently in flatwoods and central ridge, respectively. Neither species was inversely related to any EPN species, whether analyzed within or across ecoregions. Thus, whereas the community diversity of EPNs tended to be greatest on the central ridge where diversity of NF communities was the least, we found little additional evidence that NF infection rates influenced the regional occurrence of EPN species. Indeed, even within each ecoregion, the positive relationships between nematodes and fungi measured by stepwise regression suggest that EPNs may be an important resource for NF population growth, even if the NF measured here have little effect on the regional spatial distribution of the nematodes. Although *Steinernema* sp. tended to occur in sites with low incidence of most NF species, there were no significant correlations between the nematode and any NF species nor with the total NF communities in the sites. The dominant EPN *H. indica* was predictably, inversely related to its competitors *S. diaprepesi* and *Steinernema* sp. in the flatwoods, but significant relationships

between EPN species and NF species in flatwoods were all positive. A notable exception to this pattern occurred for an infrequently detected NF, *A. musiformis*, which was inversely related to the two dominant EPN species on the central ridge, *S. diaprepesi* and *H. indica*. However, *Catenaria* sp., *H. rhossiliensis* and *P. lilacinus* on the central ridge were positively associated with four EPN species between them. Put simply, the evidence from citrus orchards and their environs suggests that NF as a guild prey on EPNs at similar rates everywhere.

Arthrobotrys oligospora was the most frequently detected nematode-trapping species in both regions, consistent with numerous other reports from a broad range of habitats (Duddington, 1954; Shepherd, 1955; Persmark et al., 1996; Koppenhöfer et al., 1997; Durand et al., 2005; Farrell et al., 2006; Wachira et al., 2008). Similarly, Mitsui (1985) and Gray (1987) noted that net formers in general were the most frequently encountered species in numerous surveys on the occurrence of nematode trapping fungi. However, the constricting ring former *A. dactyloides*, as well as *P. lilacinus* and *Catenaria* species were equally abundant in nematode samples from these citrus groves. The more even detection of various NF guilds than reported previously may reflect the sampling method designed to measure predation as distinct from saprophagy (Pathak et al., 2012).

Other studies have shown positive relationships between nematodes and the endoparasitic, as well as the more parasitic nematode-trapping fungi, but only non-significant correlations with the more saprophytic nematode-trapping fungi (Jaffee et al., 1993; Persmark and Nordbring-Hertz, 1997). As noted previously, sampling to detect predation rather than saprophytic behavior in these fungi may be why positive relationships were evenly divided between obligate and facultative NF species in this study. A number of studies have also linked endoparasitic fungi to the regulation of some invertebrate communities in freshwater lakes and streams: mosquito larvae by *Coelomyces* (Whisler et al., 1974, 1975), nematodes by *Catenaria anguillule* (Gray, 1985), eggs of Chironomidae by various *Catenaria* species (Martin, 1981), and *Daphnia* sp. by the chitrid *Polycaryum* leave (Johnson et al., 2006). Martin (1984) showed that the population size of midges was controlled largely by *Catenaria* sp. and other factors in littoral communities. In contrast to those reports, our findings are consistent with those of Jaffee

et al. (1996) who found no relationship between spatial patterns of NF and those of *H. marelatus* which regulate ghost moths (*Hepialus californicus*) and the damage they cause in bush lupines. Jaffee et al (2007) also reported that resident nematodes and trapping fungi increased when dead moth larvae were added to the soil but this increase in fungus did not affect the probability of nematode mortality.

Our study implicated some abiotic factors as potentially affecting the composition of NF communities in the citrus orchards. Gray (1988) studied relationships between soil chemistry and NF in agricultural fields and noted that the effects of nutrient elements on NF are likely often indirect, mediated by relationships between nutrients and numbers of nematode prey. None of the variables that explained significant variation in NF species (AWC, EC, pH, P, K, or B) occurred at especially different levels in either ecoregion (Campos-Herrera et al., 2013), consistent with the lack of regionality in most NF species. Available water capacity was strongly associated with *Catenaria* sp., which requires moisture for zoospore function. Freeman et al. (2009) found chytrids to be the dominant group in high elevation fungal communities where soils are saturated for long periods due to snow cover. The positive association of *P. lilacinus* and AWC was consistent with the greater occurrence of that species in flatwoods orchards, whereas *A. oligospora* was also associated with AWC, but not with either region. Electrical conductivity (EC) and potassium (K) were significantly related (inversely and directly, respectively) to 5 of the 6 fungi. Salinity may have caused some of the relationships between NF and EC, but EC was also inversely correlated with pH and fungi often function better in acidic environments. Gray (1985) reported that the ring and knob-forming species were associated with soils with a low pH and the species with unmodified adhesive hyphae were isolated more frequently from soils with a higher pH. Therefore, low detection of NF at high EC may have been due to preference for low pH as was seen in *A. musiformis* and *G. gephyropagum*. This would unlikely be the case for *Catenaria* sp., however. Sayre and Keeley (1969) reported that in *C. anguillulae* infection rates of nematodes were highest at pH 9 and maximum growth rates for *C. anguillulae* in culture were between 8 and 9 (Nolan, 1985).

Phosphorus (P) content could have affected the *Catenaria* sp. and *A. oligosphora* infection rates. Phosphorus is an essential element for plant health that is vastly scarce in utilizable form (mainly

353 orthophosphates). The limited availability of P is due to the binding of phosphate anions with other
354 elements. Several studies revealed that chytrids can convert inorganic phosporous (phosphate ion) into
355 organic compounds (Willoughby 1962; Murray and Lovett 1966; Nolan 1970; Hassan and Catapane
356 2000; Gleason et al. 2006). It was also reported that available soil phosphorus may be a determinant of
357 chytrid community composition and diversity (Letcher et al. 2004). Midgley et al. (2006) showed that in
358 addition to insoluble phosphorous, chytrids can use DNA as a source of phosphorous and they speculated
359 that limited availability of phosphorus may influence the chytrids found in any habitat.

360 Lauber et al. (2008) reported that phylogenetic distance between fungal communities was
361 correlated with soil P concentrations and the C:N ratio. Their analyses of the relative abundances of
362 specific fungal taxa indicated that P-rich soils contained more Ascomycota and fewer Basidiomycota. In
363 our results, *A. oligospora* was the only ascomycete that correlated strongly with P content. The other four
364 ascomycetes showed little or no correlation with phosphorus content but were significantly positively
365 associated with potassium. Kumar et al (2005) reported that application of fertilizers (urea, di-ammonium
366 phosphate (DAP) and muriate of potash) reduced the population of nematode trapping fungi in the soil. In
367 Florida citrus orchards, phosphorus is supplied in form of ammonium phosphates (MAP or DAP),
368 superphosphate and NPK compound fertilizers that might be detrimental to sensitive fungi like *A.*
369 *dactyloides*. *Purpureocillium lilacinus* enhanced the availability of soil phosphorus in the presence of
370 calcium phosphates *in vitro* (Hernández-Leal et al, 2011). The lack of reported relationships between
371 phosphorus and other ascomycetes in our study may also reflect the sampling method.

372 The association of *A. dactyloides* with potassium is in accordance with Gray (1988) where
373 constricting ring forming species were correlated with soils containing high levels of K. In general,
374 nematophagous fungi in this study (except *G. gephyropagum*) are associated with major soil nutrients (P
375 and K) that also support large number of nematode hosts for the fungi (Gray, 1988). Eayre et al. (1987)
376 and Eayre et al. (1983) showed that K ions stimulated germination of *H. rhossiliensis* conidia and
377 infection of nematodes, but were unable to show a dose response to K or to increase nematode infection
378 by addition of KCl in field trials.

5. Conclusion

Few naturally occurring EPNs have been shown to be significant regulators of pests, and variables that modulate EPN services are generally poorly understood (Stuart et al., 2015; Lewis et al., 2015). By contrast, the EPNs that inhabit Florida citrus orchards are good targets for conservation tactics because they are especially virulent to *Diaprepes* root weevils on the central ridge where damage to citrus by the insect is least (Duncan et al., 2003; Futch et al., 2005). Moreover, controlled studies (Duncan et al., 2013; El-Borai et al., 2016) confirmed that relationships between abiotic soil properties and EPNs detected in citrus orchards and natural areas (Campos-Herrera et al., 2013, 2016) are potentially important in modulating the species composition of EPN communities. However, the possibility that NF communities extend a trophic cascade affecting citrus trees (NF→EPN→Diaprepes root weevil→citrus) differently in different ecoregions was not supported by this or a previous survey (Campos-Herrera et al., 2013). *Purpureocillium lilacinus*, the only species that predominates in the region where weevil damage is greatest, was not significantly related to EPNs across or within ecoregions The observation that *Steinernema* sp. tended to occur in sites depauperate of most NF species may have the most relevance to the question of EPN conservation. That species and *S. diaprepesi* are the most virulent to *Diaprepes* root weevil (El-Borai et al., 2012) and *Steinernema* sp. is the best adapted of the two to flatwoods conditions (Campos-Herrera et al., 2013, 2016; El-Borai et al., 2016). However, unlike *S. diaprepesi* which is virtually ubiquitous in orchards on the central ridge, *Steinernema* sp. occurs only rarely in flatwoods. Cultural practices that mitigate the biotic or abiotic constraints to *Steinernema* sp. could represent a profitable tactic for *Diaprepes* root weevil management.

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586

587 **Figure legends**

588 **Figure 1.** Results from Redundancy analysis (RDA) for the citrus regional survey (n = 53), considering
589 abiotic variables and entomopathogenic nematodes as explanatory variables. Together the first two
590 axes explain 50.9% of the variation in nematophagous fungi composition. The NF species are
591 represented by solid red arrows and are abbreviated as follows: *Arthrobotrys dactyoides* (*Ad*), *A.*
592 *oligospora* (*Ao*), *A. musiformis* (*Am*), *Gamsylella gephyropagum* (*Gg*), *Catenaria* sp. (*Cat*),
593 *Purpureocillium lilacinus* (*Pl*). The explanatory variables represented by solid black arrows ($P \leq$
594 0.05) are as follows: Sd (*Steinernema diaprepesi*), EC (electrical conductivity), P (Phosphorus), K
595 (Potassium), B (Boron), AWC and pH. The explanatory variables represented by dotted blue
596 arrows ($0.05 \geq P \leq 0.09$) are as follows GWD (ground water depth), OM (Organic matter), Cu
597 (Copper), Hi (*Heterorhabditis indica*) and sand.
598

599 **Figure 2.** Detection frequency (proportion positive sites) of the seven nematophagous fungi (NF) species
600 in both surveys involving natural areas (n = 91) and citrus orchards (n = 53), discriminating
601 between central ridge (CR) and flatwoods (FW) eco-regions. The species are abbreviated as
602 follows: *Arthrobotrys dactyoides* (*Ad*), *A. oligospora* (*Ao*), *A. musiformis* (*Am*), *Gamsylella*
603 *gephyropagum* (*Gg*), *Catenaria* sp. (*Cat*), *Purpureocillium lilacinus* (*Pl*), and *Hirsutella*
604 *rhossiliensis* (Hr). Values represent average ± SEM.
605

606 **Figure 3.** Principal components analysis (PCA) of the nematophagous fungi (NF) DNA recovered from
607 nematode samples from this survey of citrus orchards and from natural areas that were reported by
608 Campos-Herrera et al. (2016). Data from both surveys were pooled for analysis. Symbols are mean
609 and standard errors of square root transformed raw data. CR = central ridge and FW = flatwoods.
610
611 **Figure 4.** Ecological indices for the entomopathogenic nematodes (EPN) and nematophagous fungi (NF)
612 associated with nematodes in two ecoregions of the survey of citrus orchards and of natural areas
613 reported from by Campos-Herrera et al. (2013, 2016). Indices include species richness (S),
614 diversity (H') and evenness (J'). Values above and below dashed line are highest in central ridge
615 and flatwoods regions, respectively.
616
617 **Figure 5.** Linear relationships between the SADIE-derived association indices (Xa) for nematophagous
618 fungi (NF) species and ground water depth and Xa for NF species and EPN speciesAbbreviations
619 for EPN are *Heterorhabditis indica* (Hi), *H. zealandica* (Hz), *S. diaprepesi* (Sd), and *Steinernema*
620 sp. (Sx), and for NF are *Arthrobotrys dactyoides* (*Ad*), *A. oligospora* (*Ao*), *A. musiformis* (*Am*),
621 *Gamsylella gephyropagum* (*Gg*), *Catenaria* sp. (*Cat*), *Purpureocillium lilacinus* (*Pl*), and
622 *Hirsutella rhossiliensis* (Hr).
623
624 **Figure 6.** Mean and standard error of nematophagous fungi (NF) infection rates (normalized between 0-
625 1) in flatwoods sites with and without the entomopathogenic nematodes (EPN) species
626 *Heterorhabditis indica* (Hi), *Steinernema diaprepesi* (Sd) and *Steinernema* sp. (Sx). Panels A, C, E
627 are from the orchard survey and panels B, D, F are from the survey of natural areas.
628

Figures
Figure 1

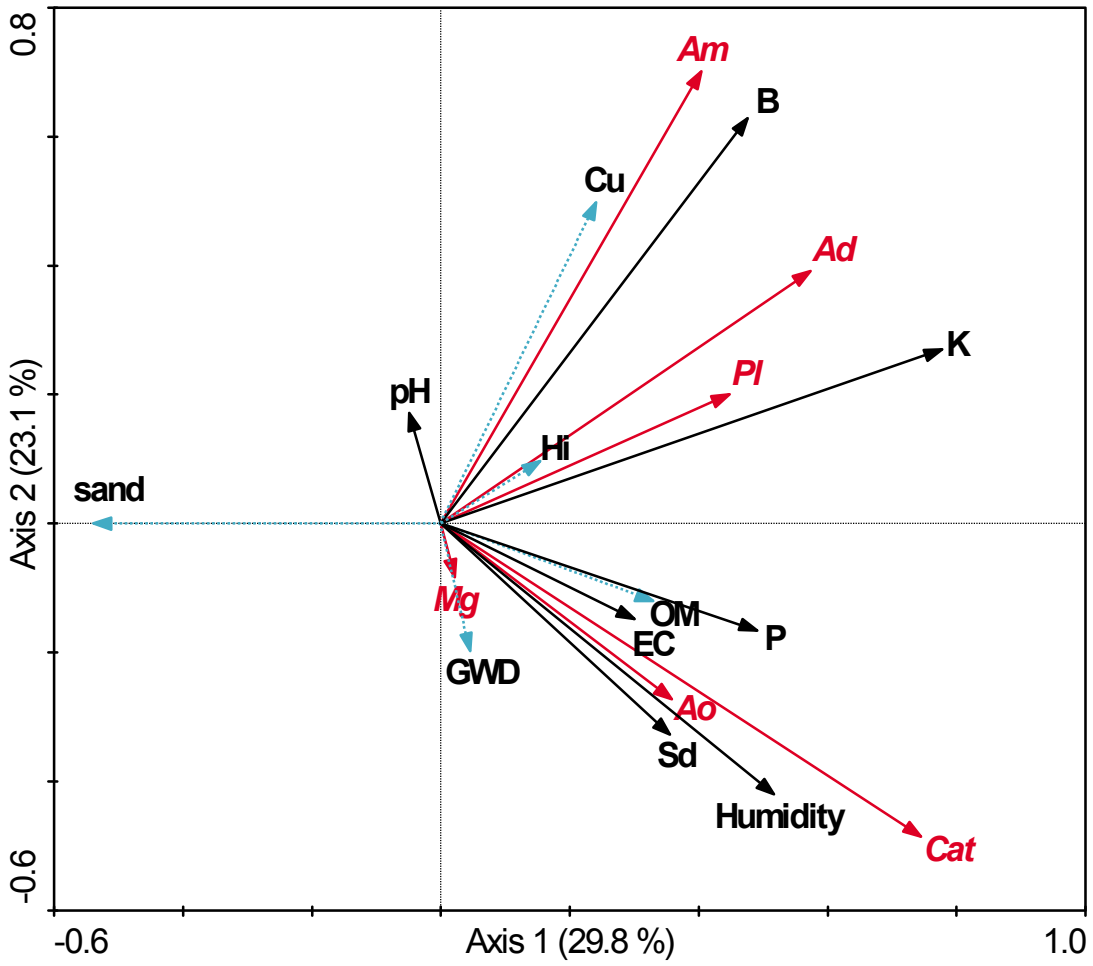


Figure 2

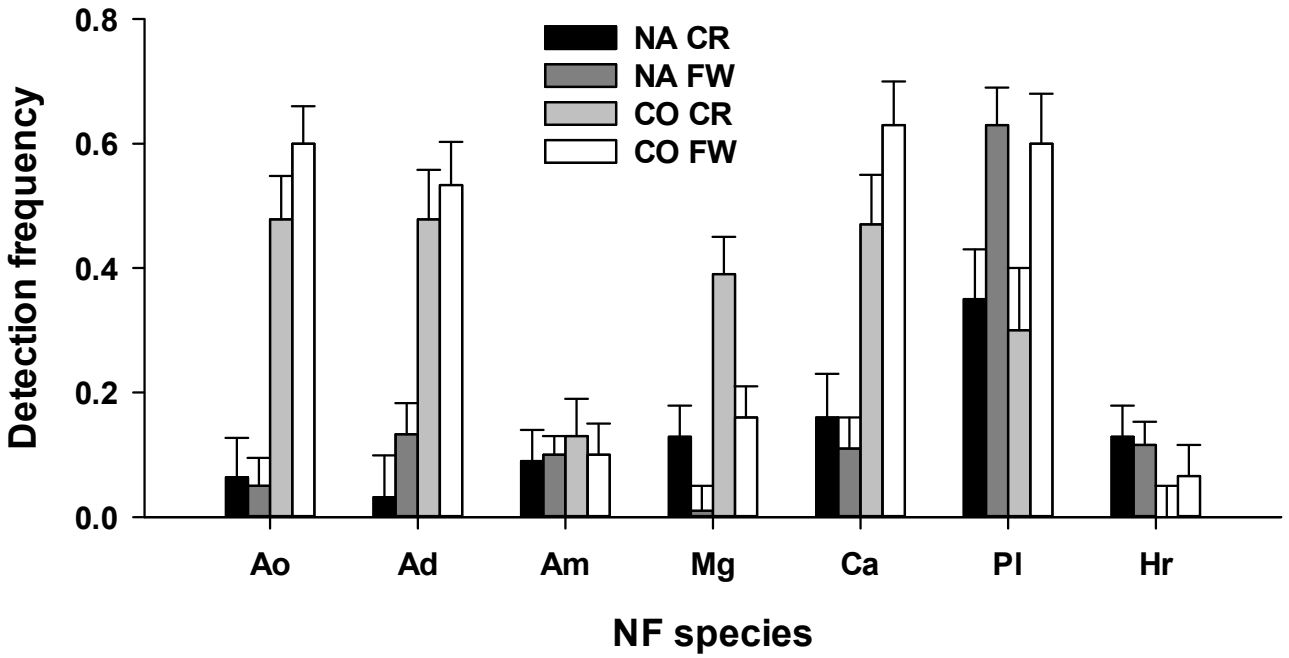

0.8
0.6
0.4
0.2
0.0
Detection frequency
NA CR
NA FW
CO CR
CO FW
Ao
Ad
Am
Mg
Ca
Pl
Hr
NF species

Figure 3

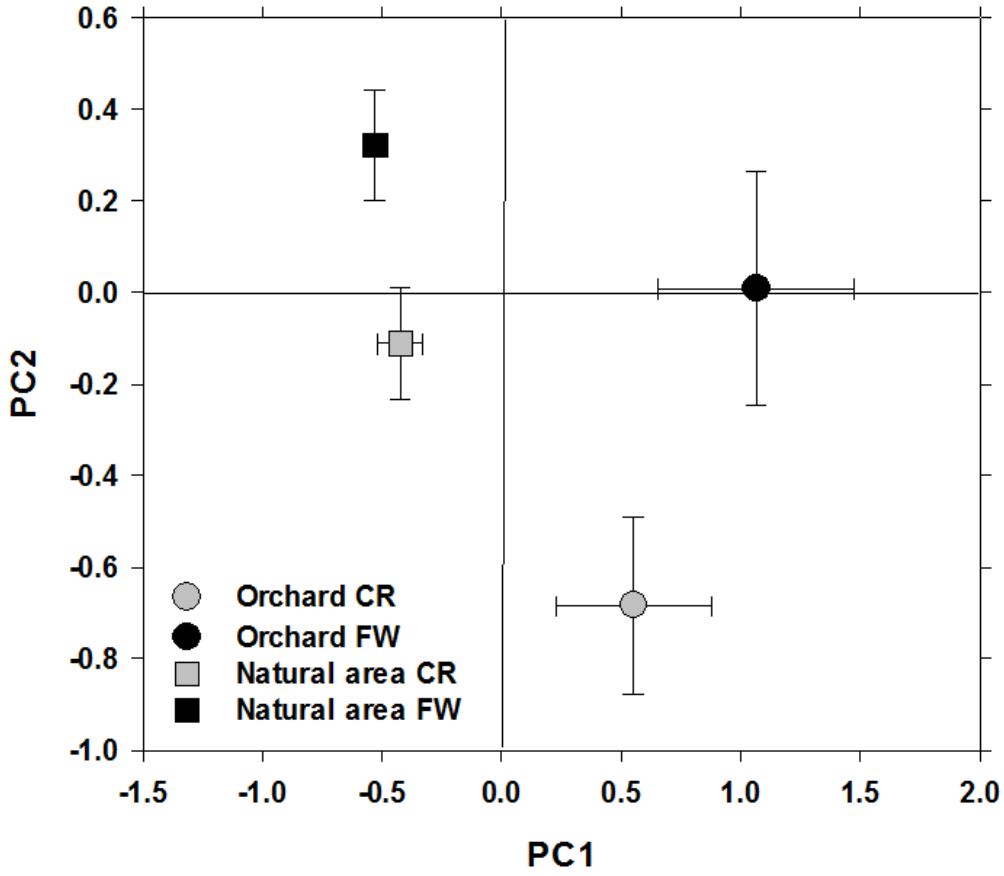

0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
PC2
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
PC1
Orchard CR
Orchard FW
Natural area CR
Natural area FW

Figure 4

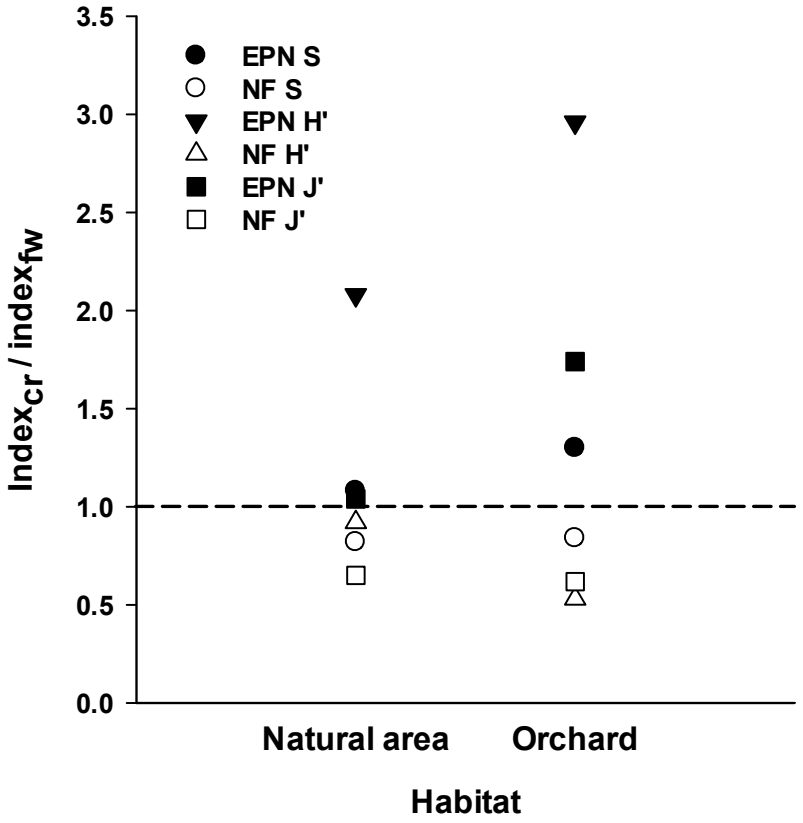

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EPN S
NF S
EPN H'
NF H'
EPN J'
NF J'
Index$_{cr}$ / index$_{fw}$
Natural area
Orchard
Habitat

Figure 5

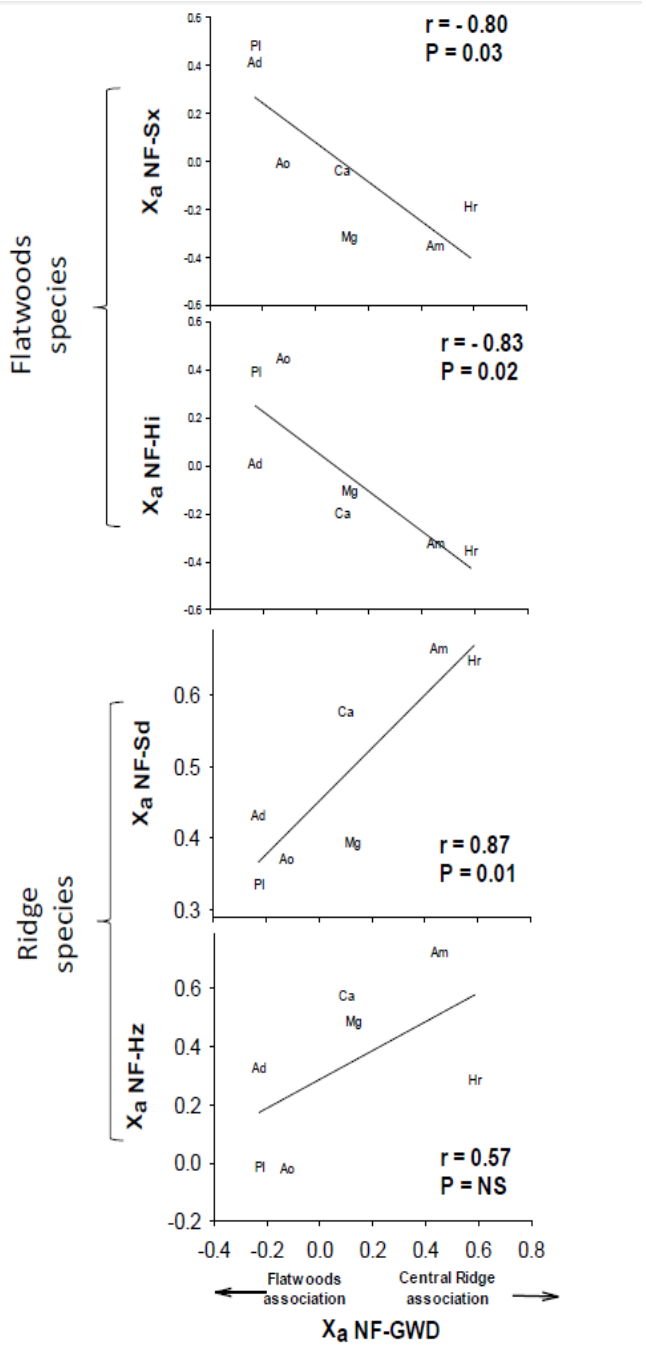
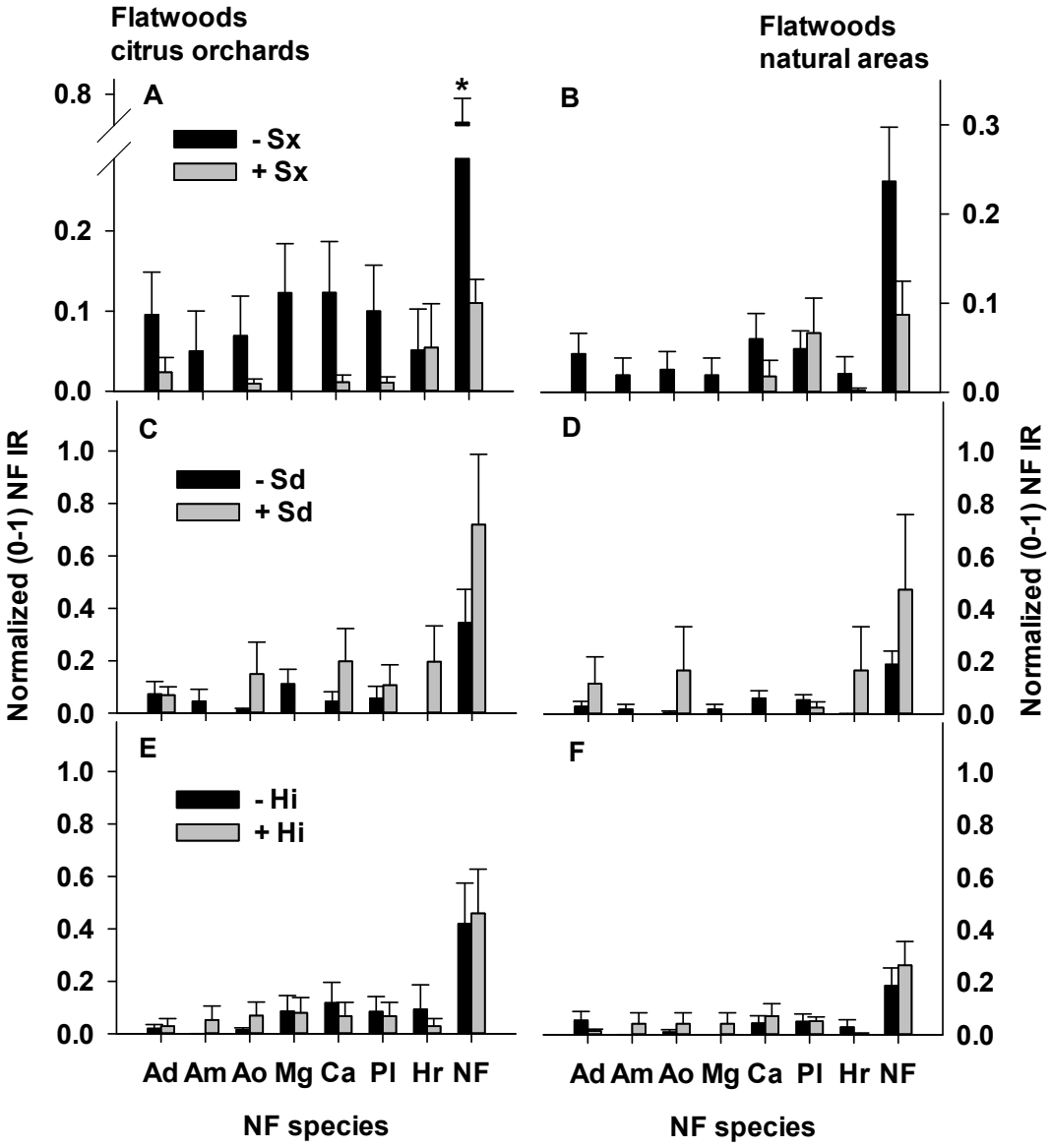

Flatwoods species
Ridge species
Xa NF-Sx
Xa NF-Hi
Xa NF-Sd
Xa NF-Hz
r = - 0.80
P = 0.03
r = - 0.83
P = 0.02
r = 0.87
P = 0.01
r = 0.57
P = NS
Pl
Ad
Ao
Ca
Hr
Mg
Am
Flatwoods association
Central Ridge association
Xa NF-GWD

Figure 6


Flatwoods
citrus orchards
Flatwoods
natural areas
A
B
C
D
E
F
- Sx
+ Sx
- Sd
+ Sd
- Hi
+ Hi
*
0.8
0.2
0.1
0.0
0.3
1.0
0.8
0.6
0.4
0.2
0.0
Normalized (0-1) NF IR
Ad Am Ao Mg Ca Pl Hr NF
NF species

Tables

Table 1. Frequency of detection of seven species of nematophagous fungi (NF) for the two region of the citrus production in Florida. Results of the T-test analysis (df = 51) for assessing differences between regions ($P \leq 0.05$).

	Frequency (%)			Abundance (ng/uL)		
NF species	Central ridge	Flatwoods	(*P*)	Central ridge	Flatwoods	(*P*)
A. dactyloides	50.0	51.6	n.s.	0.0332 ± 0.0225	0.0551 ± 0.0278	n.s.
A. musiformis	13.6	9.7	n.s.	0.00003 ± 0.00002	0.00725 ± 0.00725	n.s.
A. oligospora	50.0	58.0	n.s.	0.00085 ± 0.00044	0.00041 ± 0.000265	n.s.
G . gephyropagum	41.0	16.1	0.02	2.65 ± 1.30	1.065 ± 0.563	n.s.
Catenaria sp.	50.0	61.3	n.s.	0.00007 ± 0.00006	0.00037 ± 0.00019	n.s.
H. rhosiliensis	0.0	6.5	n.s.	0.0	0.0018 ± 0.00165	n.s.
P. lilacinus	32.0	58	0.03	0.00043 ± 0.00024	0.00115 ± 0.000378	< 0.01

Table 2. Ecological indices estimated for the nematophagous fungi (NF) occurrence in citrus orchards in two ecoregions of Florida. Results of the T-test analysis (df = 51) for assessing differences between regions ($P \leq 0.05$).

	Central Ridge	Flatwoods	T value (*P*)
Richness, S	2.6 ± 0.244	3.0 ± 0.226	n.s.
Diversity, H’	0.30 ± 0.073	0.56 ± 0.08	2.32 (0.02)
Evenness, J’	0.34 ± 0.066	0.55 ± 0.063	2.25 (0.03)

Table 3. T-values from step-wise regressions of nematophagous fungi (dependent variable) on environmental and biotic variables found significant in redundancy analysis (RDA). Significance is denoted by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). Fungi abbreviated as follows: *Arthrobotrys dactyoides* (*Ad*), *A. oligospora* (*Ao*), *A. musiformis* (*Am*), *Gamsylella gephyropagum* (*Gg*), *Catenaria* sp. (*Cat*), *Purpureocillium lilacinus* (*Pl*), and *Hirsutella rhossiliensis* (Hr).

	Ad	Am	Ao	Gg	Ca	Pl
AWC	-0.08	-0.93	2.14**	0.35	9.34***	2.29**
EC	-3.47***	-1.69*	-2.62**	-0.65	-3.27***	-4.10***
pH	0.48	-2.71***	0.91	-1.84*	2.59**	1.32
P	0.22	-0.73	2.80***	-0.28	1.99**	-1.08
K	5.34***	1.74*	1.72*	0.90	5.34***	5.59***
B	-0.21	6.95***	-1.96**	-0.92	-2.24**	-2.87***
Sd	1.39	-1.04	-0.23	-0.40	2.15**	0.00
Variation explained	48 %	66 %	23 %	4 %	78 %	36 %

Table 4.

Results of stepwise multiple regression of entomopathogenic nematodes (EPNs) on abiotic and biotic edaphic factors using data pooled from surveys of citrus orchards and adjacent natural areas. Significance is denoted by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

	Dependent variables					
	Central Ridge			Flatwoods		
Independent variables	*S. diaprepesi*	*H. indica*	*H. zealandica*	*S. diaprepesi*	*H. indica*	*Steinernema* sp. *glaseri*-group
R^2	55.5	43.3	45.8	84.2	57.5	23.5
Abiotic factors				-	-	-
Citrus vs Natural	-6.97***	-	-	3.62**	-	-
Elevation (masl)		-	-	-	-2.52*	-
GWD (m)		-	-	4.42***	-	-
Sand content (%)	-3.31**	-	-	-	-	-
Silt content (%)	-	-	-	-	-	-2.66**
Clay content (%)	-	-	-	-2.63**	-	-
CEC	-	-	-	-	2.56*	-
pH	-	-	-	-	6.44***	-
K	-	2.83**	-	-	-	-
P	-	-	2.9**	-	-	-

B	-	-	-	-4.16***	-	2.4*
Ca	-	-	-		-4.11***	-
Mg	-	-	-	10.63***	-	-
Cu	-	-	-	4.05***	-	-
Mn	-	-	-	-2.45*	-	-
Fe	-	-	-	-2.42*	-	-
Biotic factors						
EPN and FLN						
H. indica	-	-	-	-2.11*	-	-2.33*
Acrobeloides-group	-	-	2.11*	-	-	3.19**
Members of the citrus-EPN soil food web						
P. nicotianae	-	4.81***	-	7.04***	-	-
A. musiformis	-2.71**	-2.0*	-	-	-	-
A. oligospora	-	-	-	7.94***	-	-
Catenaria sp.	3.42**	-	-	-	-	-
H. rhossiliensis	-	3.36**	5.29***	8.01***	-	-
P. lilacinus	-	2.32**	-	-	-	-
G. gephirophagum	-	-	-	-	2.43*	-