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Using fine-scale relatedness to infer natural enemy movement

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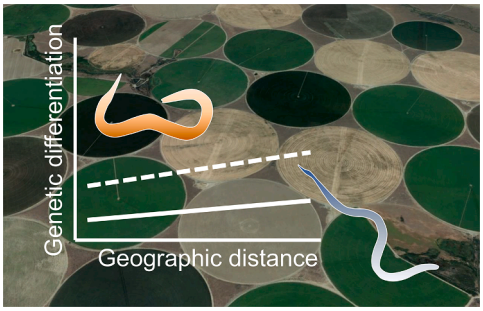
HIGHLIGHTS

- Restriction Site Associated DNA Sequencing (RAD-seq) revealed fine-scale genetic relationships among entomopathogenic nematodes.
- *Heterorhabditis bacteriophora* and *Steinernema feltiae* grew increasingly less related with greater distance between collection sites.
- Yet, intraspecific genetic diversity at a site could be high and distant strains could be closely related.
- RAD-seq provides a powerful approach to inferring natural enemy movement within agricultural landscapes.

GRAPHICAL ABSTRACT



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ABSTRACT

Natural enemies often move among habitats to track prey and resources. Indeed, biocontrol often depends on natural enemies dispersing into crops after disturbances such as tillage and pesticide applications. However, the small size of many natural enemies makes it difficult to observe such movements. Here we used genetic relatedness among entomopathogenic nematodes, tiny, soil-dwelling, and thus cryptic natural enemies, to infer their movement across an agricultural landscape. We collected strains of two nematode species, *Heterorhabditis bacteriophora* and *Steinernema feltiae*, by placing sentinel hosts into eight irrigated *Solanum tuberosum* fields across arid central Washington State. We then used restriction site associated DNA sequencing to generate single nucleotide polymorphisms and infer relatedness among strains across our study sites. We identified 3,367 and 138,286 polymorphic loci for *H. bacteriophora* and *S. feltiae*, respectively. Genetic differentiation for both species increased with greater distance between sites, although there was considerable variation. While strains collected from the same field were generally more closely related than those from different sites, for both species, strains from different fields were sometimes quite closely related. Altogether, our results suggest a surprising amount of genetic similarity among nematodes from distant sites, despite the presumably limited distances that can be

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traversed by individual worms. This is consistent with the nematodes moving, at least occasionally, between far-apart locations. More generally, we suggest that recent advances in population genomics are providing powerful new tools for mapping natural enemy movement across broad landscapes.

1. Introduction

Natural enemies often move widely across agricultural landscapes (Bianchi et al., 2006; Tscharntke et al., 2007). This is because any single cropping field generally cannot consistently provide the resources needed to maintain populations over longer time periods (Chaplin-Kramer et al., 2011; Begg et al., 2017). After all, tillage and planting, crop harvest, the application of insecticides or other agrichemicals, and other management practices will periodically, often dramatically, reshape ecological conditions in a field to render it more or less suitable for natural enemies (Duelli et al., 1990; Gurr et al., 2017). Natural enemies also move among sites as they track local differences in prey or other resources, such as overwintering sites (Tscharntke et al., 2016). This leads to the observation that natural enemies are sometimes more abundant in very heterogeneous landscapes providing greater diversity of resources over space and time (Lichtenberg et al., 2017; Karp et al., 2018). Indeed, many conservation biological control efforts rely on natural enemy movement from shelter, nectar resources, and alternative prey that are provided alongside agricultural fields, into the crops themselves (Gurr et al., 2017). The scale at which resource diversity benefits biological control in a particular field, in turn, reflects whether natural enemies disperse primarily over small distances, for example by walking, versus large distances through flight or passive movement in weather fronts (Tscharntke et al., 2007).

While conservation biological control often depends on a detailed understanding of the scale at which natural enemy species move among habitats, this information can be difficult to obtain. Invertebrate natural enemies are often small, such that affixing tags or other physical marks needed for mark-recapture studies is impractical (Behura, 2006). While some natural enemies can be chemically marked, for example by applying proteins that are later detected using antibodies, but it can be cost prohibitive to dust enough individuals to make successful recapture at distant sites at all likely (Steffan et al., 2001; Hagler and Jackson, 2001; Hagler, 2019). Genetic approaches provide an interesting additional possibility. For example, identification of sequence differences among repetitive DNA sequences, known as "microsatellites", has shown geographic differences among predator populations consistent with restricted gene flow and thus populations that are spatially isolated (e.g., Brouat et al., 2003; Hufbauer et al., 2004; Anton et al., 2007; Sethuraman et al., 2015). However, it can be time consuming to identify many useful microsatellites, often limiting the degree of genetic resolution between populations (Li et al., 2020). More powerful are approaches in the emerging field of "population genomics" that reveal sequence differences at hundreds or thousands of points along a study organism's genome, allowing fine-scale differentiation (Sethuraman et al., 2020). For herbivorous insects, these techniques have proven capable of inferring movement of insects within agricultural landscapes (e.g., Fu et al., 2020), although relatively little work of this type has been conducted with insect natural enemies (Sethuraman et al., 2020).

Here, we adopt a population genomics approach to infer landscape-scale movement patterns for two species of entomopathogenic nematodes, *Heterorhabditis bacteriophora* (genome size ~77 Mb) and *Steinernema feltiae* (genome size ~83 Mb). Both are generalists that attack a range of host insects in the soil, such that they are broadly useful in biological control (Kaya and Gaugler, 1993). These nematodes enter hosts through the mouth, anus, spiracles or cuticle (Dowds and Peters, 2002) before releasing symbiotic bacteria that infect the insect, killing it to be used as food for the nematodes' reproduction (Kaya and Gaugler, 1993). Entomopathogenic nematodes move relatively short distances through the soil while seeking hosts (Grewal et al., 1997; Shapiro-Ilan and Brown, 2013), such that conservation biocontrol efforts generally examine in-field practices, such as reduced tillage, to build densities of these natural enemies (e.g., Shapiro et al., 1999; Millar and Barbercheck, 2002; Hoy et al., 2008). However, movement over longer distances also can occur whenever nematode-infested soil is moved from one site to another on animals or farm equipment (e.g., Shapiro-Ilan and Brown, 2013). So, re-establishment of entomopathogenic nematodes at a site following disturbance, for example in a crop field following soil fumigation, might benefit from nematode conservation at both field and farm scales (Tscharntke et al., 2007; Gurr et al., 2017). Here, we used a population genomics approach to examine relatedness of *H. bacteriophora* and *S. feltiae* nematodes collected from commercial potato (*Solanum tuberosum*) fields spread across southcentral Washington State, USA. Potato in this arid region is grown under irrigation, such that potato fields represent "islands" of relatively high soil moisture presumably favorable to entomopathogenic nematode survival and movement, often separated by large stretches of dry shrub-steppe habitat that might be less favorable (Wright et al., 1987). So, our central hypothesis was that entomopathogenic nematodes of each species collected from a single field would be relatively closely related, with genetic differentiation increasing for worms collected from fields that were increasingly far away from one another.

2. Methods

2.1. Field collection of nematode strains

We used sentinel host insects to collect entomopathogenic nematodes from eight commercial potato fields in the irrigated growing region of southcentral Washington State, USA (Table S1; Fig. 1). Our sentinel hosts consisted of twenty *Galleria mellonella* larvae (Nature's Way, Ross, OH) placed in a mesh bag made from aluminum window screen and sealed at the top with duct tape. Ten traps were placed in each field, spaced 10 m apart along a single linear transect. Five traps at each site were planted at ca. 5 cm depth, and five at ca. 10 cm depth, to allow collections across soil strata where the nematodes might be active (Kaya and Stock, 1997). All traps were retrieved 48 h later and returned to the laboratory, before extraction from any infected sentinel larvae over the following 2 weeks using White traps (White, 1927). Species were identified using morphological keys of Kaya and Stock (1997), and confirmed by aligning our RAD sequences with each species' reference genome (Table S2). Nematodes from each infected host were then transferred to separate distilled-water-filled 600 ml tissue flasks with a drop of Triton X-100 (Dow Chemical Company, Midland, MI), and stored at 12 °C. Each of these separate nematode "strains" was cycled through *G. mellonella* every 6 months.

2.2. DNA extraction and RAD sequencing

High molecular weight genomic DNA was purified from approximately 10,000 infective juveniles of each nematode strain following the methodology of Donn et al. (2008), with the exception that 100 μl Tris-EDTA buffer was used to elute DNA. Quality of DNA was examined using a Qubit Fluorometer (Life technologies, Grand Island, NY). DNA samples were sent to Florogenex (Eugene, OR), who generated and performed RAD sequencing following the methods described in Baird et al. (2008) and Etter et al. (2012). Briefly, genomic DNA was digested with restriction enzyme *Pst1* and ligated to sequencing adaptors and sample barcodes to build a DNA library of each nematode strain. DNA libraries were sequenced on an Illumina Hiseq 2000 with single-end 100 bp

reads. Raw reads in FASTQ formats were generated and deposited in NCBI Short Read Archive with BioProject accession number PRJNA236113.

2.3. Variant calling and outlier identification

We used Fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to verify sequencing quality, and then short reads of the same species were aligned to their respective genomes (Wormbase-ParaSite, BioProject PRJNA13977 and PRJNA353610 for *H. bacteriophora* and *S. feltiae* respectively; Howe et al., 2016; Fu et al., 2020) using Bowtie2 (Langmead and Salzberg, 2012) with the "sensitive" preset. Thereafter, the mpileup program in samtools (Li et al., 2009) was used to generate a pileup file from all SAM files. The generated pileup file was used as the input in VarScan (v2.3.7, Koboldt et al., 2012) to call single nucleotide polymorphisms (SNPs). To accommodate the scenario of pooled sequencing, we specified a higher minimum depth of coverage of 20x, and a minor allele frequency of 0.01 to detect rare variants in the pool. Resulting variant call format (VCF) files were filtered to remove insertion-deletions (indels) and any SNPs that were not biallelic using vcftools (Danecek et al., 2011). Resulting VCF files contained 6,622 SNPs from 16 pools for *H. bacteriophora* and 196,812 SNPs from 18 pools for *S. feltiae*. For analyses of population structure and isolation by distance, SNPs were excluded that showed extreme levels of differentiation among populations (taken as evidence of being under selection). These outlier SNPs were identified using the 'pcadapt' R package (Privé et al., 2020). The false discovery rate of outliers was constrained to 0.1% using the 'qvalue' R package (Storey et al., 2020). After removing outliers, 3,367 SNPs and 138,286 SNPs remained for *H. bacteriophora* and *S. feltiae*, respectively.

2.4. Population structure and isolation by distance analysis

Population structure was assessed with principal coordinates analysis of pairwise F_{ST} using the 'ape' R package (Paradis and Schliep, 2019). We also assessed population structure using principal components analysis of variant allele frequencies and obtained nearly identical results, so we only show results from principal coordinates analysis of pairwise F_{ST}. Pairwise F_{ST} was calculated with the 'poolfstat' R package (Hivert et al., 2018), specifying a pool size of 20,000 haploid sequences (pools consisted of ca. 10,000 diploid individuals). Differences in mean pairwise F_{ST} between species and between pools within the same field or pools collected from different fields were compared using a two-way ANOVA. Neighbor-joining trees were constructed based on Nei's genetic distance using the 'aboot' function in the poppr R package (Kamvar et al. 2014), and node support was assessed using 500 bootstraps.

We examined evidence of isolation by distance (wherein genetic differentiation increases with increasing geographic distance between populations, resulting from a stepwise pattern of gene flow among populations over a landscape; Wright, 1943) using a Mantel test (Oksanen et al., 2019) run with 100,000 permutations. Geographic distances between pools were calculated as geodesic distances with the 'geodist' R package (Padgham and Sumner, 2020). Lastly, we visualized patterns of isolation by distance by plotting transformed pairwise F_{ST} (i.e., $F_{ST}/(1 - F_{ST})$) against natural log-transformed geographic distance.

3. Results

3.1. Nematode strains

In total, we collected 16 strains of *H. bacteriophora* and 18 strains of *S. feltiae* from the eight potato fields that we sampled (Fig. 1). We collected multiple strains of *H. bacteriophora* from the same potato field at 4 sites, and multiple strains of *S. feltiae* from the same potato field at 5 of the sites (Table S1).

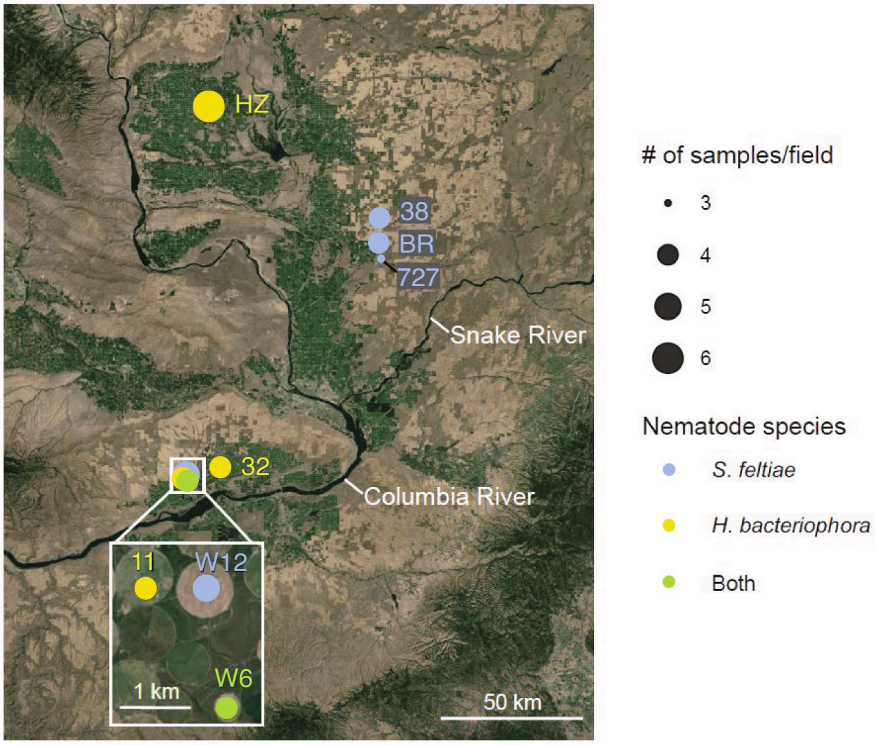

Fig. 1. Sampling map. Ten traps, each containing twenty waxworm larvae, *Galleria mellonella*, were set up in each field (See Materials and Methods). The number of traps recovered with nematode infection and nematode species were labeled as the size and color of the circles. Among four traps recovered from field W6, two traps were *Heterorhabditis bacteriophora* and the other two were *Steinernema feltiae*. All nematodes collected from each trap were considered a single strain.

3.2. RAD-tag sequencing and variant calling summary

Illumina sequencing yielded a total of ~132 million reads for *H. bacteriophora* and ~265 million reads for *S. feltiae* (Table S1). The average number of reads per pool for *H. bacteriophora* was ~8 million, of which ~82% were successfully mapped to the *H. bacteriophora* reference genome (Table S1). The average number of reads per pool for *S. feltiae* was ~15 million, of which ~68% were successfully mapped to the *S. feltiae* reference genome (Table S1). After stringent filtering of variants, we kept 3,367 and 138,286 SNPs for *H. bacteriophora* and *S. feltiae*, respectively.

3.3. Population structure and isolation by distance

In both nematode species, strains broadly separated by geographic origin along the first two axes in a principal coordinates analysis of pairwise F_{ST}, but did more so for *H. bacteriophora* than *S. feltiae* (Fig. 2). Pairwise F_{ST} values for comparisons of strains collected from different fields were generally greater than pairwise F_{ST} comparisons of two strains collected from within the same potato field (two-way ANOVA, *p*-value < 2e−16) (Fig. 3A) (Tables S3 and S4). Remarkably, for *H. bacteriophora*, the average F_{ST} of across-field populations was 15-fold greater than average F_{ST} of within-field populations (Fig. 3A). The average F_{ST} of between-field comparisons of *S. feltiae* was also greater than the average F_{ST} of within-field strain comparisons, but only by 1.4-fold. Neighbor-joining trees based on Nei's genetic distances between nematode strains recapitulated the pattern observed in principal coordinates analysis, with strains collected from the same or adjacent fields generally clustering together (Supp. Fig. 1).

Large overall differences in levels of genetic differentiation among strains were found for the two nematode species (two-way ANOVA, *p*-value < 2e−16). The average F_{ST} of *S. feltiae* populations was 3.3-fold higher than the average F_{ST} of *H. bacteriophora* populations (Fig. 3A). Particularly, at the local scale (within a field), F_{ST} of *S. feltiae* populations was 30-fold greater (Fig. 3B-C) than F_{ST} of *H. bacteriophora* populations. This was due to some extremely high values of pairwise F_{ST} among *S. feltiae* strains (e.g., F_{ST} = 0.622 between two strains within field 727), even alongside fields where genetic differentiation among strains was low (Fig. 3C) (Table S4). By contrast, there was very little population differentiation of *H. bacteriophora* populations at the local scale, with no values of pairwise F_{ST} exceeding 0.024 (Fig. 3B) (Table S3).

Despite broad variability in pairwise F_{ST} among strains within and between fields for both species, Mantel tests consistently identified significant evidence of isolation by distance (*H. bacteriophora*: Mantel r = 0.4033, p = 0.008; *S. feltiae*: Mantel r = 0.3475, p = 0.001) (Fig. 4).

4. Discussion

Individual entomopathogenic nematodes are able to actively move only relatively short distances in the soil, in search of hosts and/or favorable environmental conditions (Grewal et al., 1997). Daily movement distances for entomopathogenic nematodes have been estimated at 4 to 33 cm per day (Bal et al., 2015; Jabbour and Barbercheck, 2008), meaning that sustained movement in one direction could lead to dispersal of as much as 10 m in a month. The nematodes might also sometimes exhibit phoretic movement with the assistance of earthworms although, here again, movement would typically be over relatively modest distances of several meters (Shapiro-Ilan and Brown, 2013). However, the nematodes lack a highly dispersive stage as many predatory insects can achieve as winged adults, or spiders achieve by "ballooning" on silk threads. So, our prediction was that we would be able to detect a clear gradient of decreasing relatedness among nematode strains with increasing distance from one collection site to another. Indeed, this was generally the case for both nematode species, as we detected statistically-significant relationships between genetic and geographic distances in both cases. Comparable levels of isolation by distance have also been found in insects, but over much greater geographic distances (40–3,000 km; e.g., Schmidt et al., 2017; Cordeiro et al., 2019; Driscoe et al., 2019), while an absence of isolation by distance can occur for invasive insects over vast spatial scales (Perry et al., 2020) and for native insects even at fine spatial scales (e.g., <2 km versus <5 km in our study; Kahnt et al., 2018). Nematode strains collected from the same field were generally more genetically similar than those collected from different fields, although this difference was more clearly apparent for *H. bacteriophora* than for *S. feltiae* (Figs. 3 and 4). This could result from heterorhabditids having hermaphrodites in their life-cycle, whereas *S. feltiae* does not, perhaps leading to more clonal populations and thus reduced heterozygosity in *H. bacteriophora* (Ciche, 2007). Altogether, these patterns suggest that in-field conservation practices, such as leaving crop residue or otherwise minimizing soil disturbance, might be particularly effective means to conserve entomopathogenic nematodes (e.g., Shapiro et al., 1999; Millar and Barbercheck, 2002; Hoy et al., 2008).

At the same time, we also detected notable genetic differences among nematode strains collected from nearby sites (Figs. 3 and 4). Within fields, indeed, genetic distances among pairs of nematode strains were often as great as was seen for strains collected from our geographically most-distant sites. This can be seen, for example, when looking at the dispersion of genetic distances in fields '11' and 'HZ' for

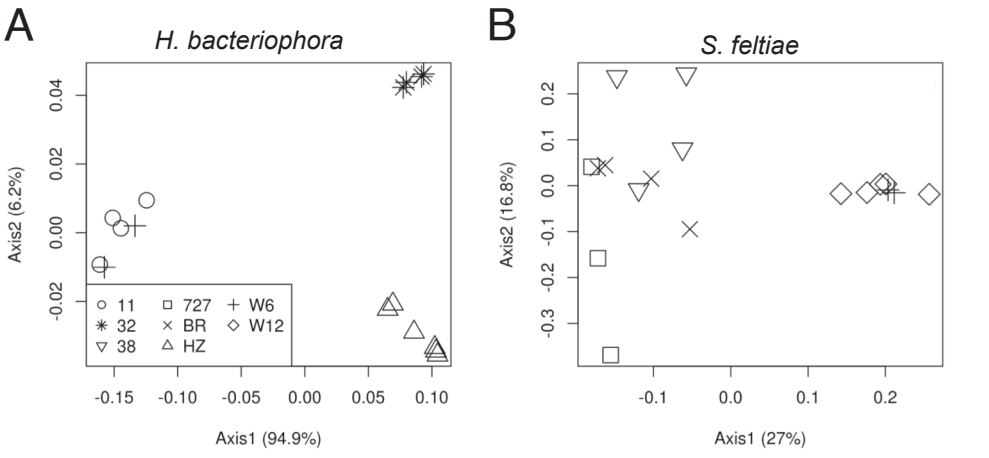

Fig. 2. Principal coordinates analysis of pairwise F_{ST} among (A) *Heterorhabditis bacteriophora* and (B) *Steinernema feltiae*. The analysis is based on 3,367 SNPs for *H. bacteriophora* and on 138,286 SNPs for *S. feltiae*.

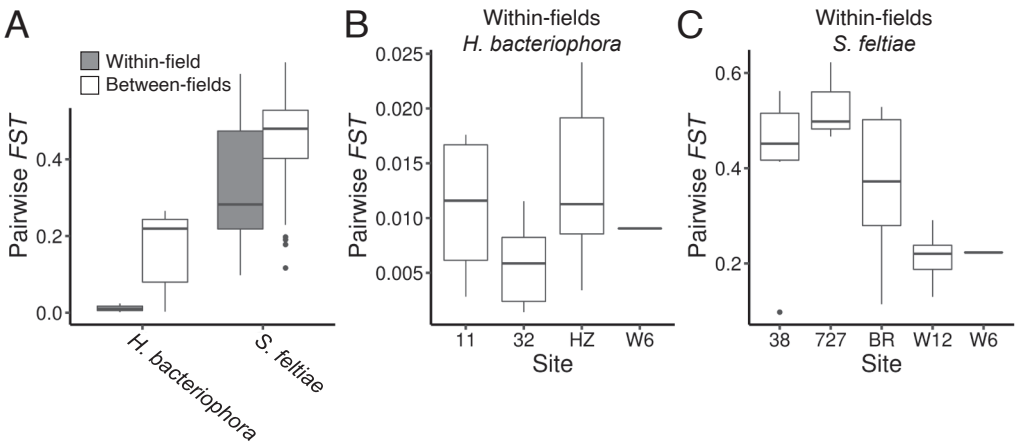

Fig. 3. Boxplots of genome-wide F_{ST} of nematode population pairs of (A) *Heterorhabditis bacteriophora* and *Steinernema feltiae* grouped by distance (whether a pair of nematode populations were collected from the same field or not). Boxplots of genome-wide F_{ST} of within-field (B) *H. bacteriophora* population pairs and genome-wide F_{ST} of within-field (C) *S. feltiae* population pairs separated by field. Note, only two populations of *H. bacteriophora* and *S. feltiae* were collected from field W6. Boxplots depict pairwise F_{ST} as medians (thick line), 25th and 75th percentiles (box edges), 95th percentiles (whiskers) and outliers (circles).

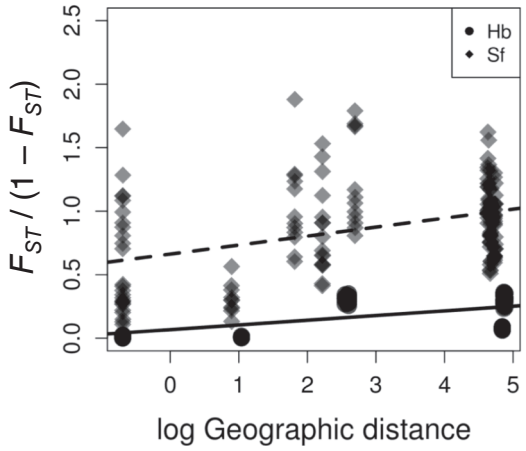

Fig. 4. Relationship between pairwise F_{ST} and geographic distance for *Heterorhabditis bacteriophora* (Hb) and *Steinernema feltiae* (Sf). Mantel tests indicated significant evidence of isolation by distance (*H. bacteriophora*: Mantel r = 0.4033, p = 0.008; *S. feltiae*: Mantel r = 0.3475, p = 0.001), and linear trend lines are included here only for visualization.

H. bacteriophora, and field 'BR' for *S. feltiae* (Fig. 4B-C). Likewise, principal coordinates analysis of pairwise F_{ST} suggested that strain pairs from relatively distant collection sites could nonetheless be quite similar genetically, especially for *H. bacteriophora* (Fig. 2). It is unclear how these nematodes, with relatively little dispersal ability, might be capable of traversing relatively large distances between cropping fields with presumably hostile dry scrub habitat in between. One possibility is that they are carried along with soil that contaminates farm implements, as equipment is moved from one field to another for tillage, harvest, or other management practices. A second possibility is that nematodes could be piggy-backing inside infected hosts, if they have entered adult insects that are not yet too ill to fly (e.g., Lacey et al., 1995; Snyder et al., 1998), or through phoretic movement when cattle or other larger animals pick up and move soil (e.g., Eng et al., 2005; Campos-Herrera et al., 2006; Shapiro-Ilan and Brown, 2013). A third possibility is that commercially available strains may have been applied as bio-pesticides at some time in the past and then formed self-sustaining populations, although to the best of our knowledge this is not a common pest-control practice in this region (Lacey et al., 2015). In any case, entomopathogenic nematodes have a genetic population structure consistent with occasional long-distance movement and establishment against a background of limited dispersal by actively moving individuals. This implies, in turn, that entomopathogenic nematodes may have an underappreciated ability to re-colonize agricultural fields from relatively distant source populations, following soil fumigation or other disturbances that reduce or eliminate local populations (e.g., Henderson et al., 2009).

There is growing evidence that biological control can grow stronger when several natural enemy species forage alongside one another (Jonsson et al., 2017; Greenop et al., 2018; Snyder, 2019). This occurs when different species forage in different ways, in different places, and/or at different times (Schmitz, 2007; Finke and Snyder, 2008; Dainese et al., 2017). That is, they occupy complementary feeding niches (Snyder, 2019). Indeed, interspecific complementarity of this type has been documented among entomopathogenic nematode species (Campbell and Gaugler, 1997; Neumann and Shields, 2006), and has been shown to improve natural pest control for several communities where entomopathogens are important components (e.g., Kaya et al., 1993; Crowder et al., 2010; Jabbour et al., 2011; Miller et al., 2020). Outside of biological control communities, it appears that intraspecific genetic diversity can bring similar benefits for resource consumption, when genetic differences code for trait differences that foster intraspecific complementarity (Hughes et al., 2008). Among the entomopathogenic nematodes that we considered here, relatively distantly related worm strains sometimes were found living within the same field. This suggests the possibility for ecologically meaningful local genetic differences within these two nematode species, but future work will be needed to determine whether this translates into a measurable improvement in biological control (Lu et al., 2016). We note that Stuart et al. (2004) found the greatest mortality of citrus root weevil (*Diaprepes abbreviates*) larvae when these herbivores were attacked by genetically-diverse mixes of the entomopathogenic nematode species *S. riobrave*, compared to those facing attack by any single genetic strain; this is consistent with benefits of intraspecific genetic diversity for pest suppression.

Successful and sustainable conservation biological control often requires that natural enemies readily move from refuges into cropping fields, and that refuges are spatially located so as to benefit the fields of the grower that has paid to establish and maintain the refuge (Gurr et al., 2017). This in turn requires a fairly sophisticated and detailed knowledge of how natural enemies move across landscapes at relatively fine scales (Tscharntke et al., 2007). Sometimes this can be achieved by physically marking natural enemies in the refuge, and then looking to see if marked individuals can later be collected within the crop (Hagler, 2019). It can be logistically burdensome and expensive, of course, to mark enough individual natural enemies to make it reasonably likely that sufficient individuals will be re-collected for conclusions to be drawn (e.g., Blaauw et al., 2016). Similarly, change in pest numbers and/or crop damage in fields near versus distant from refuges can be used to infer that natural enemies are readily moving from refuge to protection target (e.g., Tylianakis et al., 2004; Blaauw and Isaacs, 2012). Again, however, there can be logistical challenges, and it is not always

possible to tie biological control to specific natural enemy species arising out of the refuge (e.g., Lee and Heimpel, 2005; Prasad and Snyder, 2006). Here, we demonstrate that RADseq, by detailing relatively fine-scale genetic differences among pools of natural enemies, can be used to infer movement patterns across agricultural landscapes. This approach has recently been used, for example, to detail relatively localized movement patterns of mosquitoes that transmit human pathogens (e.g., Schmidt et al., 2017). We suggest that, as costs continue to fall, RAD-seq, and eventually whole-genome sequencing, will become increasingly powerful and useful tools to delineate natural enemy movement at the fine scales relevant to conservation biocontrol. This in turn will allow growers to distribute refuges in space and time to match natural enemy dispersal patterns, improving the effectiveness of conservation biological control.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2021.104662.

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