BIOAG PROJECT FINAL REPORT

TITLE: EFFECTS OF NEMATODE GENETIC DIVERSITY ON MANAGEMENT OF POTATO PESTS

PRINCIPAL INVESTIGATOR(S) AND COOPERATOR(S):

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Our project was in coordination with Andy Jensen, Research Director of the ID, WA and OR Potato Commissions; nematodes were collected on-farm in cooperation with potato growers across the Columbia Basin.

KEY WORDS

Potato, Insect Pest, Biological Control, Organic Farming, Genomics

ABSTRACT

Market forces are driving conventional Columbia Basin potato growers towards less-chemically-intensive pest management approaches such as biological control. Furthermore, organic potato production is a growing sector that relies on insecticide alternatives. Biological control by insect-killing, entomopathogenic nematodes (EPNs) is one alternative to traditional insecticides that may offer significant benefits for the natural control of pests such as the Colorado potato beetle. We have found that EPNs can be incredibly abundant in WA organic potato fields. Despite the apparent abundance of EPNs, we still know very little about their genetic diversity in WA crops, either within or among nematode species. Likely, this reflects the fact that these nematodes occur in the soil and are quite small, such that they have been greatly under-appreciated as biological control agents. In fact, we suspect that natural control of Colorado potato beetle (and other insects with subterannean stages, such as wireworms) by EPNs is a key part of biological control in WA agriculture. With support from the BIOAg program, we used modern genomics tools to begin an examination of the species and genetic diversity of EPN isolates collected from organic potato fields in WA. We also conducted experiments to determine how variation in nematode species (and strains within species) affects natural pest control. Our research addressed two of the main 2012 priorities of the BioAg program, "Biological intensive and organic approaches to sustainable management of pests" and "Innovation and diversification to increase the resiliency and sustainability of farming and food systems in the face of climate change."

PROJECT DESCRIPTION

Potatoes are the #1 dollar-value vegetable crop in WA (WSDA 2008), anchoring most crop rotations by having greater profit potential than any other crop (USDA-NASS 2008). Thus, the economic viability of many growers is largely or entirely dependent on production of high-quality potato crops, every 3-4 years in a rotation. Potatoes face a wide diversity of pests that damage plants through direct feeding (Hare 1980, 1990, Weber 2003) or by vectoring diseases (Nolte et al. 2003). In turn, conventionally-

managed potatoes are heavily sprayed with insecticides to control pests. External socioeconomic forces, however, are forcing potato growers to rethink their IPM practices. In recent years several important end-users of frozen potato products (e.g., major restaurant suppliers and restaurant chains) have required growers to develop “sustainable pest management plans” that document and justify each insecticide application, and to pass “sustainability audits” that document sustainable practices have been followed. Required information includes evidence that beneficial species were conserved and that natural pest control was factored into pest management decisions. At the same time, the organophosphate insecticide methamidophos (Monitor®, Bayer CropScience) was withdrawn from the market several years ago. This insecticide was long the backbone of pest control in WA potatoes due to the chemical’s broad efficacy and low cost. Thus, growers are being forced to move rapidly from a pest management approach based largely on zero tolerance and calendar sprays, to a more nuanced and sustainable approach focusing on pest monitoring and biological control. Unfortunately, the larger industry is poorly prepared to make this transition. Organic potato growers, a small but vibrant sector, also struggle to control insect pests with the narrow range of insecticides available to them.

Biological control by insect-killing nematodes is an ecologically-friendly and sustainable approach to pest management in many crops (Kaya and Gaugler 1993, Miles et al. 2000). Nematodes are roundworms that live in the soil; several nematode species infect and kill insects (Kaya and Gaugler 1993, Miles et al. 2000) and thus are known as "entomopathogenic nematodes" (EPNs). EPNs are abundant in potato fields, particularly on organic farms where soil pesticides are rarely available (Henderson et al. 2009, Ramirez and Snyder 2009). In turn, natural densities of nematodes can cause up to 30% mortality of a key potato pest, the Colorado potato beetle (Ramirez and Snyder 2009, Crowder et al. 2010). Biological control of potato beetles and other pests by EPNs is often particularly effective because nematodes complement tactics designed to kill pests on leaves. For example, potato beetle pupal mortality caused by nematodes (these beetles pupate in the soil) increases when beetles have been exposed to predators on potato leaves, likely because EPNs are more capable of infecting stressed hosts (Ramirez and Snyder 2009). Similarly, pests that survive pesticide applications on plant leaves are often more susceptible to EPNs than insects never exposed to pesticides (Gassmann et al. 2008).

In potatoes, however, biological control by nematodes is underutilized, in part because we know little about EPN biodiversity. EPN taxonomy is poorly known, and many "species" likely contain many cryptic species (or strains) that differ in ecologically-important ways. Several growers have expressed an interest to PI Snyder and co-PI Crowder in knowing which nematodes occur on their farms, and whether they can be deployed and/or conserved for biological control. An obvious first step in filling this knowledge gap lies in getting a fundamental grasp on EPN biodiversity, including how genetic diversity within species influences the strength of pest control.

In our BIOAg funded project, we explored the diversity of nematodes in commercial potato fields in WA. We worked with potato-grower collaborators to collect nematode strains from the broad range of climates across the Columbia Basin, and began an initial identification of the species and genetic diversity of all populations collected. In turn, we also conducted experiments to determine how nematode diversity affects pest control. Through these objectives, our research addressed two of the main 2012 priorities of the BioAg program, “Biological intensive and organic approaches to sustainable management of pests” and “Innovation and diversification to increase the resiliency and sustainability of farming and food systems in the face of climate change.

OUTPUTS

1) Quantifying intraspecific genetic diversity of EPNs in Washington potato fields.

Entomopathogenic nematodes were collected from eight different organic potato fields, using waxworm baits placed at two different depths in the soil - shallow and deep. After 2 days the waxworm baits were recovered from the soil, and nematodes were extracted from waxworms. Nematodes from each location at each depth were maintained *in vitro* individually in the laboratory.

DNA was purified from approximately 10,000 infective juveniles of each isolate using a modified phenol chloroform extraction developed by C. Bates. Briefly, nematodes were collected in 1.5 microcentrifuge tubes, and then were frozen in liquid nitrogen for 15 mins. Frozen nematodes were lysed with homemade buffer and proteinase K, then DNA was extracted using Phenol: Chloroform ammonium acetate and isopropanol. Salts were removed by washing the DNA pellets in 70% ethanol twice, and DNA was dissolved in 100 ul TE buffer. Quality of the extraction was checked with a Qubit Fluoroemeter.

Extracted DNA of each isolate (sample) together with DNA of two known pure strains of the EPN species *Steinernema feltiae* and *Heterorhabditis bacteriophora,* were sent to Florogenex, Oregon, who generated and sequenced restriction site associated DNA (RAD) tags following the methods described by Baird et al. (2008) and Hohenlohe et al. (2010). Briefly, genomic DNA was digested with restriction enzyme Pst1, then ligated to sequencing adaptors and individual sample barcodes; only a fraction of the genome, the sequences at the restrictions sites, are sequenced using this methodology. Individually barcoded RAD samples were jointly sequenced on the Illumina Hiseq 2000 platform. Reads in FASTQ formats were generated from Illumina, and RAD tags of each sample were separated based on the barcode sequences. This results in 95-bp-length RAD tags.

All the FASTQ files were run through the Fastqc program to check the quality and to search for over-representative sequences. A 5-bp over-representative sequence (TGCAG) was found at the beginning of all samples. The PRINSEQ program (Schmieder and Edwards, 2011) was used to trim this 5-bp over-representative sequence, resulting in 90-bp length reads for all samples. Trimmed sequences of each sample were aligned to unpublished reference genomes of both *S. feltiae* and *H. bacteriophora*, supplied to us from cooperating laboratories, using the Bowtie aligner (Langmead et al., 2009). A maximum of 3 mismatches within the first 72-bp of reads were allowed in each alignment, and a sum of base quality for all mismatches in one read was no greater than 105.

Table 1. Statistics of RAD tag sequences of nematode samples collected from WA organic potato fields.

Organic fields	Sample No.	Number of reads	% of reads aligned to genome: *H.bacteriophora*	*S. feltiae*
Brad-2	1	11,863,232	-	44.19%
	2	14,798,626	-	56.42%
	3	15,661,427	-	63.09%
	4	12,558,967	-	50.73%
11	5	8,169,168	80.37%	-
	6	7,056,033	79.73%	-
	7	11,991,931	80.16%	-
	8	14,509,551	73.27%	-
32	9	17,063,715	80.21%	-
	10	10,569,828	79.64%	-
	11	9,889,090	76.24%	-
	12	8,763,595	81.91%	-
38	13	24,923,916	-	48.61%
	14	35,388,207	-	26.25%
	15	35,004,321	-	48.48%
	16	16,116,115	-	59.62%
727	17	33,217,482	-	29.07%
	18	25,409,421	-	50.58%
	19	14,862,897	-	66.67%
Pat NW-6	20	12,866,925	73.28%	-
	21	13,279,590	-	64.69%
	22	6,627,861	-	62.87%
	23	5,235,527	76.87%	-
Pat NW-12	24	16,658,750	-	46.50%
	25	16,763,413	-	64.12%
	26	14,271,467	-	68.43%
	27	16,999,563	-	66.21%
	28	8,766,340	-	37.42%
Hintz	29	7,814,811	77.48%	-
	30	4,132,064	83.42%	-
	31	10,562,976	79.20%	-
	32	13,889,015	83.04%	-
	33	10,193,376	80.97%	-
	34	3,585,552	79.03%	-
Control	35	4,695,790	81.17%	-
	36	17,867,432	-	55.16%

- indicates less than 2 % of the raw reads were aligned to a given genome

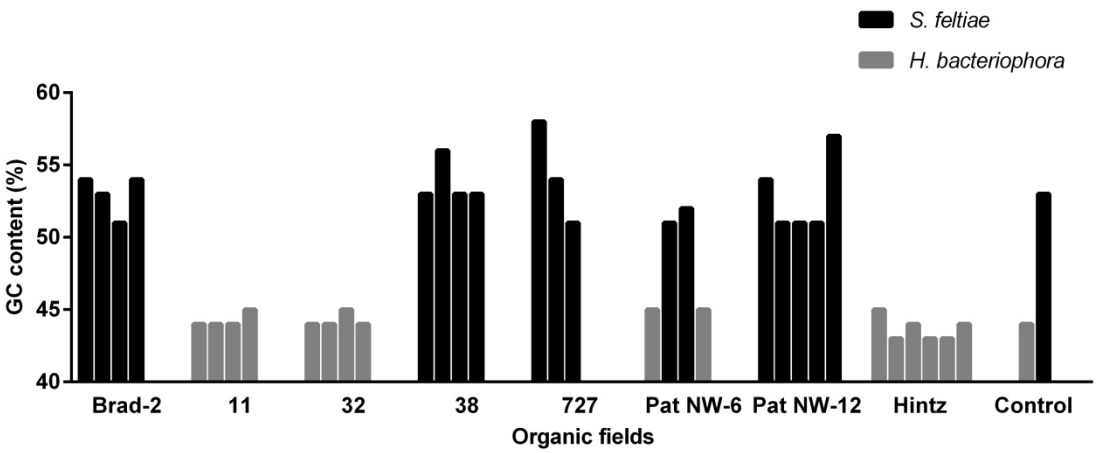

Figure 1. GC content of all 36 samples, including 34 nematode samples collected from 8 various organic fields and two pure strains obtained from the vendor. The columns from left to right represent sample number 1 to 36 in the RAD tag sequencing.

Based on the alignments and GC content (the percentage of nitrogenous bases on a DNA molecule that are either guanine or cytosine) analysis (see Figure 1 and Table 1 for statistical analysis) of each sample, we were able to identify two species in 34 samples, excluding pure strains (Table 1). Samples 1-4, 13-19, 21-22, and 24-28 were *S. feltiae*, with GC content ranges from 51% to 58% (Figure 1). The rest of the samples were *H. bacteriophora*, with GC content ranges from 43% to 45% (Figure 1), lower than GC content of *S. feltiae*. Nematode populations at each sample site were homogenous for either *H. bacteriophora* or *S. feltiae,* with the exception of field sample Pat NW-6, in which both species were found. Sequence alignment map (SAM) files were generated from alignments of each sample to each reference genome (Table 1).

Single nucleotide polymorphisms (SNPs) were called from grouping of SAM files of *S. feltiae* samples and SAM files of *H. bacteriophora* samples using SAMtools (Li et al., 2009). A total of 2,814 SNPs were culled from the *H. bacteriphora* samples and 114,668 SNPs were culled from *S. feltiae* samples. Interpreting our sequence data requires alignment with high-quality reference genomes. Unfortunately, we have found that the *S. feltiae* and *H. bacteriophora* genomes provided to us by other researchers are of exceptionally low quality, with poor genome coverage and thus weak alignment with our RAD-TAG sequences (see Table 1). This makes it difficult to annotate our SNPs and correlate them with specific genes, such that we cannot generate the convincing preliminary data that we need to pursue federal grants. We have recently received funding through the 2013 BIO-Ag competition that will allow us to create high-quality sequences for both nematode species. Throughout the spring semester we have been working to generate highly-inbred cultures of an *S. feltiae* strain that appears to be strongly complementary in its insect-killing abilities to several other nematode strains (see below). Purified DNA has been submitted to the core sequencing facility at WSU for whole-genome sequencing.

Because the Genomics Core has initially been having technical difficulties with the DNA library's preparation for sequencing, we tried an alternative route to analyze the RAD-tag data without reference-genome alignment. To do this we aligned all of the reads to two nematode strains that generated the largest number of RAD-tag reads (32-4D and 38-6 2P) using the Bowtie aligner (Langmead et al., 2009); from this Sequence Alignment/Map (SAM) files were generated. Samtools (Li et al., 2009) was used to process the SAM files and generate Variant Call Format (VCF) files. VCF is a text file format, containing meta-genotype information of each strain for each locus. Allele frequency of each locus was calculated using in-house script. Afterwards, genetic divergence between strains was calculated from

the sum difference of allele frequency for each locus using the in-house script. Genetic distance matrices which include all pairwise genetic divergences between strains were constructed. The resulting genetic distance matrices were imported to the PHYLIP program (Felsenstein, 2005) to construct phylogenetic trees with various algorithms. Finally, the phylogenic trees were visualized in the program Integrative Tree of Life (Letunic and Bork, 2006; Figure 2).

No matter which of the two strains are used as the reference (32-4D,or 38-6 2P), we conclude that the phylogenic relationship between Pat NW 12-6 and Pat NW 6-2 is greater than that between Pat NW6-2 and Pat NW12-1, and Pat NW6-2 and Pat NW12-1 are more closely related (Figure 2). The bioassay we conducted earlier showed synergistic effect of strain pairs NW 12-1 and NW12-6, and NW 6-2 and NW12-6, on mortality of waxworm (*Galleria mellonella*, Figure 3). The results of the phylogenetic tree and the bioassays illustrate that it is more likely that two nematode strains are complementary if they are distantly related.

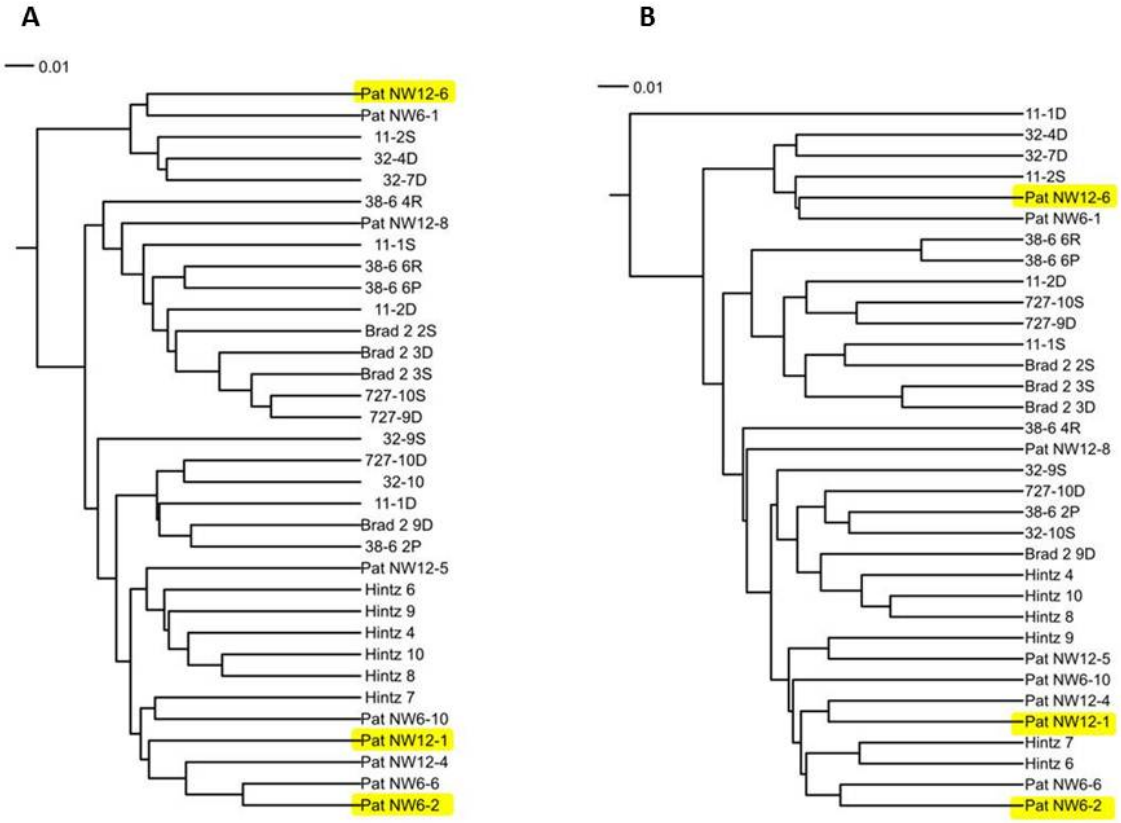

Figure 2. Phylogenetic relationship of 34 strains of entomopathogenic nematodes collected from organic potato fields in Washington. The phylogenic tree (A) was constructed based on alignment of all the reads to strain 32-4D; tree (B) was constructed based on alignment of all strains to 38-6 2P. Both phylogenetic trees were constructed with algorithm Kitsch in the PHYLIP program. The strains we tested in the bioassay are highlighted.

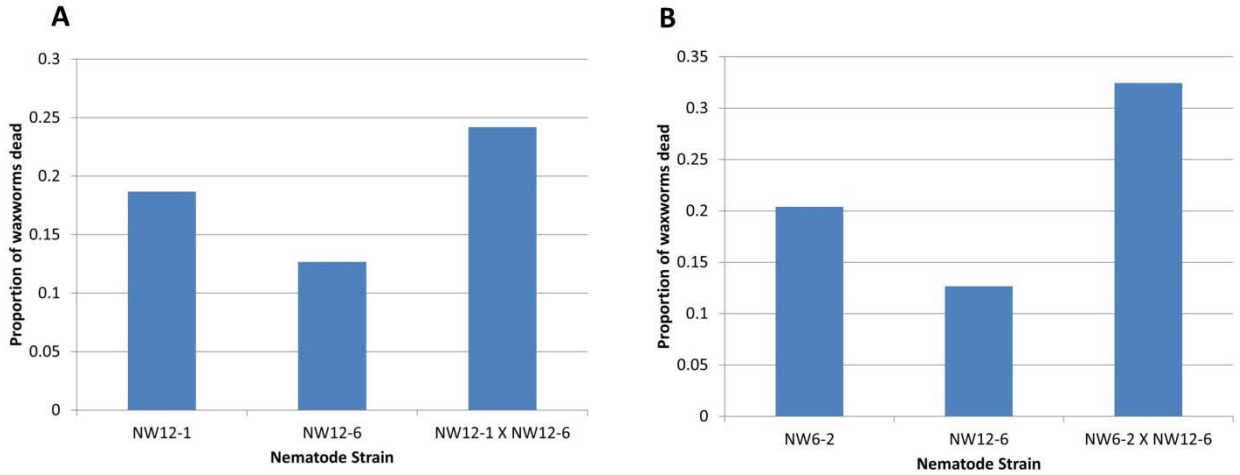

Figure 3. Synergistic effect of distantly-related entomopathogenic nematode strains on mortality of waxworms (*Galleria mellonella*). A) With the same total number of nematodes, the combination of NW 12-1 and NW 12-6 caused higher mortality on waxworms. B) With the same total number of nematodes, the combination of NW 6-2 and NW 12-6 caused higher mortality on waxworms. As shown in Figure 2 above, these synergistic nematode pairings bring together distantly-related nematode strains, consistent with our hypothesis that genetic divergence leads to greater niche differences, and thus greater complementarity, among nematode strains.

2) Comparing the pest-control effectiveness of single versus multiple EPN strains.

Our goal here was to determine whether genetically-diverse mixes of either two different nematode species, or of two genetically-different isolates of the same nematode species, killed more pest insects than any single EPN strain alone. Indeed, we have consistently found that EPN genetic diversity improves pest control. For example, pairs of two genetically-different isolates of *S. feltiae* kill far more insects than any single strain (Figure 4). Thus, EPN strains "complement" one another.

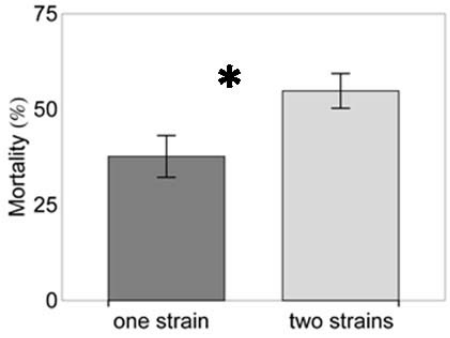

Figure 4. In soil-core microcosms, mortality of pest insects was significantly higher (Student's t-test: t=-2.262, *P* = 0.03) when they were attacked by two different strains of *S. feltiae* (right bar), compared to any single *S. feltiae* strain (left bar). Total nematode densities were constant across the two treatments, such that host-mortality differences did not reflect any difference in the total number of nematodes present. This suggests that genetically-different *S. feltiae* strains complement one another to kill more hosts than either strain could on its own.

Our ultimate goal is to use the genomics data provided by objective 1, to identify the underlying genetic basis of the strain complementarity revealed in Objective 2. If these beneficial, complementary traits could be identified, this would allow us, for example, to engineer EPN bio-pesticides that combine strains with complementary modes of activity.

Publications:

Jabbour, R, DW Crowder, EA Aultman and WE Snyder. 2011. Entomopathogen biodiversity increases host mortality. *Biological Control* 59:277-283.

Crowder, DW, TD Northfield, R Gomulkiewicz and WE Snyder. 2012. Conserving and promoting evenness: Organic farming and fire-based wildland management as case studies. *Ecology* 93:2001-2007.

IMPACTS

· Short-Term: We now have identified the species of EPN that occur in Washington organic potato fields, and have determined that significant genetic diversity exists within these populations. Furthermore, combining genetically-different EPN strains leads to higher mortality of pest insects. Thus, the genetic diversity among EPNs that organic farming promotes, seems likely to improve natural pest control.

· Intermediate-Term: Our key intermediate-term goal is to successfully compete for federal grant funding to further investigate genetic differences among EPN species, identify genes that correspond to important traits tied to a worm-strains ability to kill hosts, and develop the means to search for these traits in nematodes in potato fields. We next hope to begin the design and testing of bio-pesticides that combine beneficial and complementary nematode traits.

· Long-Term: Our ultimate goal is to provide new commercial bio-pesticides that effectively control potato beetles, and to provide a model approach for understanding why natural enemies complement one another that can be applied to other pests or cropping systems.

ADDITIONAL FUNDING APPLIED FOR / SECURED

This project has allowed PI Snyder to develop molecular biology expertise in his laboratory, and in turn this has allowed him to successfully compete for a large federal grant in this area. **We learned this fall that our USDA Organic Transitions (USDA-ORG) proposal, which includes a molecular-biology component led by BIO-Ag postdoc FU and PI Snyder, was funded at the full requested amount of $747, 955.** Snyder is PI of the new USDA-ORG grant. Also, BIO-Ag co-PIs Snyder, Elling and Fu will be submitting a proposal to USDA-NIFA's Foundational Program this fall, with preliminary data from the BIOAg project forming the core of this new proposal.

GRADUATE STUDENTS FUNDED

This project supported the research of 2 PhD students in PI Snyder's laboratory, Christine Lynch and Karol Krey. Christine successfully defended her dissertation in spring 2013 and is currently a postdoc at the University of Hawaii; Karol continues to make excellent progress toward her degree with graduation expected in 2015. This BIOAg project also provided data being used by Elliott Moon to complete his MS degree in Statistics; Elliott also graduated this past spring. Elliott recently accepted a job at a statistical consulting firm in Seattle.

RECOMMENDATIONS FOR FUTURE RESEARCH

Our next step is making use of the third-generation sequencing technology now available at the genomics core facility at WSU, to construct high-quality reference genomes for *S. feltiae* and *H. bacteriophora*. This will allow us to use our RAD-TAG data to search for genes that underlie nematode complementarity. Additionally, new sequencing technology at U Idaho will be used to compare gene expression profiles among pairs of complementary nematode strains, providing an additional, and complementary, tool to search for complementarity-related EPN genes. Our ultimate goal is to engineer EPN bio-pesticides that combine strains with complementary modes of activity, and to gain a greater fundamental understanding of how EPN strains complement one another to kill more pests.

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