1 Cultivation and domestication of highbush blueberry (*Vaccinium*
2 *corymbosum*) alters abundance, diversity and virulence of entomopathogenic
3 nematodes

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Abstract:

We investigated the interactive effect of plant domestication and perennial cultivation on abundance, diversity and virulence of entomopathogenic nematodes (EPN) associated with highbush blueberry (*Vaccinium corymbosum*). The rhizospheric soil of *V. corymbosum* plants was sampled in coupled wild and cultivated sites within the Pinelands National Reserve, New Jersey, USA from 2011-2013 at three points during each year. To isolate and assess abundance of EPN communities, soil samples were baited with wax moth larvae (*Galleria mellonella*). A subsample of EPNs isolated from the soil was identified using molecular barcoding. The virulence of three native isolates against oriental beetle, *Anomala orientalis*, larvae was assessed at multiple densities of nematodes per host. A cursory assessment of EPN diversity was based on variation in molecular barcodes because many sequences could not be identified in the genomic database GenBank. The variation among barcodes suggested that diversity was higher in rhizospheric soil associated with wild plants than that of cultivated fields. *Steinernema glaseri* was the only EPN species able to be identified using molecular methods and was the only species isolated from the six cultivated fields with EPN presence. An interaction of environment, sampling point, and year explained significant differences in EPN abundance in 2011 and 2012 when soil samples were assessed by plant. However, in 2013, soil was assessed by field rather than plant and our results show EPN abundance was higher in cultivated than in wild sites. Soil nutrients and characteristics were also assessed in 2013. A PCA analysis of soil characteristics linked higher plant nutrients to soil from cultivated fields and higher soil moisture, organic matter, boron and lower pH to wild plant-associated soil. In this study, cultivation of domesticated *V. corymbosum* increases the abundance of the more virulent EPN species against the presumed host, *A. orientalis* but appears to eliminate genetic variability from the EPN community.

1. Introduction

The development of sustainable approaches to soil conservation necessitates an increased understanding of how land management practices impact the services soil can provide for humans and others (Doran, 2002). Agricultural production of plants is known to change the abundance and diversity of communities of aboveground insect herbivores and their predators and parasitoids (Matson et al., 1997; Poelman et al., 2008). It is difficult, however, to determine the true effect of plant domestication and land alteration for agricultural use on interactions in the agroecosystem without investigation of and comparison to undisturbed wild populations of plants; this is especially true for associated soil communities held in delicate balance with one another.

Nematodes are diverse and prevalent throughout soil communities worldwide. Within this taxon many feeding habits are found that occur at multiple trophic levels making nematodes an excellent bioindicator of disturbance to the soil ecosystem (Bongers and Ferris, 1999; Bongers, 1990; Yeates et al., 1993). Thus, the diversity of nematode species present is often used to evaluate the effects of land management practices (Freckman and Ettema, 1993; Millar and Barbercheck, 2002; Morise et al., 2012). The effect of altered soil structure through agriculturally focused land management on nematode communities is well studied and the effects of disturbance for this purpose (e.g., tillage, perennial or annual cultivation) are relatively clear (Erb and Lu, 2013; Govaerts et al., 2007; Sánchez-Moreno and Ferris, 2007; Vonk et al., 2013). In spite of this, the disturbance of soil is only a single factor in the alteration of land for agricultural production.

Alongside soil disturbance, plant domestication and the subsequent establishment of large plant monocultures are two other notable modifications to the land caused by agricultural production. Increasingly, plant genotype and species identity is being linked to the diversity and functionality of soil communities (Bezemer et al., 2010; Kostenko et al., 2015; Van der Putten et

al., 2001; Wardle et al., 2003; Zak et al., 2003). Plant domestication is largely based on the artificial selection and enhancement of human-desired traits. The consequent changes to a plant's genome are substantial due to selection from a relatively small population of plants (i.e., genetic drift) which causes continued bottlenecking as a limited diversity of plant stock is used for breeding to accentuate specific traits (Chen et al., 2015). This can cause indirect and sometimes negative effects on the plant's ability to respond and defend itself against belowground attacks (Chen et al., 2015; Degenhardt et al., 2009). As knowledge of the connection between above and belowground interactions expands, the effect of cultivation and plant domestication on soil organisms has become a point of interest (Palomares-Rius et al., 2015; Rivera et al., 2015; Sánchez-Moreno and Ferris, 2007). While the impact of plant domestication has been assessed previously, usually indirectly, it is often hard to access wild populations of plants at their point of origin to directly study the effects of domestication and cultivation. This study investigates the effect of domestication and cultivation on nematodes that feed at the third trophic level, i.e., entomopathogenic nematodes (EPN) with the hypothesis that the dynamics of the belowground arena would mirror effects found in the aboveground arena with higher trophic levels being more responsive to disturbance (Jonsson et al., 2012).

EPNs are obligate parasites of soil-dwelling insects and other arthropods. In natural systems, EPNs can have significant top-down effects on insect populations (Strong et al., 1996) and thus, in agricultural systems, are considered promising biological control agents for insect pest control (Denno et al., 2008; Gaugler et al., 1997). EPNs are active in the soil only as infective juveniles (IJs) that carry with them a symbiotic bacterium released inside an insect host to cause septicemia. The IJ is a special dauer-like stage that is non-feeding but persists in the soil until a host is located. Upon locating a host, the IJ must then enter the host through any opening (e.g.,

mouth, anus, spiracles etc.). Once inside the host's body cavity, the bacterium is released and the success of the EPN is dependent on the ability of the bacterium-nematode complex to overcome the insect's immune system. A successful infection will result in up to three EPN life cycles occurring in the cadaver as they feed on the multiplying symbiotic bacteria and host tissues broken down by the bacteria. Once resources in the cadaver are depleted, hundreds to hundreds of thousands of IJs exit into the surrounding soil and search for a new host. This search is directed by a variety of cues such as carbon dioxide and other volatile organic compounds emitted by insects (Lewis et al., 1993, 2009) and by plant roots (Turlings et al., 2012). Because EPN respond to cues from soil insects and plant roots, we hypothesized that diversity and functionality of EPN communities would diverge between wild and cultivated plants of the same species.

We used the highbush blueberry (*Vaccinium corymbosum* L.) agroecosystem in southern New Jersey, USA to explore the effects of plant domestication on belowground interactions. This system is quite unique in that *V. corymbosum* was first domesticated and grown commercially there in the early twentieth century. As a result of its recent domestication, wild and cultivated multispecies hybrids of *V. corymbosum* are still considered the same species (Bian et al., 2014; Eck, 1965). In contrast, many domesticated plants are such severe mutants of their parental lines they can no longer function as the same species (i.e., cannot successfully cross-pollinate one another to produce viable offspring). Relatively shortly after commercial production began, legislation was put into effect that protected the wild plants in the unique ecosystem surrounding this region. The legislation was established in 1978 as the United States National Parks and Recreation Act, which denoted the area as the "Pinelands National Reserve" (boundaries shown in Figure 1). This spurred the later and local 1979 New Jersey Pinelands Protection Act, that regulates the boundaries, land management and minimum standards for land use within the

Pinelands National Reserve (Pinelands Commission, 2011; Pinelands Comprehensive Management Plan, 2014). This legislation acknowledges the economic importance and cultural connection of blueberry and cranberry production in this region by classifying areas within the reserve as “Special Agriculture Production Areas”. These two acts mandated the preservation of wild stands in this region and uniquely allowed for the production of domesticated cultivars nearby protected wild stands, making this an ideal system to study the interactive effects of cultivation practices and plant domestication on EPN communities. The objective of this study was to compare EPN diversity and functionality of EPN communities in wild and domesticated *V. corymbosum* plants within the Pineland National Reserve according to plant phenology over multiple years.

2. Material and Methods

2.1 Field selection and soil sampling

Field sites were selected within the boundaries of the Pinelands National Reserve (Burlington and Atlantic Counties, New Jersey, USA) based on two criteria: 1. A history of high pheromone trap captures of adult male oriental beetles, *Anomala orientalis* Waterhouse (Coleoptera: Scarabaeidae), the most prevalent soil-dwelling pest of blueberry (Dean Polk, Rutgers Fruit IPM Coordinator, Rutgers Cooperative Extension), and 2. Presence of wild *V. corymbosum* growing in a wooded field border within 30 m of the edge of the commercial field. Five sites were initially selected for this study in 2011. Based on the unexpectedly high EPN activity found in 2011, an additional five sites were added in 2012 to expand the study.

At each of the five sites in 2011 and ten sites in 2012 and 2013, a 15 m × 15 m plot was selected at random in both the commercial and wild *V. corymbosum* stands. Sites with coupled sampling plots in both areas were established and sampled according to the phenology of the

commercial plants: at bloom (early May), fruit set/harvest (early July) and post-harvest (early September). At each sampling point, eight vertical soil cores were taken with an Oakfield sampler (30.5 cm length × 2.1 cm diameter) 15-30 cm concentrically from the base of the main trunk of ten plants randomly selected within the plots. In 2011 and 2012, soil from each plant (~800g per plant) was stored in individual plastic bags and held at 8° C for no more than 48 h until processing. To further assess EPN diversity with molecular methods, a follow up sampling effort was conducted in 2013 using the same ten sites as in 2012 but combining the soil from the ten plants sampled per plot in one plastic bag (~8000 g).

2.2 Laboratory processing of soil samples

The soil from each plant (2011-2012) or field (2013) was homogenized by hand in the lab before experimental set up. Wax moth larvae (*Galleria mellonella* L., Lepidoptera: Pyralidae) used for baiting were obtained from a commercial source (Big Apple Pet Supply, Boca Raton, Florida, USA) and used within 10 days of receipt. To estimate the relative abundance of EPN, a 200 g subsample from each plant was exhaustively baited with five last instar wax moth larvae in a deep-well Petri dish (25 mm H × 100 mm D) (Koppenhöfer et al., 1998) at room temperature (22-25° C). Exhaustive baiting refers to the process of baiting subsamples and checking them until there are no infections in the baiting larvae. The baited subsamples were checked every 5 days, which included the removal of infected larvae and their subsequent replacement with fresh larvae until the dish produced no infected larvae for two consecutive checks. For each dish, the status of every larva was recorded during each check and marked as: infected (with EPN), alive, or dead but not infected with EPN (including fungal and bacterial infections). Infected *G. mellonella* larvae retrieved from baiting dishes were placed on modified White traps (Stock and

Goodrich-Blair, 2012) to collect emerging IJs for subsequent reinfection of *G. mellonella* larvae, in fulfillment of Koch's postulates. Infective isolates were stored in tissue flasks at 8° C (Stock and Goodrich-Blair, 2012).

In 2013, two 200 g subsamples from each plot were baited according to the same methods as described above and resulting fresh EPN isolates were used for molecular identification. After the post-harvest sample (early July), 500 g from each plot was taken to the Rutgers Soil Testing Laboratory to determine pH, soil moisture, organic matter, soil textural class and total extractable (Mehlich 3 method) NH_4NO_3 , NO_3^- , phosphorous, and potassium as well as plant micronutrients (Ca, Mg, B, Mn, Cu, Zn).

2.3 *Estimating EPN diversity between wild and cultivated sites*

Initially, characteristics of the infected cadaver (e.g., color and degradation process) and IJs were used for cursory identification to family. As this did not reveal notable differences in diversity (all appeared to be Steinernematids), it became necessary to use molecular methods. To address the genetic diversity of isolates, the molecular operational taxonomic unit (MOTU) (Floyd et al., 2002) was used to estimate diversity because it is suited to identification of EPNs extracted from soil as isolates through soil baiting with *G. mellonella* (Chen et al., 2010). This technique removes the need for explicit species level identification by additional biological or morphological means, utilizing differences in sequence identity (i.e., molecular barcodes). The resulting sequences are from a genomic region (18S ribosomal DNA) stable enough across life stages to use DNA from IJs rather than rearing to obtain adults from the dissection of infected insects. The downside to this technique is that in the case of a new species, additional sequencing and expert morphological and biological assessment is needed. Multiple representative isolates

were chosen for sequencing from cultivated and natural sites with high EPN activity. The number of individual MOTUs was used to estimate diversity because a subsample of extracted EPNs was sequenced. A diversity index was not used because we were not able to identify all samples in the subsample to species.

To use this method, the 5' end of the small subunit ribosomal RNA (SSU) was amplified from individual IJs from a subset of isolates and sequenced. Then, NCBI's GenBank BLASTn search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to identify species matches. Top matches from the search ($\geq 99\%$ match) were considered good candidates for taxonomic identification. One of the downsides of the baiting approach is that a multi-species complex may outcompete each other in the baiting dish. Thus, in 2015, the six sites with the most EPN activity in the wild and cultivated plots were screened for other known EPN species occurring in New Jersey using quantitative PCR to confirm there was no bias or outcompeting of species in the traditional baiting methods (Rivera et al., unpublished data).

2.3.1 Single nematode digestion and PCR

Methods of DNA extraction and PCR amplification of SSU rRNA were obtained from Floyd et al. (2002) with changes in PCR master mix according to available resources. To extract DNA, an individual IJ from a stored isolate was put in 20 μ L of 0.25 M NaOH in a 0.2 mL tube and left at room temperature ($24^{\circ}\text{C} \pm 2$) for 3-16 hours. Then, the tubes were heated at 99°C for 3 min and then neutralized by adding 4 μ L of 1 M HCl, 10 μ L of 0.5 M Tris-HCl (pH 8.0), and 5 μ L of 2% Triton X-100. A 20 μ L polymerase chain reaction was used to amplify 18S: 200 μ M dNTPs, 1X PCR reaction buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KC), 2.5mM MgCl_2 , 50 nM each primer, 1U Taq polymerase and 1 μ L template DNA (or sterile water for negative

control). The SSU primers used were: SSU18A-4F (5'-
GCTTGTCTCAAAGATTAAGCCATGCATG-3') and SSU26Rplus4 (5'-
AAGACATTCTTGGCAAATGCTTTTCG-3') (Morise et al., 2012). Amplification of the 18S
locus was initiated with a 2-min denaturation at 94° C followed by 35 cycles each involving
denaturation at 94° C for 10 s, annealing at 55° C for 30 s, and extension at 68° C for 1 min in an
Applied Biosystems™ Veriti® 96-Well Thermal Cycler. Products (5 µL) of this reaction were
visualized on agarose gels stained with ethidium bromide.

2.3.2 DNA purification and sequencing

PCR products were purified using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) for
PCR product cleanup according to manufacturer protocol. Samples were prepared according to
vendor guidelines and sent to GenScript (Piscataway, NJ, USA) for sequencing with the SSU18A-
4F/SSU26Rplus4 previously mentioned. Sequencher 5.2 (Gene Codes, Ann Arbor, MI, USA) was
used to align and edit sequences. A multiple sequence alignment containing 18S sequences from
both wild and cultivated isolates (“cultivated”, “NJXBB1”, “NJXBB2”) was created in BioEdit
version 7.1.3.0 (Hall, 1999) using the ClustalW algorithm version 1.8 (Thompson et al., 1994)
with default parameters.

2.4 Virulence of native *Steinernema glaseri* and wild isolates against *A. orientalis*

2.4.1 Soil, insects and nematodes

Soil used in this assay was an acidic loamy sand (82% sand, 12% silt, 6% clay; 1.8%
organic matter, pH 3.9) from a commercial blueberry farm in Burlington County, NJ, USA. The
blueberry field soil was sieved (4 mm) to remove large particles, pasteurized for 3 hours at 70° C

and then allowed to air dry before use. To prepare for use, the dry soil was brought to 10% (w/w) moisture (-25 kPa soil water potential). After addition of nematode treatments the final soil moisture was 11% (w/w) (-10 kPa). Third-instar *A. orientalis* larvae used in this study were collected from turfgrass areas (Rutgers Horticultural Farm II, North Brunswick, NJ) and kept individually in 24-well tissue culture plates at 8° C for 6-14 weeks in pasteurized sandy loam soil at approximately 10% moisture. Four nematode isolates were used in this study. Because the sequences of the isolates from the cultivated areas were a very close match ($\geq 99\%$) to *Steinernema glaseri* (Steiner) and very similar among one another, only one cultivated (“Cultivated”) isolate was tested (Fig. 3.2). Due to variation in sequences among the isolates from the wild environments, two isolates (“Wild 1” and “Wild 2”) from wild plots were used in this study for comparison; sequences of which are also shown in Figure 3.2. In addition to these, the *S. glaseri* NC (SgNC) strain (originally obtained from the lab of Dr. Harry Kaya in 1998 and kept in culture) was used as a positive control as it has been shown previously to be effective at parasitizing *A. orientalis* larvae in this same study set up (e.g., Koppenhöfer and Fuzy, 2006). All batches of native IJs were produced from the same original stock batch isolated from the field. SgNC was reinfected from a lab culture of this strain. All IJs were used within 1 week of harvest from reinfected *G. mellonella* and kept at room temperature (21 – 24° C) until use. Third-instar plum curculio (*Conotrachelus nenuphar* Herbst; Coleoptera: Curculionidae) were obtained from the lab of Dr. Tracy Leskey.

2.4.2 Assay set up and experimental design

To assess virulence of the native isolates against third instar *A. orientalis* larvae, an assay was set up at room temperature (21 – 24° C) in 30 mL plastic cups (34 mm inner diameter × 33

mm height). Each cup was filled with 25 g of blueberry field soil with perennial ryegrass, *Lolium perenne* L., in the soil for larval food. Larvae were held at room temperature for at least 24 hours before placement in the cups and replaced if they did not completely bury themselves in the soil after 2 hours.

Each isolate including SgNC was represented by 60 cups with 20 cups at each of three densities of IJs per larva: 50, 150, 450 in 50 μ L of water. An untreated control using only water (50 μ L) was represented by 20 cups in each run of the experiment. The assay was run twice with a new batch of IJs each time. In a separate test, third instars of *C. nenuphar*, a native pest species prevalent in commercial *V. corymbosum* fields that is not a root-feeder but pupates in soil, were exposed to the same treatments using the same method but only replicated 10 times in pasteurized silica sand at 10% w/v moisture. This test was not repeated because no infections resulted.

2.5 Statistical Analysis

Analyses were performed using the statistical software packages SAS v. 9.3 (SAS Institute, Cary, NC, USA) and R (R Core Development Team, 2014). The principal components analysis (PCA) was conducted using JMP software 3 (SAS Institute, Cary, NC, USA). To determine differences in EPN abundance between wild and cultivated plots in 2011 and 2012, a mixed linear model (Littell et al., 2006) was constructed with “environment” as the treatment (wild or cultivated), year, sample date and their interactions as fixed effects. Site was nested in treatment and considered as a random effect. The data was also sorted by year and sample to explore further the nature of the interactive effects.

The effect of isolate and IJ density on mortality of larvae was determined using the GLM procedure. In both analyses, a pairwise t-test using the Tukey-Kramer method was employed for

least squares means separation when relevant effects were significant. Data from 2013 were also analyzed with a mixed linear model. The model was constructed with treatment (wild or cultivated) and sample date as fixed effects, and site as a random effect, using the lme4 package in R. EPN abundance data were log transformed to improve normality, and significance was assessed using type II sums of squares. Effect of isolate and density on mortality of larvae was determined using the GLM procedure. In both analyses, a pairwise t-test using the Tukey-Kramer method was employed for least squares means separation when relevant effects were significant. Additionally, we were interested in the relationships between the abiotic soil characteristics measured (clay, sand, silt, organic matter, pH, soil moisture, B, Ca, Cu, Fe, K, Mg, Mn, NH_4^+ , NO_3^- , P, Zn) and treatment. Toward that end, a PCA was used with data on soil characteristics first transformed into z-scores to standardize the units (Shaw, 2003).

3. Results

3.1 EPN Abundance

In 2011 and 2012, the interactive effect of year, sample time, and environment (treatment) ($F_{(4/870)} = 4.99$, $P = 0.0006$) combined was highly significant (Fig. 2). EPN abundance showed a trend towards higher abundance in the soil of the cultivated plants than the soil of wild plants ($F_{(1/18)} = 4.35$, $P = 0.0516$). Despite the lack of significance of treatment overall, two major visual trends appear in the data, which is presented by sample, year and treatment in Figure 2. Firstly, even though overall EPN abundance was higher in 2011 ($F_{(1/870)} = 22.00$, $P \leq 0.0001$), seasonality had no effect on abundance in the cultivated environment regardless of year (Fig. 2), with no significant differences between overall sample times when interactive effects of treatment and year were not included. In the wild environment, however, abundance tended to be higher (3x in

2011, 1.5x in 2012) in the first sample of year but this effect was statistically significant only in 2011 (Fig. 2). There was no interaction between year and environment ($F_{(1/870)} = 0.05$, $P = 0.8219$), but sampling time also interacted significantly with environment ($F_{(2/870)} = 8.15$, $P = 0.0003$).

To further describe the nature of these trends, the data was also analyzed by year and sample. Analysis of these data by sampling time showed no significant effect of environment in either year in the first sample (bloom) ($F_{(1/18)} = 0.63$, $P = 0.4394$), but significantly higher EPN abundance in the cultivated than the wild environment in samples 2 (fruit set) ($F_{(1/18)} = 16.91$, $P = 0.0007$) and 3 (post-harvest) ($F_{(1/18)} = 7.61$, $P = 0.0129$) (Fig. 2). When data were analyzed within each year, sampling time had no effect on abundance in the cultivated environment regardless of year; in the wild environment, however, abundance tended to be higher in the first sample of year (8-16x in 2011, 3-4x in 2012) but this effect was statistically significant only in 2011 ($F_{(2/286)} = 5.72$, $P = 0.0037$) (Fig. 2).

In 2013, when soil was combined by site rather than by plant, treatment had a significant effect on EPN abundance ($\chi^2 = 4.89$, $df = 1$, $P = 0.027$), with greater mean numbers of EPN infections per sample found in cultivated plots (0.97 ± 0.25) compared with wild plots (0.43 ± 0.22) (Fig. 3). However, there was no significant effect of sampling time on EPN abundance ($\chi^2 < 0.01$, $df = 1$, $P = 0.991$).

3.2 EPN Diversity

Cultivated soils had a marked lack of diversity of EPN. Of the 10 coupled sites sampled in 2012 and 2013, six cultivated plots and five of the wild sites were positive for EPN presence. In the subset of isolates sequenced, 100% from the cultivated fields were identified as *Steinernema*

glaseri using a BLASTn search. Since the subset of isolates was selected based on morphological or life history characteristics that represented the variation seen over the three years of extracting from soil, we assume that most if not all of the isolates from the cultivated soils were *S. glaseri*, and this notion was later confirmed by screening for 10 other nematode species using quantitative-PCR (Rivera et al. unpublished data). Samples from cultivated fields being the same species had very little variation between them, therefore only a single representative sequence is presented in Figure 4. Isolates from the five wild sites positive for EPN presence had such distinct variability that none of them could be reliably identified using the BLASTn search. Although comment and analysis on the evolutionary implications are beyond the scope of the present study, shown also in Figure 4 is an example of variation between two sequences from EPN isolated from wild plants from two different sites. These could not be reliably identified using a BLASTn search because their first match was only 96% to *S. glaseri*. The potential divergence of cultivated and wild populations is evident in the multiple sequence alignment (Fig. 4) with the most marked differences occurring between positions 182 and 200, including a 8bp gap corresponding to a shift in the reading frame.

3.3 Virulence of native isolates

Mortality of third instar *A. orientalis* was significantly affected by EPN density and isolate. Density had a significant effect on mortality only for the ‘Wild 2’ isolate ($F=261.15$, $df=2$, $P<0.0001$) (Fig. 5). Percent mortality did differ significantly, however, between isolates (Fig. 5). All native EPN isolates caused less mortality in *A. orientalis* than the NC strain (78%). The cultivated strain caused greater mortality (46%) in *A. orientalis* than the ‘Wild 1’ strain (32%) but was equally as effective as the ‘Wild 2’ strain (42%).

3.4 Effect of abiotic soil characteristics in wild and cultivated environments on EPN abundance

The PCA model used explained 58.4% of the variance (42.5% for axis 1, and 15.9% for axis 2) and revealed that the soil characteristics of sites were markedly clustered by environment (Fig. 6). More specifically, moisture, organic matter, and to a lesser extent silt, pH, and B, were correlated with wild sites, and the other 12 abiotic characteristics, particularly plant macro and micronutrients, were strongly positively associated with cultivated sites.

4. Discussion

To the best of our knowledge, the present study is the first to link the interactive effect of plant domestication and altered soil structure to the abundance, diversity and virulence of EPN. The overall conclusion from the present study is that the recent perennial cultivation of highbush blueberry in its primordial environment appears to promote EPN abundance and to some extent EPN virulence against *A. orientalis* larvae but potentially decreases substantially the diversity of EPN communities.

EPN abundance in per-plant soil samples from cultivated fields showed a strong trend for higher abundance in 2011 and 2012. In 2013, when soil was consolidated by site rather than plant in this same study, EPN abundance also was higher in the cultivated fields. Although results from 2013 are consistent with trends from the previous years, it is our belief that it is more useful to examine variation on a per plant basis. In the case of this study, the significantly higher abundance of EPN in cultivated sites in 2013 could be the populations of just a few of the ten plants sampled in the site. If this is true, it falsely explains the nature of the variation across an area by favoring redundancy unlinked to a spatial area that is massive in scale for such small

organisms; it also neglects the potential for indirect connection of EPN with plants (Dicke and Baldwin, 2010; Gols, 2014; Hiltbold et al., 2014; Turlings et al., 2012). Furthermore, evidence from previous studies suggests plants are the architects of soil communities (Bezemer et al., 2010; Eisenhauer and Reich, 2012), especially with relation to EPN as they provide nutrition to their insect hosts (Barbercheck et al., 1995). This is also relevant when considering the life history of EPN and the exodus of tens of thousands (or more) of IJs into the soil from a depleted host cadaver; it would be more likely to find higher abundance in an area where a cadaver was recently depleted than one where a cadaver has not been present for an extended amount of time.

Since fields with high *A. orientalis* pressure were selected, the increase in abundance of EPNs in cultivated sites could be attributed to greater host availability. However, the number of larvae per plant was not measured to avoid destruction of grower plants, and therefore this assumption remains speculative. Limited soil sampling around both wild and cultivated plants did not reveal any significant host numbers other than *A. orientalis* larvae (Rivera, pers. observations). Also, in a previous study investigating the efficacy of EPN for *A. orientalis* management in commercial *V. corymbosum* fields in the same area in 2003 and 2004, no other potential host species were detected among the roots even though the plants were uprooted and the entire root mass destructively searched (Polavarapu et al., 2007). Since that study was conducted, the plum curculio has also become established in the *V. corymbosum* growing areas in New Jersey. As it pupates in the soil, it could potentially serve as another host for EPN. But in our laboratory experiments, no EPN infections were observed. It is possible that the large IJ size may have contributed to the lack of infection in this host since mature *C. nenuphar* larvae are only 6 – 9 mm in length and the EPNs extracted from wild and cultivated *V. corymbosum* soil are quite large (average IJ length $\times$ maximum diameter: $1200 \times 43 \mu\text{m}$). However, *S. glaseri* readily infects

second-instar *A. orientalis*, which are similar in size to mature *C. nenuphar* larvae, at an equal density to third-instar (Koppenhöfer et al., 2004).

As with the higher abundance and diversity of plant parasitic nematodes associated with cultivated plants in this system (Rivera et al., 2015), it is similarly likely the EPN abundance is indirectly linked to increased plant health. The cultivated highbush blueberry sites sampled are genotypic monocultures, propagated from the same rootstock, that are irrigated and fertilized for fruit production and thus, plants in the cultivated arena are larger and more uniform (i.e., provide more of a better resource for insect herbivores). Evidence for this was found in the PCA analysis (Fig. 6) where the majority of the plant nutrients assessed were correlated with the cultivated sites rather than the wild sites. Soil moisture and organic matter explained a significant amount of variation attributed to wild sites (Fig. 6) and soil moisture is very important to EPN function. However, since the agricultural fields are irrigated, the moisture level in soil of cultivated fields is unlikely fall below what is needed for EPN activity during the growing season.

The increased abundance of EPNs in the cultivated environment appears to be negatively related to diversity. While cultivated fields had more abundant populations of EPNs, *S. glaseri* was the only species identified from cultivated fields and was isolated from six of the ten cultivated sites sampled. Conversely, isolates from the five wild sites positive for EPN presence had such distinct variability that none of them could be identified using the BLASTn search. They are likely undescribed species and have been submitted for description to taxonomists. The higher diversity of EPN seems to mirror the variability in virulence as the two wild isolates tested differed significantly in their ability to infect *A. orientalis* larvae, the presumed host (Fig. 5). This variability could impact the function of EPN communities beyond just what was tested here. For example, the coexistence of such a diverse EPN assemblage could be attributed to niche

partitioning by factors not tested such as larval age and presence of other potential hosts (Ettema, 1998; Finke and Snyder, 2008; Hutchinson, 1959; MacArthur, 1970).

The presence, high abundance and distribution of *S. glaseri* among cultivated fields is only somewhat surprising. *S. glaseri* was first discovered in a golf course approximately 50km away in Haddonfield, NJ, USA (Glaser and Fox, 1930). A large-scale, statewide effort followed to inoculate *S. glaseri* as a means to limit the spread of the then recently introduced Japanese beetle, *Popillia japonica* Newman. However, it is likely that this massive undertaking was doomed from the start as the researchers were still unaware of the nematode's symbiotic bacterium, *Xenorhabdus poinarii*, and likely limited infectivity via use of antibiotics in mass-production of the nematodes. Additionally, both the initial inoculation and the subsequent sampling efforts to monitor *S. glaseri* establishment 50 years later (Gaugler et al., 1992) seem to have overlooked the Pinelands National Reserve. The sites sampled in the present study and the sites with previously confirmed *S. glaseri* populations are shown in Figure 1A. Thus, the prevalence of *S. glaseri* in this area was surprising as it was one of the species found less frequently in extensive sampling of the state of New Jersey (Gaugler et al., 1992). Taking into account the biology and ecology of *S. glaseri*, this finding is not surprising as the species is very competitive with other EPN species, persistent (Koppenhöfer et al., 1996) and highly mobile (Grewal et al., 1994; Lewis et al., 1995).

The poor virulence of the isolate from cultivated blueberry fields when compared to the much-studied SgNC strain against *A. orientalis* larvae was unexpected (Fig. 5) mostly because it was assumed EPN of the same species, which carry the same bacterial symbiont, would be similar in their ability to infect a specific insect. There is, however, evidence for differences among *S. glaseri* strains in their ability to infect *A. orientalis* larvae. This variability is, at least in part, due to the presence or absence of surface coat proteins that aid in overcoming the encapsulation by the

immune system of *A. orientalis* larvae (Li et al., 2007). These results beget speculation on what benefit these *S. glaseri* isolates may gain from being only marginally effective at killing what appears to be the most common potential host.

It seems unlikely that evolutionary pressures on EPN would select for both higher virulence and higher persistence of *S. glaseri*. After all, this would mean the EPN would quickly exhaust their host resource population and have to persist long enough for the area to be recolonized by another host or adapt to another host. Perhaps the transitioning of once wild land into land for cultivation causes genetic drift in local EPN populations that in this case selected for higher persistence but lower virulence. In fact, isolates extracted from soil samples in 2012 are still infective three years later in 2015 (Rivera, personal observation). Otherwise, the cultivated isolate may have adapted to the cultivated arena over time and thus, became more virulent to *A. orientalis* larvae than the wild isolates. EPN populations that are highly persistent and virulent to a specific insect host may also suggest a connection to specific and highly linked insect-plant dynamic (Preisser et al., 2006). Additional research is needed to elucidate the connections between the belowground community at large and EPN in order to understand what drives the persistence, virulence and diversity of EPN communities.

Over time, it is possible that members of the belowground third trophic level in perennially cultivated environments can become more adapted to the available hosts. Their survival in a disturbed system depends on this. However, it seems unlikely such systems will recover the diversity found in the natural associations of soil and plant. The results presented from this study imply that the third trophic level responds similarly to the redundancy of monoculture by selecting and promoting the abundance of the most successful strain.

470

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Fig. 1. Distribution of entomopathogenic nematode sampling sites in wild and domesticated stands of *Vaccinium corymbosum* within the Pineland National Reserve (PNR) in New Jersey, USA. Panel A shows sites positive for *Steinernema glaseri* (solid black circles) in an extensive sample of the state of New Jersey (Gaugler et al. 1992), and sites used in this study with positive infections (solid black triangles). Panel B shows a more detailed map of the location within the PNR.

Fig. 2. Abundance (mean +SEM) of entomopathogenic nematodes (number of infected bait insects) in rhizospheric soil of cultivated and wild highbush blueberry (*Vaccinium corymbosum*) in soil sampled in 2011 (11) and 2012 (12). Soil sampling occurred at three different times correlating with the phenology of the cultivated plants: bloom (S1), fruit set (S2), and post-harvest (S3). Means with same letter within sampling time did not differ significantly ($\alpha = 0.05$).

Fig. 3. Abundance (mean +SEM) of entomopathogenic nematodes from rhizospheric soil associated with sites containing wild and cultivated highbush blueberry (*Vaccinium corymbosum*) in 2013. Means with same letter did not differ significantly ($\alpha = 0.05$).

	130	140	150	160	170	180
Cultivated- <i>S. glaseri</i> (99%)	CAAGCGAAAC	GCCTGTTTTA	AGCACTCTAA	TTAATTCAAA	GTAAACTCGT	CAGTCACGAC
Wild- NJBBX1 (96%)	CAAGCGAAAC	GCCTGTTTTA	AGCACTCTAA	TTAATTCAAA	GTAAACTCGT	CAGTCACGAT
Wild- NJBBX2 (96%)	CAAGCGAAAC	GCCTGTTTTA	AGCACTCTAA	TTAATTCAAA	GTAAACTCGT	CAGTCACGAC
	190	200	210	220	230	240
Cultivated- <i>S. glaseri</i> (99%)	GAACACCCCG	TGAGGAGCAC	TCGCCAAAAA	CCGACAAATG	AGAGCAACAC	GAAGCAGTAG
Wild- NJBBX1 (96%)	G-ACGCC---	---GAAGCA-	TCACCAAAAA	CCGACAAAAG	AGAGCAACAC	GAAACAGTAA
Wild- NJBBX2 (96%)	G-AAGCC---	---GAAGCC-	TCGCCAAAAA	CCGACAAAAG	AGAGCAACAC	GAAGCAGTAA
	250	260	270	280	290	300
Cultivated- <i>S. glaseri</i> (99%)	TCGCTCCAAG	AGCGAACC GC	CTCGCGTCGC	GAAGATCCAA	CTACGAGCTT	TTTAACCGCA
Wild- NJBBX1 (96%)	TCGCTCCAAG	AACGAACCGT	CTCGCGTCGC	GAAGATCCAA	CTACGAGCTT	TTTAACCGCA
Wild- NJBBX2 (96%)	TCGCTCCAAG	AACGAACCAC	CTCGCGTCGC	GAAGATCCAA	CTACGAGCTT	TTTAACCGCA

Fig. 4. Portion of the multiple sequence alignment comparing 18S sequences from nematodes collected in cultivated vs. wild soil. Alignment exported from BioEdit version 7.1.3.0. Polymorphic positions are highlighted in gray. Percentages represent sequence similarity to *S. glaseri* (GenBank Accession# FJ040422.1) obtained from a BLASTn search.

Fig. 5. Percent mortality of *Anomala orientalis* third instar larvae when exposed to different rates and strains of entomopathogenic nematodes endemic to wild and cultivated highbush blueberry (*Vaccinium corymbosum*) in the Pineland National Reserve. Three native isolates were tested. *Steinernema glaseri* (NC Strain) was also included as a positive control. Means with the same letter did not differ significantly ($\alpha = 0.05$). Panel shows larval mortality when exposed to three densities of three native EPN isolates (lowercase letters) and shows larval mortality by isolate (uppercase letters).

Fig. 6. Biplot showing the first and second axes of ordination from the principal components analysis of soil characteristics and total extractable nutrients (loadings represented by arrows) by treatment (circles = cultivated sites, triangles = wild sites).