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Fine-scale spatial genetic structure of a fungal parasite of coffee scale insects

Doug Jackson *, Ash T. Zemenick, Brian Malloure, C. Alisha Quandt, Timothy Y. James

Department of Ecology and Evolutionary Biology, University of Michigan, 2019 Kraus Nat. Sci. Bldg., 830 North University, Ann Arbor, MI 48109, USA

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ABSTRACT

The entomopathogenic fungus *Lecanicillium lecanii* persists in a highly dynamic network of habitat patches (i.e., a metapopulation) formed by its primary host, the green coffee scale *Coccus viridis*. *Lecanicillium lecanii* is an important biological control of both *C. viridis* and the coffee rust, *Hemileia vastatrix*. Successfully managing this biocontrol agent will depend on an increased understanding of the characteristics of its dispersal, as migration between occupied and unoccupied patches is essential for the persistence of this metapopulation. In the present study, we employ a population genetics approach, and show that in our study system, a coffee farm in the Soconusco region of southern Mexico, *L. lecanii* is characterized by clear spatial genetic structure among plots within the farm but a lack of apparent structure at smaller scales. This is consistent with dispersal dominated by highly localized transport, such as by insects or rain splash, and less dependence on longer distance dispersal such as wind transport. The study site was dominated by a few multi-locus microsatellite genotypes, and their identities and large-scale locations persist across both study years, suggesting that local epizootics (outbreaks) are initiated each wet season by residual propagules from the previous wet season, and not by long-distance transport of propagules from other sites. The index of association, a measure of linkage disequilibrium, indicates that epizootics are primarily driven by asexual, clonal reproduction, which is consistent with the apparent lack of a teleomorph in the study site and the presence of only a single mating type across the site (MAT-1-2-1). Although the same predominant clonal genotypes were found across years, a drastic difference in genotypic diversity was witnessed across two sites between the two years, suggesting that interclonal selection was occurring. In light of the dispersal limitation of *L. lecanii*, spatial structure may be an essential axis of management to ensure the persistence of *L. lecanii* and preserve the ecosystem services provided by this versatile biocontrol agent in this and similar coffee farms.

1. Introduction

Due to the ubiquity of populations that are distributed across networks of habitat patches, and the difficulty of empirically assessing such metapopulations, the persistence of metapopulations has proven to be a topic of great theoretical interest (Hanski, 1999; Levins, 1968). Persistence of metapopulations is ultimately a matter of two factors: the local extinction rate and the rate of migration between patches. Therefore, translating the insights gained by this theory to ecosystems of interest requires knowledge of extinction and migration rates under field conditions. This can be difficult to obtain for more cryptic organisms with short life cycles, such as fungi and bacteria, which, despite their microscopic propagules, also exhibit dispersal limitation (Peay et al., 2010).

Molecular population genetics provides powerful tools to infer dispersal patterns (Broquet and Petit, 2009) and obtain evidence of migration between patches. The distribution of alleles at multiple spatial scales, and the extent to which these distributions adhere to or depart from panmixia, can provide information about the connectedness of metapopulation habitat patches and the persistence of populations within these patches. These methods are particularly powerful for microorganisms, which are not as amenable to other techniques for ascertaining migration characteristics (e.g., tagging, visual observations, etc.).

In the present study, we apply a population genetics approach to gain insight into the dispersal characteristics of *Lecanicillium lecanii*, an important biological control of two potential pests of coffee in southern Mexico: the green coffee scale, *Coccus viridis*,

* Corresponding author.
E-mail address: dougjack@dougjackson.net (D. Jackson).

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and coffee rust, *Hemileia vastatrix* (Jackson et al., 2012a; Vandermeer et al., 2009).

The primary host of *L. lecanii* in the study site is *C. viridis*. Nearly all of the biomass of *L. lecanii* in the farm is generated by infections of *C. viridis*, while infections of *H. vastatrix* account for a minuscule fraction of *L. lecanii* biomass, and are therefore likely to play a negligible role in the population dynamics of *L. lecanii* in this system. Populations of *C. viridis* can reach in the thousands on a single coffee plant (Perfecto and Vandermeer, 2006); these large concentrations provide the abundance and density of hosts necessary for *L. lecanii* to proliferate in local epizootics, which can infect and ultimately kill nearly 100% of the *C. viridis* populations on a coffee plant (Jackson et al., 2009; MacDonald et al., 2013).

The distribution of *C. viridis* itself is a consequence of its interaction with a second organism: its mutualistic partner *Azteca sericeasur*, an arboreal-nesting ant that provides protection from predators to *C. viridis* while feeding on the carbohydrate-rich honeydew that *C. viridis* excrete. *Azteca sericeasur* creates carton nests in shade trees, which form a canopy over the coffee plants, and occasionally nests in the coffee plants themselves. In our study system, a coffee farm in the Soconusco region of southern Mexico, *A. sericeasur* nests are distributed in a spatially clustered pattern, despite the shade trees being distributed in a pattern that is statistically indistinguishable from a uniform distribution (Vandermeer et al., 2008). These clusters of nests, and the associated clusters of large *C. viridis* populations in the coffee plants surrounding these nests, form a spatially-extended landscape of resource-rich habitat patches for *L. lecanii* to exploit.

This landscape of habitat patches in which the *L. lecanii* metapopulation exists is temporally variable due to the pronounced wet-dry seasonality of the region. In the wet season, conditions are favorable for the scale insects (Jackson et al., 2014a) and for *L. lecanii* (Reddy and Bhat, 1989), and this is the season during which ongoing epizootics are most prevalent. In the dry season, the scale populations contract (Jackson et al., 2014a) and conditions are unfavorable for the fungus, which is almost exclusively found as dry, residual infections on *C. viridis* cadavers (unpublished data).

Thus, for *L. lecanii* to persist in this system, it must function as a metapopulation in a landscape of habitat patches that are separated spatially and which expand and contract in a highly dynamic fashion throughout the course of a year. Persistence of *L. lecanii* is a function of two factors: first, its ability to avoid extinction in a given site (defined as a cluster of coffee plants populated by *C. viridis* surrounding an *A. sericeasur* nest or tight cluster of nests), either via infected cadavers that persist through the dry season or by a pool of propagules in the soil (Jackson et al., 2012b) or other environmental reservoir; and second, the dispersal ability of the fungus, which determines its migration rate and thus its ability to infect newly formed populations of *C. viridis* or rescue patches from which the fungus has been locally extirpated.

Understanding the influence of dispersal on the persistence of the *L. lecanii* metapopulation, and by extension the robustness of this metapopulation to management actions that may alter the spatial structure of the system, such as the pruning or felling of shade trees, is important for the management of *C. viridis* and *H. vastatrix*, which can become significant pests if they escape control (Vandermeer et al., 2010). Despite this, relatively little is known about the dispersal of *L. lecanii* under field conditions. Outstanding questions include: Is there apparent spatial genetic structure within a landscape, and if so, is it consistent with wind dispersal or smaller-scale processes such as rain splash or insect dispersal? Is dispersal a consequence of clonal reproduction, as is suggested by the fact that a teleomorph (the sexual reproductive stage) of *L. lecanii* has yet to be observed in this system? To what extent are local epizootics initiated by long-distance dispersal from external sources versus residual propagules from the previous year's epizootics? Is the system dominated by a few successful genotypes or by a diverse and variable suite of genotypes?

To begin to answer these questions, and to help illuminate the extent to which migration between patches is promoting persistence of this metapopulation, we employed a population genetics approach based on a nested, hierarchical sampling across two years. From these data, we were able to identify unique genotypes; determine their spatial distribution within and across years; calculate the amount of genetic variation that was partitioned at a variety of spatial scales; test for clonality and evidence of sexual recombination; and test for spatial autocorrelation.

2. Material and methods

The study was performed at Finca Irlanda, a coffee farm in the Soconusco region of southwest Chiapas, Mexico (15°11′ N, 92°20′ W), the site of a 45-hectare plot that has been under continuous and intensive study for over a decade (Perfecto et al., 2014, 2003; Perfecto and Vandermeer, 2008; Vandermeer et al., 2010, 2008, 2002; Vandermeer and Perfecto, 2006). Annual rainfall is ca. 4500 mm, and the elevation ranges from ca. 900–1150 m. The farm encompasses a total area of approximately 300 ha, and is managed as a commercial polyculture (Moguel and Toledo, 1999), with 30–50% shade cover and almost 100 tree species in total, primarily *Inga* spp. The total number of trees with dbh > 10 cm in the 45 ha study plot was 7294, or approximately 162/ha, in 2010; and 6145 (ca. 137/ha) in 2011. The total number of *A. sericeasur* nests in the study plot was 624 and 581 in 2010 and 2011, respectively. Large populations of *C. viridis*, on the order of 1000–2000 individuals per coffee plant (Perfecto and Vandermeer, 2006), are typically found only directly adjacent to an *A. sericeasur* nest on a few coffee plants (generally less than 5).

Samples of infected *C. viridis* individuals were collected in 2010 and 2011. To determine how genotypic variation was partitioned at multiple spatial scales, we employed a nested design: samples were collected from five sites (sites A-E), from four coffee plants at each site, and from 10 branches chosen haphazardly at different heights within each coffee plant. Our sites were defined as the coffee plants surrounding a single *A. sericeasur* nest or group of nests in adjacent shade trees. Nested sampling is an effective method for obtaining information about the effect of spatial scale, particularly for clustered populations such as this (Storfer et al., 2007).

Our sampling scheme was designed to balance coverage with sampling effort, allowing us to obtain a representative sample of the unit of interest (infected *C. viridis* individuals) across nested spatial scales while accounting for the sparse distribution of *C. viridis* populations in the plot and the substantial time and effort involved in hiking between sites across difficult terrain. The number of branches sampled within each plant (10) was also influenced by the tendency of *C. viridis* individuals to cluster on a small number of branches within a given plant and the average number of branches per plant found previously in a representative sample of coffee plants (n = 430, mean = 31.3, SD = 17.4).

To obtain five sites with four plants hosting sufficient scales infected by *L. lecanii*, it was necessary to select two sites from outside the perimeter of the 45 ha plot (Fig. 1). It was not possible to sample the same plants in both years because individual plants are periodically pruned, cut back to the stump, or replaced between seasons. Also, epizootics of *L. lecanii* may not occur on the same plants in subsequent years. Due to these constraints, only two plants were available in one site (Site B) in 2011. Because an additional plant with sufficient infected scales was available, we also opportunistically sampled an additional plant at Site A in 2011.

We removed infected scales from leaves and berries of the coffee plants using forceps that were surface-sterilized with 95%

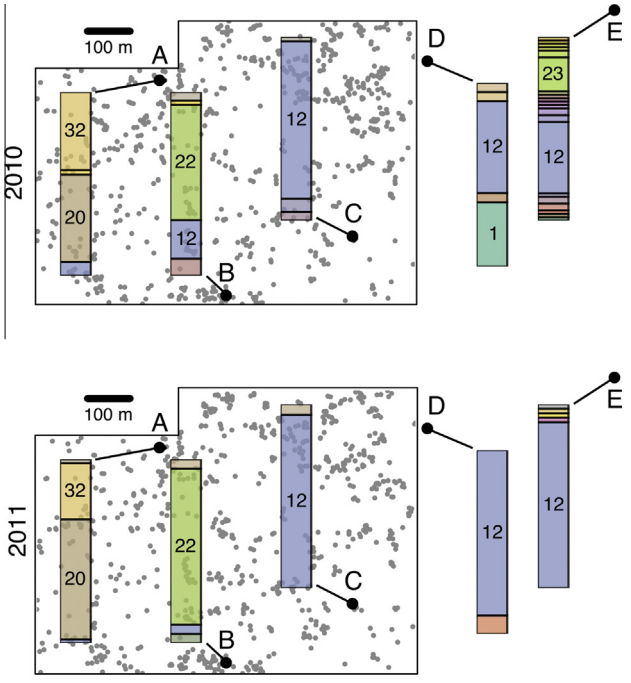

Fig. 1. Distribution of genotypes at the scale of the 45 ha plot in 2010 and 2011. Bar plots show the fraction of samples at each site belonging to each of 39 total genotypes, indicated by color code and by number where space permits. Sites (A–C) were located within the 45 ha plot and sites (D and E) were outside. Gray dots show the locations of *Azteca sericeasur* nests, which are often associated with large populations of *Coccus viridis* and associated epizootics of *Lecanicillium lecanii*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ethanol and flamed between each sample. Infection of scales by *L. lecanii* is readily apparent, as the mycelium of the fungus forms a characteristic white mat from which the common name of the fungus, "white halo fungus," derives. Samples were transferred to individual vials containing 2X CTAB buffer (James et al., 2008) for transport back to the laboratory in the United States. DNA was extracted by grinding with a microfuge tube pestle and extraction with chloroform/isoamyl according to James et al. (2008) and resuspended in 25–50 µl of H_2O.

The genome from a strain (UM487) collected in 2009 was sequenced to develop microsatellite markers. We first obtained a pure culture by repeated sub-culturing on potato dextrose agar. We then grew this culture in liquid medium and extracted DNA from it using the CTAB extraction protocol (James et al., 2008). A paired-end sequencing library with mean fragment size of 650 bp was then constructed by the U. Michigan DNA sequencing core using NEBNext Reagents for DNA Sample Preparation (New England BioLabs, Ipswich, MA, USA). The library was run on one half of a lane of an Illumina GAIIx analyzer to generate approximately 13.2 million paired-end reads of length 154 bp. Because of lower quality towards the ends of the reads, they were uniformly trimmed to 108 bp. We then assembled the genome using the trimmed reads with the software Velvet v. 1.1.04 (Zerbino and Birney, 2008) using k-mer = 49. Gene model predictions were created using the Maker annotation pipeline (Cantarel et al., 2008). Information supplied to Maker included a SNAP hmm (Korf, 2004) trained on *Fusarium graminearum*, which was also set as the species model for AUGUSTUS v 3.1 (Stanke et al., 2006), and EST data from the following hypocrealean taxa: *F. graminearum*, *Nectria haematococca*, *Trichoderma reesei*, *Trichoderma virens*, *Metarhizium robertsii*, *Tolypocladium inflatum*, *Tolypocladium ophioglossoides*, *Cordyceps militaris*, and *Beauveria bassiana*. Annotation of transposable elements was performed using RepeatMasker v 4.0.6 with organism set to "fungi" (Smit and Green, 1996), and custom repeat contents were estimated using RepeatScout v 1.0.3 and scripts associated with that package (Price et al., 2005). The genome assembly and annotations have been deposited in NCBI/Genbank under the accession LUKN00000000. Completeness of the *L. lecanii* genome was estimated using CEGMA (Parra et al., 2007, 2009).

Microsatellites were obtained from the assembled genome using the script MIcroSAtellite identification tool (MISA) (Thiel, 2003). After an initial screen of loci for repeatability and polymorphism, a subset of 10 loci were targeted for population analysis. The primers for PCR amplification are shown in Table S1. We used a labeling strategy employing a M13 overhang on the forward primers to incorporate the fluorophore 6-FAM into the amplicons (Schuelke, 2000). The PCR reaction consisted of locus-specific forward and reverse primers and a 6-FAM 5′ labeled M13 primer (5′-TGTAAAACGACGGCCAGT) in the same reaction. PCR was performed using ExTaq proofreading DNA polymerase (TaKara, Otsu, Japan) with 12.5 nM forward primer, 12.5 nM 6-FAM labeled M13F primer, 416 nM reverse primer in a 6 µl reaction with 1 µl genomic DNA. PCR amplification conditions were: 94 1C (5 min), then 30 cycles at 94 1C (30 s)/56 1C (45 s)/72 1C (45 s), followed by 8 cycles 94 1C (30 s)/53 1C (45 s)/72 1C (45 s), and a final extension at 72 1C for 10 min (Schuelke, 2000). One microliter of the PCR product was then added to 12 ml formamide and 0.1 ml ROX GS500 standard (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730 capillary sequencer at the University of Michigan Sequencing Core. The resulting chromatograms were analyzed using the software GeneMarker (Soft Genetics LLC, State College, PA), and allele sizes were extracted.

In some cases, the chromatograms revealed that individual scales had been infected by at least two different genotypes of *L. lecanii*, as indicated by individual loci with signals from two alleles. In such cases of multiple infection, there is no way to know for certain which of the two alleles at a given locus should be assigned to which genotype. Following the principle of parsimony, we assigned one set of alleles to the most common genotype consistent with the alleles observed at the 10 loci, and the remaining sequence was assigned to a second genotype. We then treated these two inferred genotypes as separate samples for all further analyses.

Mating type was estimated by PCR analysis of the MAT locus using primers designed to be specific to the MAT1-1-1 alpha-box gene (LL-MAT-1-1-1-F: 5′-GCCGTCGAAGATACCGAATA and LL-MAT1-1-1-R: 5′-AGTGGACGGTGTCGTGATCT) or to the MAT1-2-1 HMG gene (LL-MAT1-2-1-F: 5′-CAAGAGCCACATTTGTCGAA and LL-MAT1-2-1-R: 5′-GTCTGGTGTTGCTGATGCTG). Primers for MAT1-2-1 were designed using the sequence of UM487, and the MAT1-1-1 primers were designed using GenBank acc. # AZHF01000005 bases 535,854-537,341. Amplification of the MAT regions was conducted using GoTaq Green 2X Master Mix (Promega) with 0.5 µM forward and reverse primers and 5 µl DNA in a 12.5 µl reaction. Amplicons were run on a 1% agarose gel and the presence or absence of the appropriate sized band was used to infer mating type.

We used the R (R Core Team, 2014) package *Hierfstat* (de Meeûs and Goudet, 2007; Goudet, 2004) to test for population genetic structure using fixation indices, which are analogous to Wright's F-statistics. The nested study design allowed for testing of genetic differentiation of sites within the total population and of plants within each site. Since samples were taken from the same sites during both study years, we treated year as a crossed factor (a factor that occurs at the same rank in multiple units across a single level of the hierarchy, and thus cannot be treated as a nested factor; de Meeûs and Goudet, 2007). Because we were unable to

sample the same plants in both years, we could not test the effect of year as a crossed factor at the plant level.

We tested for spatial structure at the within-plant, within-site, and within-farm (within the 45 ha plot) scales using permutation tests. At the within-plant scale, the average physical distance between samples with the same genotypes was calculated for each plant, with distance based on the heights of the samples. If samples with identical genotypes cluster together spatially, as would be expected if the fungus tends to spread sequentially from branch to branch within a plant (e.g., because of small-scale dispersal of spores by insects), the average distance between similar genotypes should be less than would be expected by chance. The observed average distances were compared to the null expectation of a random distribution of genotypes within the plant by randomly permuting the sample heights, thereby generating a synthetic distribution of genotypes with no spatial structure. This permutation was repeated 1000 times. A *P*-value was calculated as the fraction of these permutations for which the average distance between genotypes was equal to or less than the observed distance – roughly speaking, the probability that clustering of like genotypes as extreme as the observed clustering could have occurred by chance.

We performed a similar analysis at the within-site and within-farm scales, except the physical distance at these scales was defined as the Euclidean distance between plants (for the within-site analysis) or sites (for the within-farm analysis) based on their Cartesian coordinates.

To test for clonal reproduction, we assessed linkage disequilibrium by calculating the index of association, I_A (Brown et al., 1980), using the R package *Poppr* (Kamvar et al., 2014). The index of association assesses how likely individuals that share an allele at one locus will also share alleles at other loci (Agapow and Burt, 2001). Missing data were treated as novel alleles, which is the most common and conservative assumption (Kamvar et al., 2014), though the results were found to be insensitive to this choice. Significance was assessed using a permutation test with 999 permutations.

We used the four-gamete test for the occurrence of historical recombination via sexual reproduction (Hudson and Kaplan, 1985). If all four possible combinations of two alleles at two loci occur (e.g., AB, A′B, AB′, A′B′), it can be inferred that a recombination event must have occurred, assuming that the mutation rates are not high relative to recombination rates. We applied this test to every possible two-locus combination of our complete 10-loci data set, and to every possible four-gamete combination of alleles for each pair of loci.

3. Results

In the two study years, a total of 327 infected scales were successfully genotyped with microsatellite markers. Of these, 269 were infected by a single genotype, and 58 were multiply-infected by fungi with two distinct genotypes, resulting in an effective total of 385 samples. In every case, the assignment of alleles detected in doubly-infected individuals resulted in two genotypes that had already been observed in singly-infected individuals, i.e., the assignment was consistent with the scale having been infected by two fungi with genotypes that were already known to exist in the population.

Overall, the diversity was low, with a mean of 3.1 alleles per locus averaged across years (see Table 1). A total of 32 unique genotypes were present in the 2010 samples. The total number of samples genotyped ranged from 20 to 54 per site in 2010 and from 20 to 49 per site in 2011 (Table 2). No samples were successfully genotyped from plant d of Site D in 2010 (Fig. 2). Genetic diversity was substantially reduced in 2011, falling to a total of 12 unique genotypes. The per-site totals in 2010 were 4, 5, 4, 5, and 20 unique genotypes for Sites A through E, respectively (Fig. 1, Table 2). The total counts in Sites A through E in 2011 were 4, 4, 2, 2, and 5 unique genotypes (Fig. 1, Table 2). The greatest decrease in diversity between years was in Site E, which decreased from 20 unique genotypes to 5.

Table 1
Mean numbers of genotypes and alleles per locus. A total of 39 unique genotypes were identified in the study.

	Mean number of genotypes		Mean number of alleles per locus	
	2010	2011	2010	2011
Per year	32	12	3.3	2.9
Per site	7.6	3.4	1.5	1.4
Per plant	3.6	2.0	1.4	1.2

Table 2
Per year and per site number of samples successfully genotyped, unique genotypes, and mean number of alleles per locus.

Year	Site	Samples genotyped	Unique genotypes	Mean number of alleles per locus
2010	A	40	4	1.4
	B	43	5	1.3
	C	42	4	1.3
	D	20	5	1.7
	E	54	20	2.0
2011	A	49	4	1.3
	B	20	4	1.8
	C	35	2	1.1
	D	41	2	1.3
	E	41	5	1.7

Qualitatively, at the farm level there was a pronounced spatial structure that remained consistent across years (Fig. 1). Site A, on the western side of the 45 ha plot, was dominated by genotypes 20 and 32 in both years. Site B was largely populated by genotype 22 in both years, with a small fraction of genotype 12. Sites C-E, in the eastern side of the study site, were populated primarily by genotype 12. Genotypes 1 and 23 were also common in Sites D and E in 2010. The identity of the dominant genotype in each site was consistent from 2010 to 2011; in each site, the dominant genotype in 2010 constituted an even larger proportion of samples in 2011 (Fig. 1).

There was significant genetic differentiation between sites and between plants within each site in 2010, and between sites in 2011. The $F_{site/total}$ fixation index, which measures the differentiation between sites relative to the total genetic diversity, was 0.32 in 2010 and 0.41 in 2011, and was highly significant ($P < 0.001$) in both years. $F_{plant/site}$, a measure of the partitioning of genetic diversity of plants within sites, was significant in 2010 ($F_{plant/site} = 0.1$, $P < 0.05$) but not in 2011 ($F_{plant/site} = 0.01$, $P = 0.59$). These results are also evident in the qualitative patterns in Figs. 1–3: sites have distinct compositions in both years (Fig. 1), while plants within sites are clearly distinct in 2010 (Fig. 2) but not in 2011 (Fig. 3).

Fixation indices measuring the contribution of year to the partitioning of genetic diversity within each site, $F_{year/site}$, showed a significant effect of year ($P < 0.05$) in all sites except B ($F_{year/site}$ in Site A = 0.87; Site B = 0.88; Site C = 0.97; Site D = 0.77; Site E = 0.66). Site B was the site with only two plants sampled in 2011. The overall contribution of year across all sites, calculated using Fisher's combined probability test, was highly significant ($P \ll 0.001$).

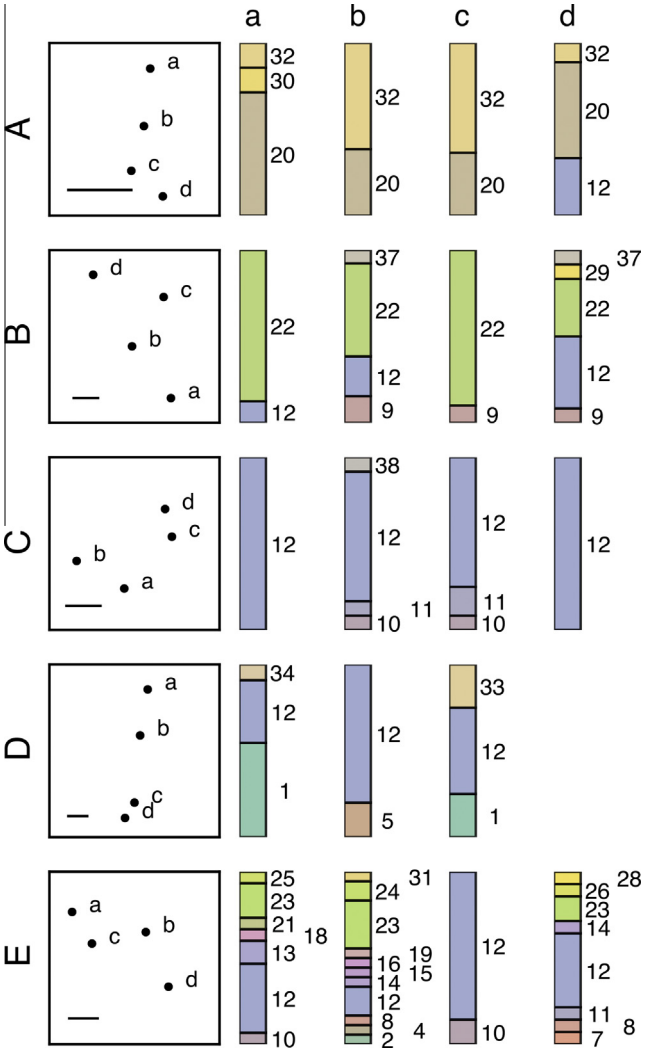

Fig. 2. Distribution of genotypes at the scale of individual sites in 2010. Plots in the left column show the spatial arrangements of plants within the sites, (A–E) located as shown in Fig. 1; the bar in the lower left of each plot is a 1 m length scale. Bar plots show the fraction of samples from each plant belonging to each of 39 total genotypes, indicated by color code and by number.

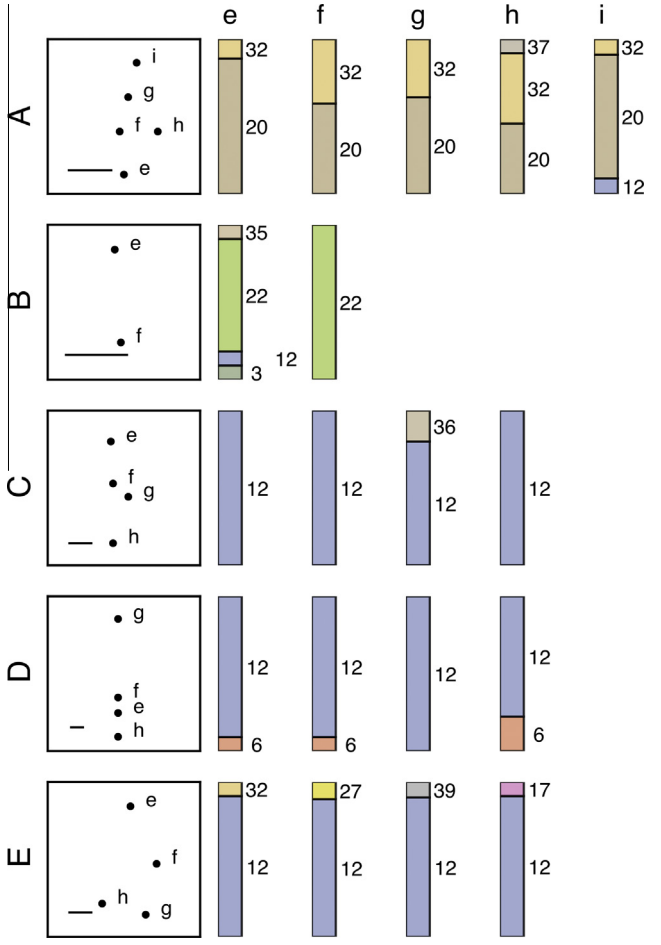

Fig. 3. Distribution of genotypes at the scale of individual sites in 2011. Plots in the left column show the spatial arrangements of plants within the sites, (A–E) located as shown in Fig. 1; the bar in the lower left of each plot is a 1 m length scale. Bar plots show the fraction of samples from each plant belonging to each of 39 total genotypes, indicated by color code and by number.

The permutation tests of spatial structure revealed no significant clustering of similar genotypes in 2010 at the within-plant scale, and the combined *P*-value using Fisher's method was also not significant. In 2011, one of the 14 plants containing multiple genotypes showed significant clustering ($P < 0.05$) and one showed marginally-significant clustering ($P < 0.1$), but the combined *P*-value was not significant. At the within-site scale, neither individual sites nor the combined test revealed significant clustering in either 2010 or 2011. At the within-farm level, however, there was significant clustering ($P < 0.001$) in both years, indicating significant spatial autocorrelation at this scale. These results are reflected in the qualitative patterns seen in Figs. 1–3: sites of similar genotypic composition tend to be found in close proximity (Fig. 1), but there is no analogous pattern apparent within individual sites (Figs. 2 and 3).

The index of association indicated significant linkage disequilibrium in 2010 ($I_A = 4.05$, $P < 0.001$) and 2011 ($I_A = 1.12$, $P < 0.001$), providing strong evidence for clonality in both years. Analyses of individual sites within years also showed evidence of clonality, with significant linkage disequilibrium detected within all sites except Sites B and C in 2010 and Site C in 2011. There were a total of 2209 possible unique combinations of pairs of alleles at pairs of loci in the data set. Of these, 47 passed the four-gamete test, i.e., all four possible gametes were present in the complete pool of genotypes, suggesting that recombination can occur, albeit at a low level.

To search for genes that may potentiate sex, we investigated the genome of strain UM487 for typical Sordariomycete mating type genes. The *L. lecanii* genome was similar in size to other Cordycipitaceae (Zheng et al., 2011; Xiao et al., 2012; Shang et al., 2016), with a genome size of 32.6 Mb and a GC content of 52.2% (Table S2). The UM487 genome assembly was highly fragmented (spread across 4758 contigs), which resulted in a lower than expected percentage of core eukaryotic genes as predicted by CEGMA (83% full and 90% partial completeness). In comparison to the recently sequenced genome of another *L. lecanii* strain, RCEF 1005, the genome of UM487 has fewer predicted genes (8206 vs. 11,030) and a lower estimated completeness which suggests that the UM487 genome is partial.

Nonetheless, we were able to identify in the UM487 genome a locus with a structure consistent with that of the MAT1 mating type locus, namely a gene encoding a HMG domain MAT1-2-1 protein (LLEC1_01563) within a genomic region bounded by a DNA lyase gene and *SLA2* (Fig. 4), characteristic of the MAT1-2 mating type in other Hypocreales (Bushley et al., 2013; Martin et al., 2011). The mating type locus contained a second gene with low

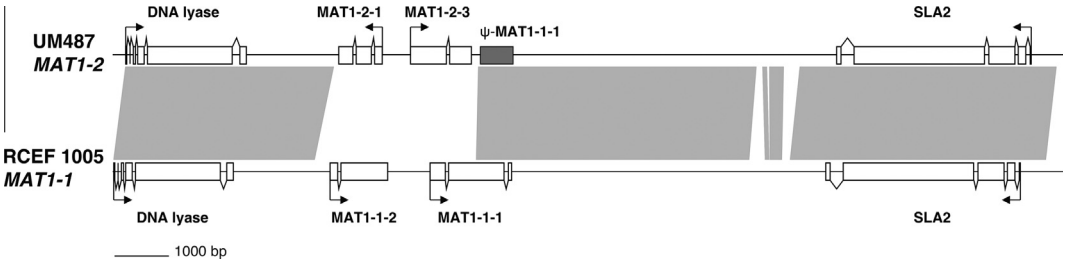

Fig. 4. Schematic showing the gene arrangement at the mating type *MAT1* locus of *L. lecanii* compared between the two mating types as inferred from genome sequences. Five significant ($P < e^{-5}$) pairwise blastn blocks are annotated as gray boxes between the mating types. The *MAT1* locus is flanked by two conserved non-mating-type specific genes, a DNA lyase and *SLA2*, a cytoskeleton assembly control protein. The *MAT1-2* mating type idiomorph is comprised of the two genes *MAT1-2-1* and *MAT1-2-3* as in *Gibberella fujikuroi* (Martin et al., 2011). The *MAT1-1* mating type idiomorph is comprised of the two genes *MAT1-1-2* and *MAT1-1-1* as observed in Cordycipitaceae (Bushley et al., 2013). A putative pseudogene (ψ-MAT1-1-1) with similarity to the 3′ end of the *MAT1-1-1* gene has also been observed in *Cordyceps takaomontana* (Yokoyama et al., 2003).

similarity but positional homology to *MAT1-2-3* observed in *Gibberella fujikuroi* (Martin et al., 2011). Furthermore, a pseudogene similar to the C-terminus of *MAT1-1-1* was found within the MAT region of UM487 as was observed in *Cordyceps takaomontana* (Yokoyama et al., 2003). No evidence of the second mating type allele (MAT1-1) was found in the UM487 genome. By comparison, the genome of *L. lecanii* strain RCEF 1005 revealed a MAT1-1 mating type with the α1-domain *MAT1-1-1* gene (Fig. 4; Shang et al., 2016), suggesting a heterothallic mating system with two mating types for *L. lecanii* as observed in most *Cordyceps* sensu lato (Zheng et al., 2013). In order to determine whether the population of *L. lecanii* on Finca Irlanda is capable of sex, we designed PCR primers specific to one or the other mating type using these two genome sequences. After screening 96 samples and 8 genotypes (including all of the dominant genotypes: 12, 20, 22, 32), only the MAT1-2 mating type was detected in our samples and not the MAT1-1 genotype, consistent with the extensive clonality estimated using microsatellite markers.

4. Discussion

The results of these population genetic analyses provide substantial insight into the spatial structure of the *L. lecanii* population and the dispersal characteristics of this insect and fungal pathogen, and allow for some answers to the four motivating questions posed in the introduction. First, the analyses revealed a clear spatial structure of genotypes among sites within the coffee farm, but no apparent structure at smaller scales, such as the within-site and within-plant scales; this is consistent with new infections being caused by smaller-scale dispersal processes such as rain splash or insect dispersal, rather than sedimentation following long-distance wind dispersal. Second, the index of association indicates clonality, as was expected given the apparent absence of a teleomorph in the study site in combination with the absence of a detected MAT1-1 mating type in our sample. This agrees with the apparent absence of the teleomorph, *Cordyceps confragosa* observed on the coffee farm. On the other hand, the perithecia of the teleomorph are cryptic and not produced on a stroma and could be easily overlooked (Mains, 1949), suggesting sexual reproduction could be cryptic and overlooked in the field. Third, the year-to-year consistency of the large-scale spatial patterns suggests that local epizootics are primarily initiated by residual asexual propagules from the previous wet season's epizootics – whether from *C. viridis* cadavers that remain on the coffee plants or via dispersal of spores from another environmental reservoir, such as the soil. Fourth, the sites sampled in this study were dominated by a small number of genotypes, and this was highly consistent across the two sample years.

Despite the clonal population structure, a very small, but non-zero, fraction of allele/loci combinations passed the four-gamete test, showing that recombination does occur. One explanation would be rare sex, but, alternatively, recombination may be the result of a parasexual process (Pontecorvo and Sermonti, 1954), which would happen through the fusion of vegetatively compatible genotypes. The observation of frequent detection of multiple genotypes on a single scale insect suggests low vegetative incompatibility and a high genotype encounter rate that could facilitate this alternative form of recombination. The interannual data also show that sexual reproduction and ascospore formation are not required for persistence of genotypes over the season, as the same clones were found between seasons. Despite the absence of the MAT1-1 mating type in our farm, we can predict that *L. lecanii* is heterothallic and that if long distance dispersal were to introduce a compatible mating type, sexual reproduction and ascospore formation would be possible. Ascospores may have a greater role in long distance dispersal, as these spores are aerially dispersed (forcibly ejected from a perithecium) whereas the asexual conidia are produced in slimy masses that may be primarily adapted for animal host dispersal.

Prior to this study, there were two equally plausible hypotheses related to dispersal of *L. lecanii* in this system. The first was that *L. lecanii* does not experience dispersal limitation at the scale of the farm, due to the strong homogenizing effects of wind dispersal. The alternative hypothesis was that *L. lecanii* experiences dispersal limitation, with propagules being limited to a spatial scale below the level of the farm. The former would lead to all genotypes being likely to be observed at any given location within the farm, and the latter would give rise to a coherent spatial population genetic structure. Our results are clearly consistent with the latter alternative, and show that the genetic structure of the population contains a strong signal of dispersal limitation at the scale of the farm. The study area can be approximately divided into two regions based on our sampling, with each region being dominated by a different subset of the overall genotype pool, and this pattern was consistent across study years. This clear pattern of spatial autocorrelation would be unlikely to arise in a well-mixed population. On the other hand, we found a predominance of particular genotypes in both years of sampling, and it may be that when viewed across multiple farms, there is little spatial structure because of the widespread dispersal of highly selected clones, which may establish and dominate due to priority effects. This would suggest more stochastic dynamics dominated by local dispersal between seasons and plants (metapopulations), yet more predictable dynamics on a broader scale dominated by selection and not dispersal limitation.

The presence of dispersal limitation is notable because it is directly linked to metapopulation persistence. According to the classical metapopulation formulation of Levins (1968), the

equilibrium patch occupancy (the proportion of occupied patches) is $1-(e/m)$, where e is the patch extinction rate and m is the migration rate. Therefore, a high equilibrium patch occupancy and a high likelihood for persistence are favored by high migration rates and low extinction rates. Dispersal limitation tends to reduce migration rates, particularly between distant patches, thereby reducing the equilibrium patch occupancy and increasing the likelihood of a population collapse.

Our data reveal evidence of dispersal limitation at the smaller spatial scale of the individual sites, as indicated by the significant $F_{plant/site}$ fixation indices in 2010, although the qualitative pattern is not as readily apparent. The statistical results show that there is partitioning of genetic diversity between individual plants, but there is no clear spatial clustering of genotypes within the site level, i.e., there is no clear pattern of genotypes being limited to a single plant or group of adjacent plants within a single site. Likewise, there was no evidence for clustering of genotypes within individual plants.

The presence of dispersal limitation at the farm scale could have profound implications for the persistence of *L. lecanii* in this system. The distribution of the primary hosts of *L. lecanii*, the green coffee scale *C. viridis*, is largely determined by the distribution of nests of the ant *A. sericeasur*, the mutualistic partner of *C. viridis*. These ant nests are distributed in a clustered pattern that shifts through time, forming a dynamic landscape of habitat patches for *L. lecanii* and *C. viridis*. With unlimited dispersal, this landscape of patches would operate as a single habitat patch for *L. lecanii*, and the disappearance and appearance of ant nest clusters would pose no threat to the persistence of the fungus: it would rapidly colonize new patches, thereby ensuring its persistence even in the face of the disappearance of other clusters of ant nests. With dispersal limitation, in contrast, the landscape of ant nest clusters operates as a network of habitat patches that the fungus inhabits as a metapopulation. Persistence in the landscape then becomes a function of the relative rates of local (patch-level) extinction and the rescue of patches by migration. These rates depend on the characteristics of the spatial structure of the landscape, such as the patch size and the spatial distribution of patches (Jackson et al., 2014a). These characteristics of the ant nest cluster landscape are a consequence of the self organization of the ant colonies (Perfecto and Vandermeer, 2008; Vandermeer et al., 2008), which is itself sensitive to management actions, such as the degree of pruning and felling of shade trees (Jackson et al., 2014b). Consequently, the apparent dispersal limitation revealed by this population genetics analysis implies that the persistence of *L. lecanii* in this system will be sensitive to management actions to a greater extent than if it were not dispersal limited.

In addition to the implications for persistence (whether *L. lecanii* is able to maintain itself in the system), dispersal limitation may have a strong influence on spatial and temporal patterns of prevalence (where and when the fungus is abundant). Surveys during the wet and dry seasons suggest that epizootics of *C. viridis* infected by *L. lecanii* are active almost exclusively in the wet season (unpublished data), which implies that the population in a site goes through a periodic bottleneck in abundance, during which the possibility of local extinction is elevated. The potential for local extinction of individual clones is also apparent in the dramatic reduction in genetic diversity from 2010 to 2011, particularly in Site E. With dispersal limitation, recovery from these local extinctions will take more time, resulting in a patchwork of occupied and unoccupied patches at the landscape level (a phenomenon which is also an essential component of classical metapopulation theory).

The conclusion that the persistence of the *L. lecanii* metapopulation may be highly sensitive to the dynamics of spatial structure is highly relevant for the successful management of coffee in this region, for two reasons. First, maintenance of *C. viridis* below pest status may require the presence of *L. lecanii* (Vandermeer et al., 2010). Additionally, *L. lecanii* is a mycoparasite that has been shown to hyperparasitize *H. vastatrix* (Alarcón and Carrión, 1994; Eskes et al., 1991), and there is evidence that *L. lecanii* may reduce the prevalence of *H. vastatrix* under field conditions (Jackson et al., 2012a; Vandermeer et al., 2009, 2010). Coffee rust is a potentially devastating disease of coffee that has been largely responsible for the abandonment of coffee cultivation in entire regions (McCook, 2006) and recently caused drastic reductions in yield across much of Latin America (Avelino et al., 2015). Elucidating the mechanisms that favor the persistence of *L. lecanii* metapopulations at significant levels could consequently serve an important role in mitigating or preventing outbreaks of this disease.

Specifically, these results suggest that management of the farm in a manner that helps maintain the spatial structure of *A. sericeasur* nests may be important for retaining or enhancing the autonomous pest control service provided by *L. lecanii*. Recent research has shown that the spatial distribution of *A. sericeasur* nests is shaped by a combination of exogeneous and endogenous factors (Li et al., 2016). By monitoring the spatial distribution of *A. sericeasur* nests and managing the factors that influence this structure, such as the density and spatial distribution of shade trees, farmers could help to maintain the habitat structure necessary for the persistence of *L. lecanii* in the system. In addition, the apparent dispersal limitation of *L. lecanii* suggests caution in the use of broad-spectrum fungicides, e.g., to control *H. vastatrix*: It is conceivable that recovery of *L. lecanii* would lag the re-establishment of *H. vastatrix* in the system, allowing the coffee rust to temporarily escape the control afforded by *L. lecanii* and rebound to even higher levels than before the intervention, similar to the phenomenon of primary pest resurgence sometimes observed following the application of insecticides or acaricides (Dutcher, 2007). Although these examples are necessarily speculative, they serve to illustrate the general point that the spatial structure of *A. sericeasur*, and the dispersal characteristics of *L. lecanii* more broadly, may be essential considerations for farmers interested in retaining the ecosystem services provided by *L. lecanii* in this and other coffee agroecosystems.

Another consequence of the dispersal limitation, albeit one that is much more speculative, is the potential it presents for an evolutionary response of the ant *A. sericeasur* to the fungus. By virtue of its attack of the scale insects, *C. viridis*, *L. lecanii* is likely one of the most important natural enemies of *A. sericeasur*; epizootics of *L. lecanii* often result in nearly 100% mortality of the *C. viridis* population in a site, thereby drastically reducing the food available to the ant colony. Therefore, reduced prevalence of *L. lecanii* would be beneficial from the ants' perspective. Since the prevalence of *L. lecanii* is the result of migration and extinction rates, and these rates are a function of the spatial distribution of the ant nests, which is in turn a function of the demographic and dispersal characteristics of the ants, it follows that the ants could evolve to exhibit a spatial distribution that reduces the ability of the fungus to spread through the landscape. This phenomenon, termed the evolution of prudent hosts, has been demonstrated in theory (Jackson, 2014), but it is uncertain that it could occur in the context of realistic environmental variability and multidirectional coevolutionary interactions.

This study demonstrates the potential utility of a population genetics approach for revealing otherwise cryptic dispersal patterns in insect pathogens that have real management implications. It also shows that spatial structure can be an important axis to consider for ecosystem conservation and for the management of agroecosystems. As the molecular technologies that enable such population genetics approaches become more affordable and readily available, this potentially rich area of investigation will become an ever more valuable and accessible tool for scientists and managers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2016.07.007.

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