1 **Spatial relationships between entomopathogenic nematodes and nematophagous fungi in**

2 **Florida citrus orchards**

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4 Ekta Pathak[a,b], Raquel Campos–Herrera[a,c,d], Fahiem E. El–Borai[a,e], Larry W. Duncan[a,*]

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6 *[a] Citrus Research and Education Center (CREC), University of Florida (UF), 700 Experiment Station*

7 *Road, Fl 33850, USA*

8 *[b] Sam Higginbottom Institute of Agriculture, Technology & Sciences, Allahabad, U.P., India*

9 *[c] Instituto de Ciencias Agrarias, CSIC, Serrano 115 Dpdo, Madrid 28006, Spain*

10 *[d] Current address: Centro para os Recursos Biológicos e Alimentos Mediterrânicos (MeditBio), Campus*

11 *Gambelas FCT Ed. 8 room 2.36.5, Universidade do Algarve, Faro 8005–139 (Portugal)*

12 *[e] Plant Protection Department, Faculty of Agriculture, Zagazig University, Egypt*

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14 * Corresponding author: Larry W. Duncan (lwduncan@ufl.edu)

15 *Citrus Research and Education Center (CREC), University of Florida (UF), 700 Experiment Station*

16 *Road, Fl 33850, USA*

17 Phone: +1 863 956111; Fax: +1 863 9564631

18   **Abstract**

19   Relationships between entomopathogenic nematodes (EPNs), nematophagous fungi (NF) and soil

20   physical and chemical properties were studied in a survey of 53 citrus orchards in central ridge and

21   flatwoods ecoregions of Florida. Seven species of NF associated with nematodes were quantified directly

22   using a real time qPCR assay. All nematophagous fungi studied except *Arthrobotrys musiformis*

23   and *Hirsutella rhossiliensis* were frequently detected (24-56%) in both regions. *Paecilomyces*

24   *lilacinus* and *Gamsylella gephyropagum* were encountered more frequently in the flatwoods (P = 0.03)

25   and on the ridge (P = 0.02), respectively. Redundancy analysis revealed seven abiotic and biotic factors as

26   significantly related to the NF occurrence. Multiple regression of fungi on these variables explained 78%,

27   66%, 48%, 36%, 23% and 4% of the variation in *Catenaria* sp., *A. musiformis*, A. *dactyloides*, *P.*

28   *lilacinus*, *A. oligospora* and *G. gepharopagum*, respectively. When the data from citrus were pooled with

29   those reported previously from natural areas and subjected to principle component analysis, the first two

30   principle components explained 43% of the variation in NF communities. The surveys (citrus vs natural

31   areas) were discriminated by PC2 ($P <0.001$) and the ecoregion by PC1 ($P <0.002$), and all but one NF

32   species were related ($P < 0.01$) to one or both components. NF communities tended to have more species

33   and greater diversity in the flatwoods, where EPN richness and diversity were the least. However, the

34   strength of associations between individual EPN and NF species as measured by SADIE reflected the

35   associations between each species and ground water depth, suggesting that ecoregion preferences affected

36   the species associations. Within each ecoregion, significant relationships between the individual NF and

37   EPN species measured by stepwise regression tended to be positive. The results did not support the

38   hypothesis that NF modulate the spatial patterns of EPN species between or within these two ecoregions.

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41   *Key word*s: Nematophagous fungi; quantitative real–time PCR; soil ecology; species–specific primers and

42   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

95   rates in the sand than the loam during four years. However, real time PCR assays of DNA from

96   nematodes isolated from soil samples showed that the NF species *Purpureocillium lilacinus* and

97   *Catenaria* sp. consistently infected nematodes at higher rates in sand than loam, whereas no NF species

98   appeared to be favored by conditions in the loam soil. Consequently, there is little evidence to date that

99   the spatial patterns of EPN species at a broad, landscape scale are modulated by variation in susceptibility

100   to NF across habitats.

101   The DNA of seven NF species recovered from samples of nematodes was identified and

102   quantified, but not reported, in a survey of EPNs in Florida citrus orchards (Campos-Herrera et al., 2013).

103   The agricultural inputs in those orchards created soil conditions very different than those of natural areas

104   in the same region reported by Campos-Herrera et al. (2016). Here we report the relationships between

105   NF and soil properties in the citrus orchards and compare them with those detected in the natural areas.

106   Results from both surveys were also combined to explore spatial relationships between NF, member of

107   the EPNs soil food web and abiotic factors. Our hypotheses were that 1) the occurrence frequency of

108   some NF species would differ between orchards and natural areas due to differences in soil chemistry and

109   management, 2) some spatial associations between species of EPNs and NF would be due to similar

110   habitat adaptations and therefore, 3) associations between NF and EPNs within a habitat might provide

111   better insights into the role played by NF in modulating EPN occurrence.

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113   **2. Materials and methods**

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115   *2.1. General survey design, sampling and soil processing*

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117   The survey area extended nearly 200 km east to west (from the gulf coast to the Atlantic coast)

118   and more than 300 km north to south (from Umatilla to Immokalee). The orchards were selected on the

119   basis that they were known to be infested by *Diaprepes abbreviatus* and that representative numbers were

120   located in each of the two ecoregions (23 sites in the central ridge and 30 in the flatwoods) in which

121 Florida's orchards occur. Sampling and sample processing methods are described in detail by Campos-

122 Herrera et al. (2013). Briefly, two samples (30 cores, 2.5-cm diam. x 30 cm deep per sample) were

123 obtained systematically at each 2-3 ha site during summer-early autumn 2009-2010. Samples were mixed

124 and nematodes extracted (Jenkins, 1964) from 2, 500-cm^3 subsamples (i.e., 2 liters of soil per site). DNA

125 was extracted using the UltraClean Soil DNA Extraction Kit (MoBio), quality was ensured (Nanodrop

126 system 1000 v.3.3.0, Thermo Scientific, Wilmington, DE, USA), and the quantity adjusted for further

127 molecular analysis. Following previous optimized protocols, seven species of NF were identified and

128 quantified using real-time qPCR approaches (Atkins et al., 2005; Zhang et al., 2006; Pathak et al., 2012).

129 Selected organisms previously reported in this survey by Campos-Herrera et al. (2013) were also

130 combined in the evaluation, which included the species specific qPCR quantification of EPNs (13

131 species), and several other nematodes, bacteria and oomycete phytopathogens, all of which are known to

132 interact with EPNs (Huang et al., 2010; Campos-Herrera et al., 2011a, 2011b, 2012; 2013)

133 (Supplementary data 1). In all the cases, primers and probes were synthesized by Integrated DNA

134 Technologies Inc. (IDT, San Diego, CA), with all TaqMan® PCR probes labelled with the fluorogenic

135 reporter dye FAM, the quencher Iowa Black™ FG and the ZEN molecule in the middle of the probe. Real

136 time qPCR runs were performed in ABI Prism 7000 (Applied Biosystem), in optical-well reaction plates

137 (USA Scientific, Orlando, FL, USA) with a final volume of 20 μL, and employing those reactive

138 materials and primers/probe concentrations, annealing temperature and number of cycles described

139 previously (Supplementary data 1). For each of the species, the identification and quantification was

140 performed using positive (standard curve), negative (sterile de-ionized water) controls and the

141 corresponding unknowns, with two technical replicates in each run.

142 Soil characteristics were described for the citrus survey (Campos-Herrera et al., 2013) and the

143 natural areas study (Campos-Herrera et al., 2016). Briefly, remaining soil was air dried and sand fractions

144 were determined by wet sieving (53 mm) followed by re-drying and processing on stacked sieves on a

145 mechanical shaker (W.S. Tyler Co., Mentor, OH). Soil pH, organic matter (OM), percentage sand, silt and

146 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

172 contributing significantly to the total spatial variability (data not shown) together with GWD. The

173 graphical results of the RDA were presented with bi-plot scaling (CANOCO 5). Stepwise multiple

174 regression analysis (Minitab 15, State College, PA) of each NF species on the independent variables

175 found to be significant by RDA was used to assess the relative explanatory contributions of those

176 variables.

177

178 *2.3. Analysis of fungal distribution and relationships in the combined citrus and natural area surveys*

179

180   The biotic and abiotic data from natural areas and citrus orchards reported by Campos-Herrera et

181 al. (2013, 2016) were combined with the NF data from citrus reported here for more comprehensive

182 analyses. NF communities were characterized by principal component analysis (PCA) of the square root-

183 transformed infection rates and subjected to ANOVA (GLM Minitab 15) to assess differences in the first

184 two principal components between 1) surveys and 2) ecoregions. For comparison, Spatial Analysis by

185 Distance Indices (SADIE; http://www.rothamsted.ac.uk/pie/sadie/SADIE-home_page_1.htm) was also

186 used to assess the degree of association between each NF species and each EPN species as well as NF

187 species and GWD (Spiridonov et al., 2007). The pooled data from the citrus and natural area surveys were

188 also subjected to RDA as described previously. Pooled data from within each ecoregion were used to

189 determine whether NF species occurrence differed (T-test, Minitab 15) in sites with or without a given

190 EPN species. Stepwise multiple regression of the pooled data within each ecoregion was used to assess

191 relationships between EPNs and NF.

192

193 **3. Results**

194

195 *3.1. Analysis of fungal distribution in the citrus survey*

196

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' $= 0.30 \pm 0.073$ vs 0.56 ± 0.08; t $= 2.32$, *P* $= 0.02$) and evenness (J' $= 0.34 \pm$

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

249 commonly detected on the central ridge (*S. diaprepesi, H. indica* and *H. zealandica*) revealed significant

250 positive relationships between EPNs and citrus sites, potassium, phosphorus, *Acrobeloides*-group, *H.*

251 *rhossiliensis, P. lilacinus, Catenaria* sp. and *P. nicotianae*, and negative relationships between EPNs and

252 sand content and *A. musiformis* (Table 4). Subsets of those independent variables explained 55%, 73%

253 and 46% of the variation in *S. diaprepesi, H. indica* and *H. zealandica,* respectively. For flatwoods sites,

254 regression of EPN species commonly encountered in the flatwoods (*H. indica, S. diaprepesi* and

255 *Steinernema* sp.) showed positive relationships between EPNs and natural areas, GWD, calcium, boron,

256 magnesium, copper, cation exchange capacity, *G. gephyropagum, P. lilacinus, Catenaria* sp.*, P.*

257 *nicotianae* and *Acrobeloides*-group, and inverse relationships between EPNs and silt and clay content,

258 elevation, boron, manganese, iron and between *H. indica* and both of the steinernematid species (Table 3).

259

260 **4. Discussion**

261

262　　　　As predicted, several NF species were detected at different frequency in nematode samples from

263 the citrus orchards than were reported from natural areas nearby these orchards (Campos-Herrera et al.,

264 2016). Each of those species (*A. oligospora, A. dactyloides, G. gephyropagum, Catenaria* sp.) tended to

265 be detected in nearly half of the samples from orchards and fewer than 10% of natural sites. It is unknown

266 whether these differences were due to the different survey times, sample processing (operator) variability

267 or orchard *versus* natural area properties. A remarkably similar trend was reported from a Mediterranean

268 region in which trapping fungi (primarily *A. oligospora*) and the endoparasitic *Catenaria anguliluae* were

269 detected (by sprinkling soil on agar and baiting with nematodes) in nearly all samples from seven citrus

270 orchards, but fewer than half of the samples from 19 nearby forests (Olivares-Bernabeu et al., 2003). If

271 the occurrence of NF is generally greater in orchards, the potential to develop conservation biological

272 control tactics for managing plant or animal parasitic nematodes with these fungi is evident. Several

273 edaphic properties measured here that reflect the use of irrigation and fertilizers were significantly related

274 to the abundance of each NF species both in the orchards and when orchard and natural area data were

275   pooled, and, therefore, merit controlled studies of their effects on these fungi (discussed below).

276   Additionally, our orchard samples were gotten only where *Diaprepes* root weevil infestations were

277   documented, whereas the natural sites were chosen without knowledge of soil arthropod host status.

278   Campos-Herrera et al., (2012, 2016) reported close associations between EPNs, free-living nematodes and

279   several species of NF, and noted that insect cadavers provide abundant resources to r-selected nematode

280   species and their natural enemies. The lack of overall species diversity in monoculture orchards compared

281   to natural areas may also have resulted in more EPN hosts in the orchards. Efron et al. (2001) reported a

282   strong temporal correlation between seasonal abundance (host availability) of the grub *Maladera matrida*

283   and steinernematids and heterorhabditids in citrus orchards in Israel.

284   We also confirmed that some spatial associations between EPNs and NF may have resulted from

285   the ecoregion preferences of each species. SADIE association indices derived from georeferenced spatial

286   patterns revealed consistently that the degree to which NF and EPN species were associated or

287   disassociated was predictable by whether each tends to occupy habitats with deep or shallow water table.

288   Nevertheless, in contrast to EPNs (Campos-Herrera et al., 2013, 2016), the regional differences between

289   NF communities measured by either SADIE or PCA were very subtle. Only *P. lilacinus* and *G.*

290   *gephyropagum* occurred significantly more frequently in flatwoods and central ridge, respectively.

291   Neither species was inversely related to any EPN species, whether analyzed within or across ecoregions.

292   Thus, whereas the community diversity of EPNs tended to be greatest on the central ridge where diversity

293   of NF communities was the least, we found little additional evidence that NF infection rates influenced

294   the regional occurrence of EPN species. Indeed, even within each ecoregion, the positive relationships

295   between nematodes and fungi measured by stepwise regression suggest that EPNs may be an important

296   resource for NF population growth, even if the NF measured here have little effect on the regional spatial

297   distribution of the nematodes. Although *Steinernema* sp. tended to occur in sites with low incidence of

298   most NF species, there were no significant correlations between the nematode and any NF species nor

299   with the total NF communities in the sites. The dominant EPN *H. indica* was predictably, inversely

300   related to its competitors *S. diaprepesi* and *Steinernema* sp. in the flatwoods, but significant relationships

301   between EPN species and NF species in flatwoods were all positive. A notable exception to this pattern

302   occurred for an infrequently detected NF, *A. musiformis,* which was inversely related to the two dominant

303   EPN species on the central ridge, *S. diaprepesi* and *H. indica*. However, *Catenaria* sp., *H. rhossiliensis*

304   and *P. lilacinus* on the central ridge were positively associated with four EPN species between them. Put

305   simply, the evidence from citrus orchards and their environs suggests that NF as a guild prey on EPNs at

306   similar rates everywhere.

307   *Arthrobotrys oligospora* was the most frequently detected nematode-trapping species in both

308   regions, consistent with numerous other reports from a broad range of habitats (Duddington, 1954;

309   Shepherd, 1955; Persmark et al., 1996; Koppenhöfer et al., 1997; Durand et al., 2005; Farrell et al., 2006;

310   Wachira et al., 2008). Similarly, Mitsui (1985) and Gray (1987) noted that net formers in general were the

311   most frequently encountered species in numerous surveys on the occurrence of nematode trapping fungi.

312   However, the constricting ring former *A. dactyloides*, as well as *P. lilacinus* and *Catenaria* species were

313   equally abundant in nematode samples from these citrus groves. The more even detection of various NF

314   guilds than reported previously may reflect the sampling method designed to measure predation as

315   distinct from saprophagy (Pathak et al., 2012).

316   Other studies have shown positive relationships between nematodes and the endoparasitic, as well

317   as the more parasitic nematode-trapping fungi, but only non-significant correlations with the more

318   saprophytic nematode-trapping fungi (Jaffee et al., 1993; Persmark and Nordbring-Hertz, 1997). As

319   noted previously, sampling to detect predation rather than saprophytic behavior in these fungi may be

320   why positive relationships were evenly divided between obligate and facultative NF species in this study.

321   A number of studies have also linked endoparasitic fungi to the regulation of some invertebrate

322   communities in freshwater lakes and streams: mosquito larvae by *Coelomyces* (Whisler et al., 1974,

323   1975), nematodes by *Catenaria anguillule* (Gray, 1985), eggs of Chironomidae by various *Catenaria*

324   species (Martin, 1981), and *Daphnia* sp. by the chitrid *Polycaryum* leave (Johnson et al., 2006). Martin

325   (1984) showed that the population size of midges was controlled largely by *Catenaria* sp. and other

326   factors in littoral communities. In contrast to those reports, our findings are consistent with those of Jaffee

327 et al. (1996) who found no relationship between spatial patterns of NF and those of *H. marelatus* which

328 regulate ghost moths (*Hepialus californicus*) and the damage they cause in bush lupines. Jaffee et al

329 (2007) also reported that resident nematodes and trapping fungi increased when dead moth larvae were

330 added to the soil but this increase in fungus did not affect the probability of nematode mortality.

331   Our study implicated some abiotic factors as potentially affecting the composition of NF

332 communities in the citrus orchards. Gray (1988) studied relationships between soil chemistry and NF in

333 agricultural fields and noted that the effects of nutrient elements on NF are likely often indirect, mediated

334 by relationships between nutrients and numbers of nematode prey. None of the variables that explained

335 significant variation in NF species (AWC, EC, pH, P, K, or B) occurred at especially different levels in

336 either ecoregion (Campos-Herrera et al., 2013), consistent with the lack of regionality in most NF species.

337 Available water capacity was strongly associated with *Catenaria* sp., which requires moisture for

338 zoospore function. Freeman et al. (2009) found chytrids to be the dominant group in high elevation fungal

339 communities where soils are saturated for long periods due to snow cover. The positive association of *P.*

340 *lilacinus* and AWC was consistent with the greater occurrence of that species in flatwoods orchards,

341 whereas *A. oligospora* was also associated with AWC, but not with either region. Electrical conductivity

342 (EC) and potassium (K) were significantly related (inversely and directly, respectively) to 5 of the 6

343 fungi. Salinity may have caused some of the relationships between NF and EC, but EC was also inversely

344 correlated with pH and fungi often function better in acidic environments. Gray (1985) reported that the

345 ring and knob-forming species were associated with soils with a low pH and the species with unmodified

346 adhesive hyphae were isolated more frequently from soils with a higher pH. Therefore, low detection of

347 NF at high EC may have been due to preference for low pH as was seen in *A. musiformis* and *G.*

348 *gephyropagum*. This would unlikely be the case for *Catenaria* sp., however. Sayre and Keeley (1969)

349 reported that in *C. anguillulae* infection rates of nematodes were highest at pH 9 and maximum growth

350 rates for *C. anguillulae* in culture were between 8 and 9 (Nolan, 1985).

351   Phosphorus (P) content could have affected the *Catenaria* sp. and *A. oligosphora* infection rates.

352 Phosphorus is an essential element for plant health that is vastly scarce in utilizable form (mainly

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

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

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

379

5. Conclusion

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382   Few naturally occurring EPNs have been shown to be significant regulators of pests, and
383 variables that modulate EPN services are generally poorly understood (Stuart et al., 2015; Lewis et al.,
384 2015). By contrast, the EPNs that inhabit Florida citrus orchards are good targets for conservation tactics
385 because they are especially virulent to *Diaprepes* root weevils on the central ridge where damage to citrus
386 by the insect is least (Duncan et al., 2003; Futch et al., 2005). Moreover, controlled studies (Duncan et al.,
387 2013; El-Borai et al., 2016) confirmed that relationships between abiotic soil properties and EPNs
388 detected in citrus orchards and natural areas (Campos-Herrera et al., 2013, 2016) are potentially important
389 in modulating the species composition of EPN communities. However, the possibility that NF
390 communities extend a trophic cascade affecting citrus trees (NF→EPN→Diaprepes root weevil→citrus)
391 differently in different ecoregions was not supported by this or a previous survey (Campos-Herrera et al.,
392 2013). *Purpureocillium lilacinus,* the only species that predominates in the region where weevil damage
393 is greatest, was not significantly related to EPNs across or within ecoregions The observation that
394 *Steinernema* sp. tended to occur in sites depauperate of most NF species may have the most relevance to
395 the question of EPN conservation. That species and *S. diaprepesi* are the most virulent to *Diaprepes* root
396 weevil (El-Borai et al., 2012) and *Steinernema* sp. is the best adapted of the two to flatwoods conditions
397 (Campos-Herrera et al., 2013, 2016; El-Borai et al., 2016). However, unlike *S. diaprepesi* which is
398 virtually ubiquitous in orchards on the central ridge, *Steinernema* sp. occurs only rarely in flatwoods.
399 Cultural practices that mitigate the biotic or abiotic constraints to *Steinernema* sp. could represent a
400 profitable tactic for *Diaprepes* root weevil management.

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Figure legends

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

Figure 2. Detection frequency (proportion positive sites) of the seven nematophagous fungi (NF) species in both surveys involving natural areas (n = 91) and citrus orchards (n = 53), discriminating between central ridge (CR) and flatwoods (FW) eco-regions. The species are 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). Values represent average ± SEM.

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

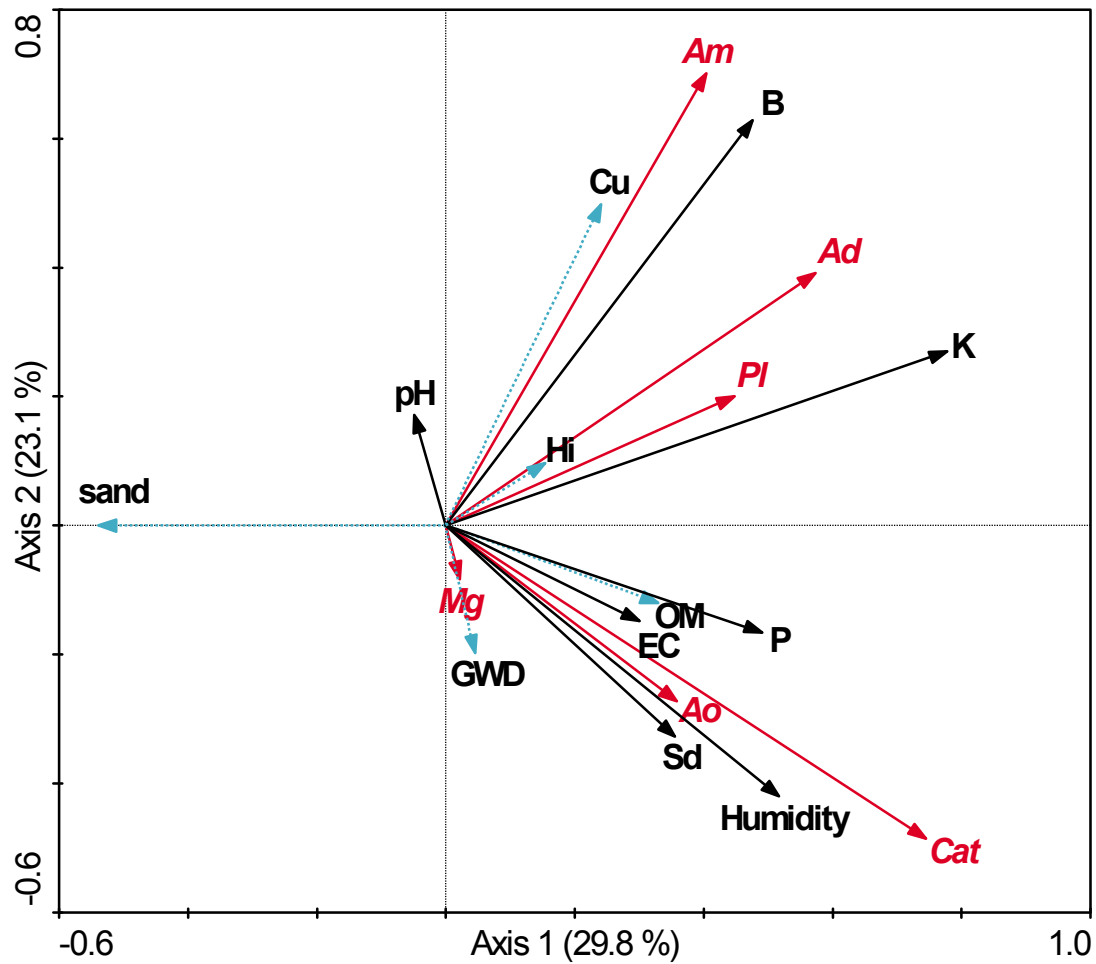627   are from the orchard survey and panels B, D, F are from the survey of natural areas.
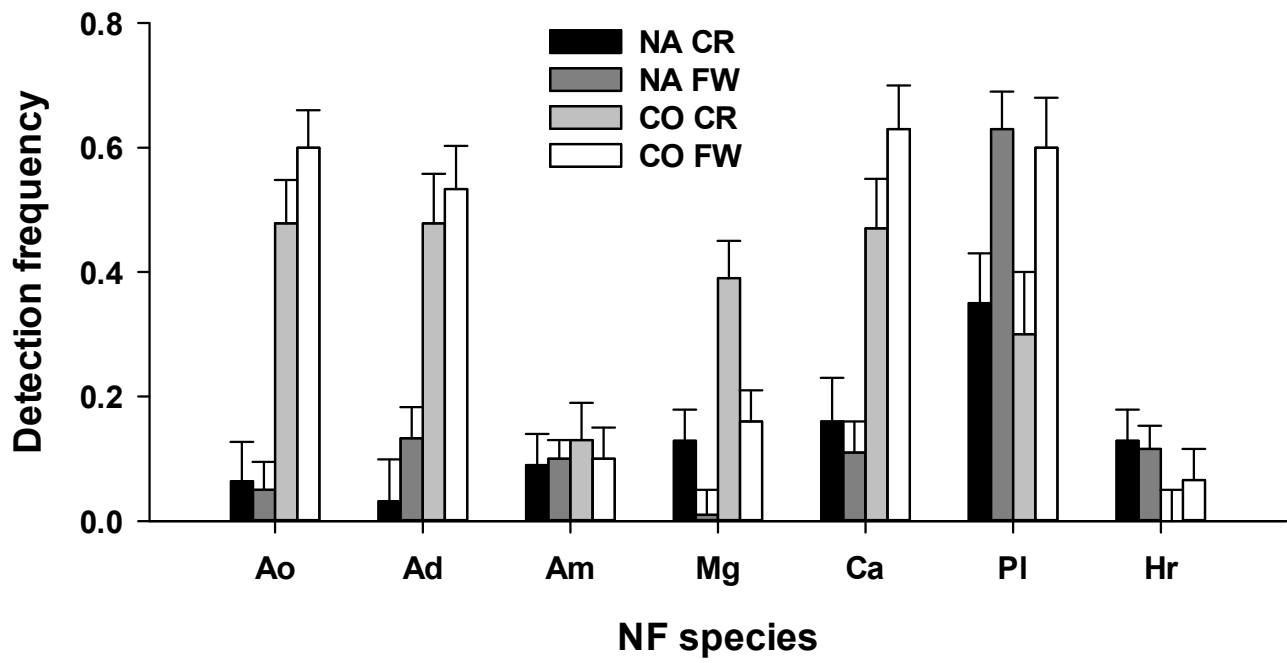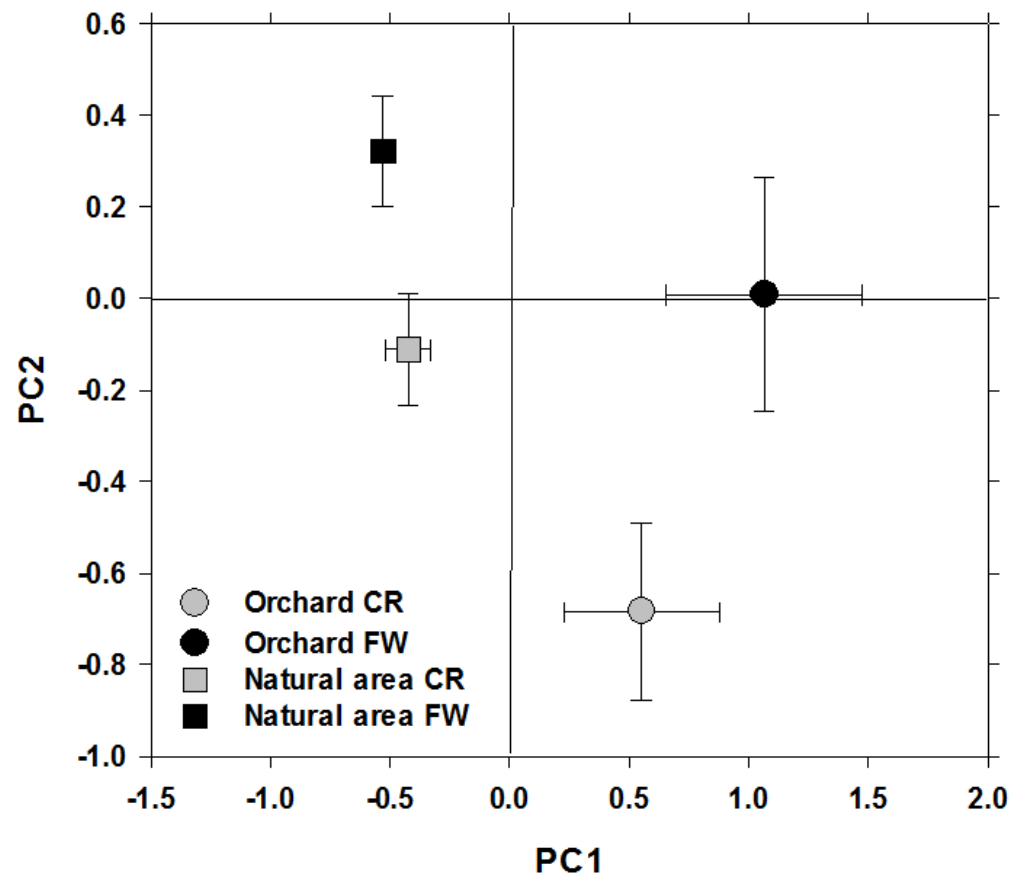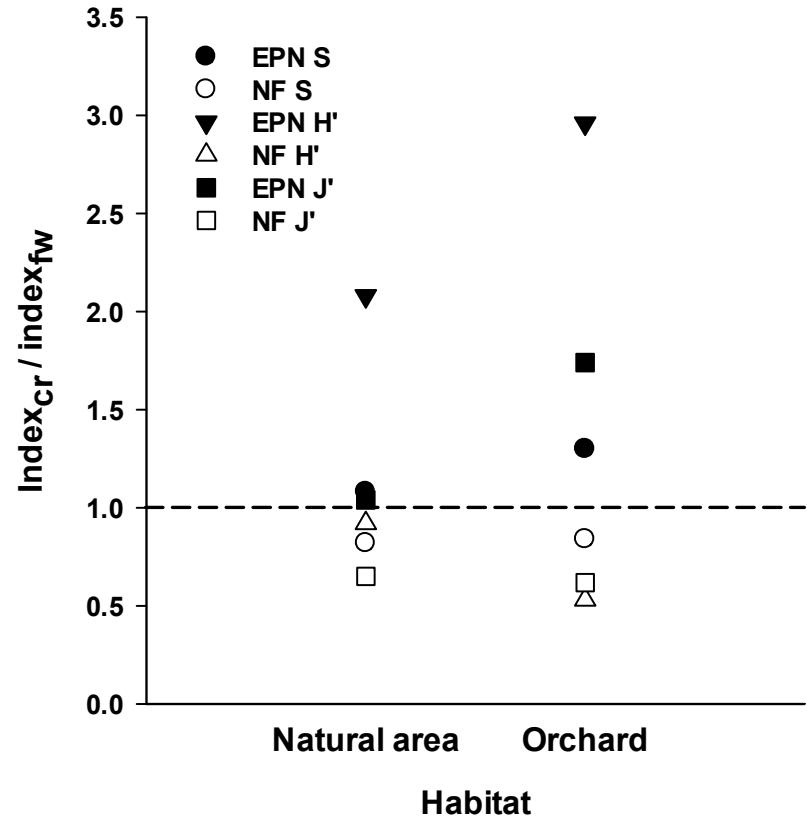
628

Figures
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5
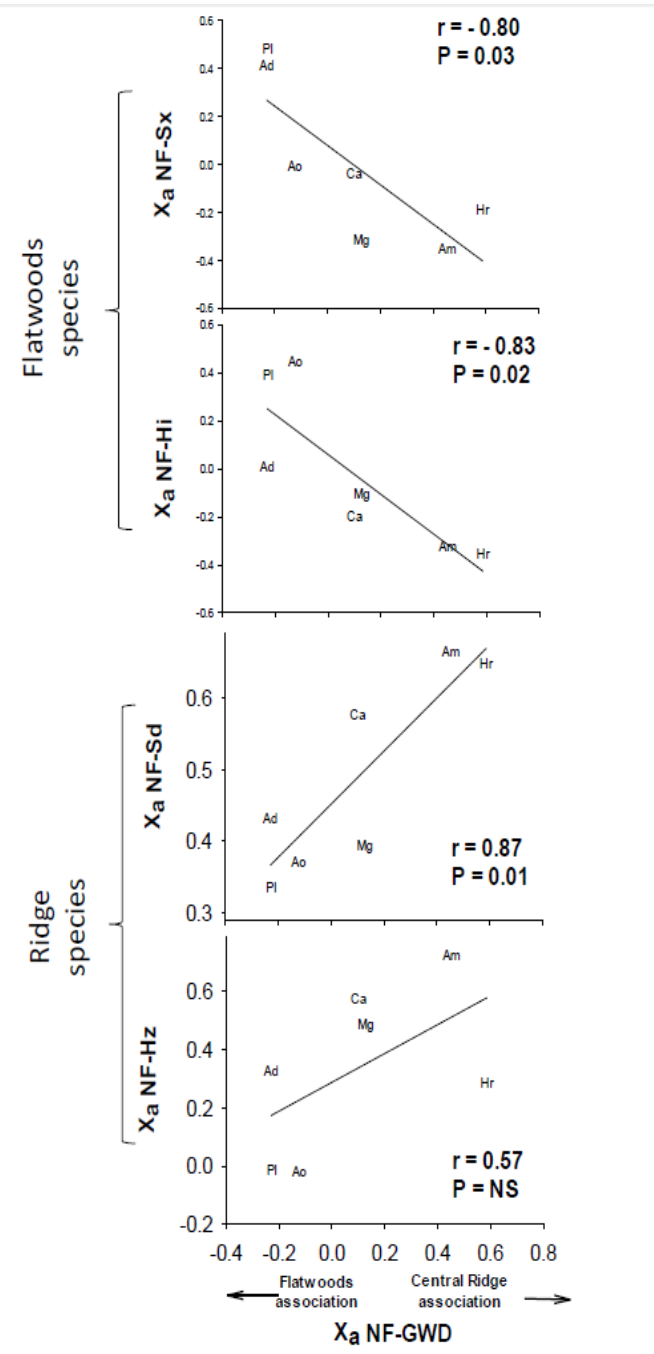
Figure 6

Flatwoods
citrus orchards

Flatwoods
natural areas

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 ($P \leq 0.05$).

NF species	Frequency (%)			Abundance (ng/uL)		
	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 *** ($P < 0.001$).

Independent variables	Dependent variables					
	Central Ridge			Flatwoods		
	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**	-	-	-	-
<mark>*G. gephirophagum*</mark>	-	-	-	-	2.43*	-