RESEARCH ARTICLE

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First genome-wide analysis of the endangered, endemic lichen *Cetradonia linearis* reveals isolation by distance and strong population structure

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PREMISE OF THE STUDY: Lichenized fungi are evolutionarily diverse and ecologically important, but little is known about the processes that drive their diversification and genetic differentiation. Distributions are often assumed to be wholly shaped by ecological requirements rather than dispersal limitations. Furthermore, although asexual and sexual reproductive structures are observable, the lack of information about recombination rates makes inferences about reproductive strategies difficult. We investigated the population genomics of *Cetradonia linearis*, a federally endangered lichen in the southern Appalachians of eastern North America, to test the relative contributions of environmental and geographic distance in shaping genetic structure, and to characterize the mating system and genome-wide recombination.

METHODS: Whole-genome shotgun sequencing was conducted to generate data for 32 individuals of *C. linearis*. A reference genome was assembled, and reads from all samples were aligned to generate a set of single-nucleotide polymorphisms for further analyses.

KEY RESULTS: We found evidence for low rates of recombination and for isolation by distance, but not for isolation by environment. The species is putatively unisexual, given that only one mating-type locus was found. Hindcast species distribution models and the distribution of genetic diversity support *C. linearis* having a larger range during the Last Glacial Maximum in the southern portion of its current extent.

CONCLUSIONS: Our findings contribute to the understanding of factors that shape genetic diversity in *C. linearis* and in fungi more broadly. Because all populations are highly genetically differentiated, the extirpation of any population would mean the loss of unique genetic diversity; therefore, our results support the continued conservation of this species.

KEY WORDS biogeography; Cladoniaceae; conservation genetics; endangered species; fungal biology; *Gymnoderma lineare*; population genetics; population genomics; rock gnome; symbiosis.

Symbiotic fungi, including lichenized species, represent some of the most ecologically important radiations on earth. However, the processes that shape genetic differentiation and gene flow in these groups remain poorly understood. Historically, two major assumptions have shaped hypotheses about symbiotic fungal population structure and evolution. First, because most fungi produce very small spores, their distribution is thought to be limited primarily by ecological suitability rather than geographic distance (O'Malley, 2007). Second, species in which no sexual reproductive structures have been observed are assumed to reproduce only asexually (Taylor et al., 2015). Phylogenetic and population genetic studies have already challenged these assumptions in fungi that do not form lichens. For instance, in the common and widespread fungus *Suillis brevipes*, there is evidence for both isolation by distance (IBD) and adaptation of coastal populations to saline environmental gradients (Branco et al., 2015). Species in *Saccharomyces* show varying levels of geographic structure in their genetic differentiation, with *S. paradoxus* showing clear evidence of IBD and *S. cerevisiae* showing

much less geographic structure (Liti et al., 2009). Taylor et al. (2015) reviewed the literature on clonal reproduction in fungi, concluding that numerous species showed evidence for recombination regardless of observed reproductive structures. Additional examples of similar observations, indicating that fungal reproduction and population genetics are more complex than previously expected, have been derived from population genetic and genomic data in other groups of non-lichenized fungi (see reviews in Grünwald et al., 2016; Peter and Schacherer, 2016).

Lichens are a major group of fungi that form obligate symbioses with algae and/or cyanobacteria and comprise >20% of all ascomycetes (Lücking et al., 2016). Despite their ecological importance and conspicuous abundance in many terrestrial ecosystems, relatively few taxa have been studied with traditional population genetics methods, and no published studies have used a genome-wide approach to assess gene flow or other population-level attributes. To date, most population genetic studies of lichens have been conducted on *Lobaria pulmonaria* and its algal photobiont *Dictyochloropsis reticulata* using microsatellite markers (Dal Grande et al., 2010; Widmer et al., 2010; Nadyeina et al., 2014b). These studies have shown that *L. pulmonaria* frequently disperses short distances via lichenized propagules (bundles of algae and fungi) and infrequently disperses long distances via sexually produced fungal spores (Werth et al., 2006), and there is evidence of adaptation and population isolation at small spatial scales (Nadyeina et al., 2014a). Population genetic patterns in another lichenized fungus, *Xanthoria parietina*, based on RAPD-PCR markers, contrast starkly with the findings for *L. pulmonaria*; in the former, high genetic diversity and very few clones were found within small areas, even among adjacent individuals (Itten and Honegger, 2010). The pattern recovered in *X. parietina* is similar to a study of *Parmelina carporrhizans* based on microsatellite loci, where high rates of migration were recovered among populations, except for isolated island populations (Alors et al., 2017). A microsatellite-based study on *Parmotrema tinctorum* and its algal symbiont found that most dispersal was clonal over short distances, similar to *L. pulmonaria*, but still found evidence for high rates of sexual reproduction in the fungus (Mansournia et al., 2012). While highly detailed, these studies of lichen population genetics represent only a fraction of this diverse group of fungi, which have evolved symbiotic relationships with algae or cyanobacteria at least seven times independently throughout the fungal tree of life (Schoch et al., 2009) and occupy every terrestrial ecosystem from the poles to the tropics (Brodo et al., 2001). Microsatellites have recently been developed for a broader diversity of lichenized fungi (Magain et al., 2010; Devkota et al., 2014; Nadyeina et al., 2014a; Lindgren et al., 2016; Lutsak et al., 2016), but these tools have not yet been utilized for population genetic analyses in lichens.

Population genomics is a promising approach to rapidly advance our knowledge of population biology in lichens because it circumvents difficulties associated with developing species-specific markers, especially since lichens are notoriously difficult and slow to culture (Crittenden et al., 1995). Among the domains of eukaryotic organisms, fungi are one of the most amenable to genomic studies, due to their generally small, compact genomes (Gladieux et al., 2014). Population genomic studies have already added substantial depth and breadth to the knowledge of basic fungal biology, allowing researchers to address questions that were once intractable about the life history and evolution of reproductive systems. For example, fungi that have only been observed reproducing asexually show genomic evidence for sexual reproduction (Tsai et al., 2008; Stefanini et al., 2016); speciation through homoploid hybridization has been shown to occur rapidly, at least in yeast (Leducq et al., 2016); and Glomerales, arbuscular mycorrhizal fungi, have highly flexible levels of ploidy in the heterokaryotic cells within species (Wyss et al., 2016). Applying these methods to lichenized fungi holds great promise to rapidly advance knowledge of lichen population biology.

The rock gnome lichen (*Cetradonia linearis* [A. Evans] J.C. Wei & Ahti) is one of two fungal species protected by the Endangered Species Act in the United States (U.S. Fish and Wildlife Service [USFWS], 2013), and one of eight lichens on the IUCN Red List (Allen et al., 2015). It is narrowly endemic to the southern Appalachians of eastern North America, where it is known from ~100 populations, most of which are located in western North Carolina (USFWS, 2013). It forms colonies on rocks, either on exposed cliffs at high elevations or on large boulders in mid- to high-elevation streams. *Cetradonia* is a monotypic genus whose position as the earliest-diverging member of the widespread and ecologically important Cladoniaceae makes its study essential for addressing hypotheses of evolution in this family (Wei and Ahti, 2002; Zhou et al., 2006). It forms colonies of simple to branched squamules with black apothecia (sexual reproductive structures) and/or pycnidia (asexual reproductive structures), frequently produced at the tips (Fig. 1). Despite having been protected by the Endangered Species Act for >20 yr, little is known about *C. linearis* beyond its distribution (USFWS, 2013). Currently, nothing is known about dispersal or population genetic structure in this species.

We tested three hypotheses concerning population-level processes in *Cetradonia linearis*: (1) most reproduction and dispersal occurs through clonal processes; (2) isolation by distance is the major force shaping the genetic differentiation, while ecological adaptation plays a minor role; and (3) the southern portion of its current extent represents a major refugium during the Last Glacial Maximum (LGM). To test these hypotheses, low-coverage, whole-genome shotgun sequencing was used to generate large quantities of genomic data from samples throughout the species' range. The resulting genome-wide single-nucleotide polymorphisms (SNPs) were used to measure genetic diversity, recombination, and clonality. Population genetic structure, connectivity, and evidence for isolation by environment were also investigated. This study is the first assessment of population genomics in a lichen, providing a baseline for comparison in this group of organisms, along with valuable information for the continued conservation of the endangered rock gnome lichen.

FIGURE 1. Morphology, habit, and habitat of *Cetradonia linearis*. (A) Large granite dome where the species occurs at the base of large, seeping rock faces (inset). (B) Stream habitat where the species occurs frequently on scattered rocks and boulders. (C) Large boulder face covered in the species, illustrating sampling protocol using sterile forceps. (D) Fertile colony on a mossy boulder in a stream. (E) Large rock outcrop hosting colonies of the species; inset shows view from the perspective of *C. linearis*. (F) Waterfall populations are very abundant; one colony is outlined by the black box. (G) Colony displaying apothecia and a potential zoochory event.


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MATERIALS AND METHODS

Study system, sampling, and sequencing

Samples were collected from 15 sites throughout the geographic and ecological range of *Cetradonia linearis* (Fig. 2). At each site, two or three squamules were taken from ≤10 distinct colonies using surface-sterilized forceps. Each colony is considered an individual in this study; two or three squamules from each colony were placed into an Eppendorf tube, together constituting a single sample, because one squamule alone would not provide sufficient DNA for next-generation sequencing. Squamules were placed in 1.5 mL Eppendorf tubes, set out to air dry for 24 h, then stored at −40°C. Samples were washed with acetone, and DNA was extracted using the Qiagen DNeasy Plant Mini Kit with the cell lysis stage extended for 4–6 h. Thirty-two samples were chosen for sequencing on the basis of DNA quality and yield, while maintaining the geographic and ecological breadth of samples. Sequencing was conducted at the Rockefeller University Genomics Resource Center. Libraries were prepared with the Nextera XT kit (Illumina) and sequenced on the Illumina NextSeq platform in Mid Output on 150 base pair (bp) paired-end read mode. All samples were sequenced at roughly equal coverage, except one sample from the Balsam Mountains, B224, which was sequenced at 5× higher coverage for assembly of a reference genome.

Quality filtering, genome assembly, and annotation

A reference genome was assembled from sample B224 and annotated after strictly filtering contaminating reads (see below). B224 reads were trimmed, adapters were removed, and overlapping read-pairs were combined using cutadapt and FLASH (Magoč and Salzberg, 2011; Martin, 2011). Read pools for all other samples were trimmed, adapters were removed, and overlapping read-pairs were combined using FLASH and Trimmomatic version 0.36 (Bolger et al., 2014). An initial assembly of B224 was built using Minia with a kmer size of 75 and an abundance minimum of 3 (Chikhi and Rizk, 2013). To filter out contaminants, including algal symbionts, the Blobology workflow and perl scripts were used (Kumar et al., 2013). Specifically, a random subset of 15,000 contigs longer that 250 bp were subjected to homology search using megablast against the non-redundant nucleotide database from GenBank, and the e-value cutoff was set to 1e-5. On the basis of these plots (Altschul et al., 1990; Appendix S1; see Supplemental Data with this article), contigs with GC content >0.6 and coverage <5 were pooled to form a set of contaminant contigs. Then all B224 reads were aligned to the contaminant contigs using bowtie2, and all reads that did not align to the contaminants were retained for reassembly. The final assembly was built using ABySS version 2.0 with the paired-end read setting and a kmer size of 41 (Simpson et al., 2009). All resulting contigs shorter than 500 bp were removed from the dataset before further analyses. Genome annotation was conducted using the MAKER pipeline (Holt and Yandell, 2011). SNAP was used for the ab initio gene predictor, and protein homology evidence was drawn from *Aspergillus niger* ATCC 1015 v4.0, *Cladonia grayi* Cgr/DA2myc/ss v2.0, and *Cochliobolus heterostrophus* C5 v2.0 (Korf, 2004; Andersen et al., 2011; Ohm et al., 2012; Condon et al., 2013; McDonald et al., 2013). For a final filtering step, all genes were blasted against the *A. niger*, *C. grayi*, and *C. heterostrophus* gene sets. Contigs were kept for downstream analysis only if the gene with the highest-scoring blast hit matched most closely with a *C. grayi* gene. Because the *C. grayi* genome was assembled from pure culture of the fungal symbiont (McDonald et al., 2013), this was an additional step that filtered any remaining contaminants from the

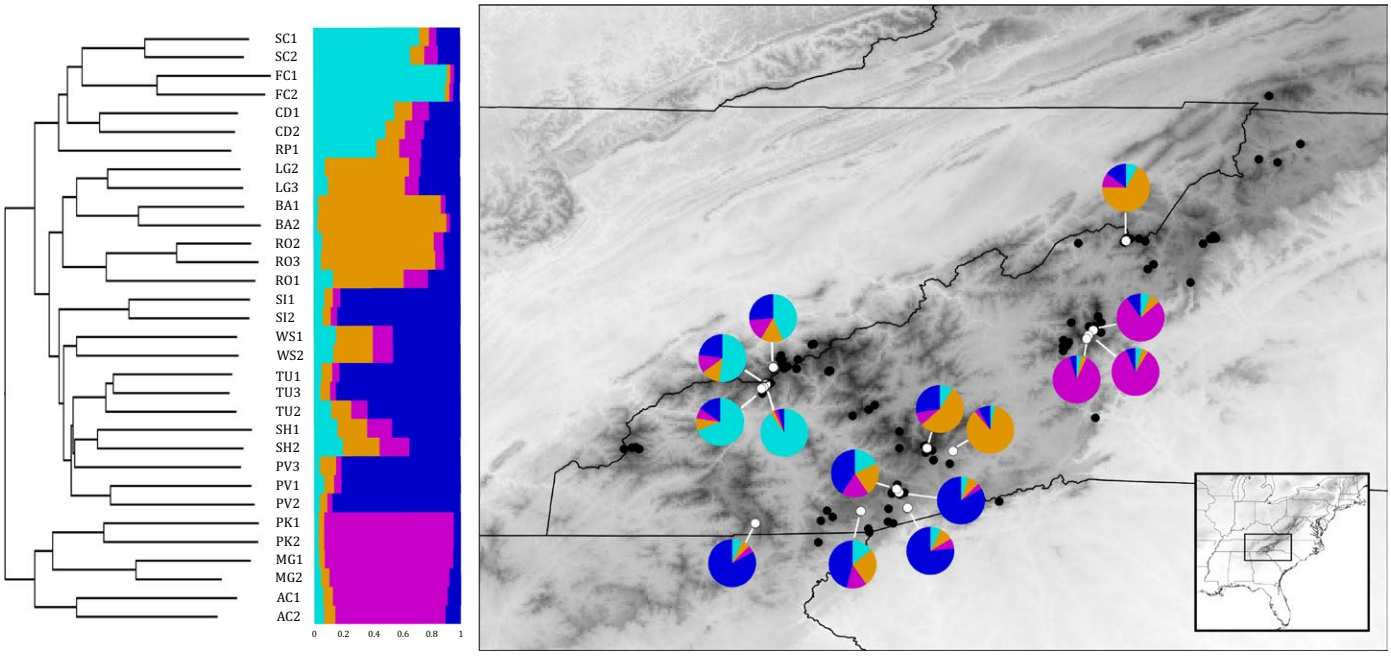

FIGURE 2. Population structure of *Cetradonia linearis*. (Left) Neighbor-joining tree showing hierarchical clustering of all sampled individuals. (Middle) Proportional cluster belonging to each individual sampled, as inferred by InStruct. (Right) Distribution of *C. linearis* and average proportional cluster belonging to each sampling site. Inset shows the larger spatial orientation of the study area.

genome. Finally, we conducted homology searches for both mating-type idiomorphs (MAT1-2 and MAT2-2) in the genome and all sampled read pools (Appendix S2).

SNP calling and descriptive statistics

Single-nucleotide polymorphisms were called for all sequenced samples using the annotated contigs as a reference genome for the fungal component. First, we used bwa (Li and Durbin, 2009) to align the reads to the contigs. Then, to call the SNPs from this alignment, FreeBayes was used with ploidy set to 2 and minimum alternate fraction set to 0.9 (Garrison and Marth, 2012). Ploidy was set to 2 because all samples were fertile and thus there were potentially two genetic individuals present (we also conducted the same analyses with ploidy set to 1, without a change in results). Average nucleotide diversity was calculated using VCFtools (Danecek et al., 2011). Linkage distance was calculated between all sites using the PopLDdecay program (https://github.com/BGI-shenzhen/PopLDdecay; accessed May 2018) and plotted in R with the nonlinear least-squares smoothing function implemented for the trendline to create a linkage disequilibrium decay chart (Gaunt et al., 2007). We corrected for linkage disequilibrium using the R package "poppr" (Kamvar et al., 2014) with a threshold of 0.2 and a 1 kb sliding window to generate a dataset of unlinked SNPs for subsequent analyses. Then pairwise F_{ST} was calculated among all sampling sites and populations with the linkage-disequilibrium-corrected dataset using BEDASSLE (Bradburd et al., 2013), a Bayesian inference program that estimates the relative influence of ecological and geographic distance on genetic distance. This program automatically excludes sites with missing data when calculating F_{ST} (Bradburd et al., 2013). The dataset was checked for clones by searching for multilocus genotypes that are >95% identical to account for sequencing and SNP calling errors using the mlg.filter function in the R package "poppr" with a threshold of 0.05.

Statistical analyses

First, the relationships among populations were explored to determine if there were phylogenetic signals for each distinct sampling site and mountain range, and at what spatial scale the relationships were clearest. Three methods were used to explore population structure: InStruct, discriminant analysis of principal components (DAPC), and a neighbor-joining (NJ) tree. InStruct is a Bayesian clustering program that infers self-fertilization rates and clusters individuals into subpopulations using a Markov chain Monte Carlo (MCMC) algorithm (Gao et al., 2007). InStruct was run with K values between 1 and 15, with five independent chains per K value. Each chain was run with a 100,000-iteration burn-in period, followed by 10,000 iterations after burn-in. Mode 2 was used to infer subpopulation structure and selfing rate. The run with the highest DIC (deviance information criterion) value was chosen to represent the population structure and inferred selfing rate. We also implemented DAPC, a multivariate approach to identifying genetically distinct clusters of individuals, with the clustering algorithm implemented to define groups, rather than designating them a priori. We set the clustering algorithm to test for 1–15 clusters, and it resulted in 10 genetic clusters being chosen, based on the Bayesian information criterion (Jombart et al., 2010). This method was specifically designed to cope with the large quantity of next-generation sequencing data and was implemented in R through the package "adegenet" version 2.0 (Jombart, 2008). An unrooted (NJ) tree was also built to infer the relationships among individuals, based on bitwise distances, using the R package "ape" (Paradis et al., 2004).

The influence of geographic and ecological distance on genetic distance was investigated using two approaches. First, a partial Mantel test with 10,000 permutations was used to test for correlation between genetic distance (measured as pairwise F_{ST}), geographic distance (Euclidean distance in kilometers), and a set of four environmental variables. The set of four environmental variables were habitat (boulder in stream vs. exposed rock outcrop) and three non-collinear variables from the widely used Worldclim dataset: mean temperature of wettest quarter (BIO8), mean temperature of warmest quarter (BIO10), and annual precipitation (BIO12) (Hijmans et al., 2005). These last three variables were retained after removing all correlated climatic variables from Worldclim (see below). The habitat variable was based on field observations of the species made while collecting samples. Second, a Bayesian approach as implemented in BEDASSLE was used to estimate the contributions of geographic and ecological distance to genetic distance (Bradburd et al., 2013). The same set of ecological and geographic distance variables were used as input data, along with allele sample sizes and frequencies in all samples. An initial Bayesian analysis, run for 1 million generations, indicated that the effect sizes of BIO8 and BIO12 were very close to zero, and these were removed from the dataset. A second analysis was run retaining the habitat and BIO10 as environmental variables for 5 million generations with a sample frequency of 10. A third analysis was conducted retaining only the habitat as the environmental variable and was run for 10 million generations with a sample frequency of 10. Trace plots were examined for convergence, and mean marginal densities and 95% confidence intervals (CIs) were calculated for αE:αD for each environmental variable, with the first 50% of generations treated as burn-in and removed.

To identify the number of unique recombination events among individuals in the dataset, we used RDP4 version 4 (Martin et al., 2015). RDP4 uses multiple recombination detection methods to analyze large alignments. We tested for recombination among all individuals and multiple subsets of the data based on the clades recovered in the NJ tree, including each mountain range, major clusters detected by InStruct, and smaller clades within mountain ranges. Our input alignment consisted of the full SNP dataset (not corrected for linkage disequilibrium) with sites with >25% missing data removed, with separations between contigs marked by exclamation points (Milgroom et al., 2014; RDP4 Instruction Manual). We used the default settings with the Bonferroni correction and a P value cutoff of 0.05 (Milgroom et al., 2014; RDP4 Instruction Manual). We further considered only recombination events detected by two or more analysis methods. All unique recombination events for each set of individuals analyzed were recorded, along with the number of unique recombination events detected after removing any recombination events flagged as potentially being due to other evolutionary processes besides recombination.

Reproductive morphology

To determine whether reproductive structures were observable in *Cetradonia* specimens, fertile samples were dissected to search microscopically for trichogynes. Trichogynes are specialized hyphae that receive spermatia (conidia) to begin sexual reproduction, and their remnants should be observable at the base of apothecia,

sexual structures that produce fungal spores that result from meiosis. Thin sections of five squamules from three sampling sites were cut by hand with a razor blade through the squamule and base of the apothecia and mounted on slides. Sections were stained with phloxine and cleared with potassium hydroxide before examination under a compound microscope (Appendix S2).

Species distribution modeling

Species distribution modeling was used to investigate whether the sites with the highest genetic diversity were located in an area that was likely a refugium during the LGM. To model past distributions, we first built a species distribution model (SDM) to predict the probability of a species' presence across the landscape for the present, then projected this SDM to past climates. Species distribution modeling was conducted using Maxent version 3 (Phillips et al., 2006; Phillips and Dudik, 2008) after steps were taken to reduce sampling bias and calibrate the model. First, localities were thinned by a 5 km radius to reduce sampling bias by randomly excluding one of two localities when they fell within that radius, as implemented in the R package "SpThin" (Aiello-Lammens et al., 2015). After thinning, 42 of the 101 original localities were retained and used for all further analyses. The Worldclim dataset of 19 bioclimatic variables was used for the environmental data at a resolution of 10 arc seconds for the present and 2.5 arc minutes for the LMG. All autocorrelated variables were first removed, leaving mean temperature of wettest quarter (BIO8), mean temperature of warmest quarter (BIO10), and annual precipitation (BIO12) (Hijmans et al., 2005). These three variables were clipped to the extent of the species' known range, with a small buffer, for environmental variables from the present and LGM. Two modeling parameters were tuned to identify the best level of complexity for the model: feature classes define the allowed shape of the environmental variable response curves, and the regularization multiplier controls for complexity, with higher values increasingly penalizing complexity (Scheglovitova and Anderson, 2013). The best modeling parameters were chosen on the basis of Akaike's information criterion corrected for small sample size (AIC_c; Warren and Seifert, 2011). Model tuning was implemented using the R package "ENMEval" with the "blocks" setting (Muscarella et al., 2014). The final model was built and projected using all thinned localities, with the regularization multiplier set to 3.5 and with linear, quadratic, and hinge response curves allowed.

RESULTS

High-coverage, whole-genome shotgun sequencing of one individual of *Cetradonia linearis* was obtained and used to assemble a reference genome. Whole-genome shotgun sequencing of 31 additional individuals was mapped to this genome, and the resulting SNPs were used to infer the population structure, biogeographic history, and mating system of the species.

Cetradonia linearis *reference genome*

Multiple steps of stringent quality and contaminant filtering resulted in the production of a high-quality, partial reference genome. The original read pool from the sample used to create the reference genome, sample B224, contained 55 million reads, for a total of 16 Gb. The mean PHRED quality score was 33. After trimming, filtering for low-quality base calls, and merging paired ends, there were 44 million merged reads (where two paired-end reads overlapped and merged into a single sequence) with a total of 5.6 Gb of sequence, and 8.3 million read pairs that did not overlap with a total of 2.1 Gb of sequence. The initial assembly using Minia built 32,669 contigs. When contigs under 500 bp were excluded, the total assembly length was 105.5 Mb and the N50 (a measure of the quality of the assembly, calculated as the length of the smallest contig, such that 50% of the nucleotides are incorporated into equal size or longer contigs) was 3,814 bp. After filtering contaminants, 41.9 million merged reads with 5.4 Gb of sequence remained, as well as 7.6 million paired-end reads with 2.0 Gb of sequence. These filtered reads were then assembled, which built 17,199 contigs with a total length of 40.0 Mb and an N50 of 6,093. This assembly was then annotated with protein homology data from *Aspergillus niger* ATCC 1015 version 4.0, *Cladonia grayi* Cgr/DA2myc/ss version 2.0, and *Cochliobolus heterostrophus* C5 version 2.0 and ab initio prediction using SNAP. Then only contigs for which the annotated gene with the best blastp score against *C. grayi* and *A. niger* proteins most closely matched *C. grayi* were retained for the final reference genome to be used in all downstream analyses. This reference genome comprised 2,703 contigs with a total length of 19.5 MB, a contig N50 of 10,095 bp, and an average coverage of 54.7 X. CEGMA (Parra et al., 2007) analysis of conserved gene content showed that 74% of universally conserved genes are present in our assembly, suggesting that our assembly is ~74% complete. Consistent with this, our assembly was 53–70% as large as the three genomes available for other species in the Cladoniaceae (28 Mb-37 Mb; Armaleo and May, 2009; Park et al., 2013). The MAT1-2 idiomorph was located in the reference genome and in 14 of 32 read pools (Appendix S2). No MAT1-1 genes were located in any samples. Because only one mating type was discovered, we preliminarily determine that the mating system of *C. linearis* may be homothallic and specifically unisexual (Wilson et al., 2015).

Cetradonia linearis *population structure*

To call SNPs, all read pools were aligned to the reference genome. A total of 126,662 SNPs were identified. After correcting for linkage disequilibrium, 10,026 SNPs remained. This large reduction in SNPs after correcting for linkage disequilibrium suggests a low rate of recombination. Examination of the linkage disequilibrium decay plot further supports the hypotheses of low recombination rates, because the linkage remained high even as distance increased (Fig. 3). In the dataset used for subsequent analyses, the average SNP coverage was 66% and the coverage per population ranged from 52.6% to 98.6% (Table 1). Nucleotide diversity (π) within sampling sites ranged from 0.084 for one site in the Great Smoky Mountains to 0.18 for one site in the Black Mountains. When the samples were grouped by mountain range, nucleotide diversity ranged from 0.148 to 0.338 (Table 1). Pairwise F_{ST} values between sites ranged from 0.312 to 0.730 (Appendix S3). The recombination analysis with RDP4 detected 218 recombination events in the entire dataset, or 141 when events possibly attributable to other evolutionary processes were removed. The number of recombination events within mountain ranges and clusters ranged from 16 events in the Balsam Mountains to 115 in the Black Mountains (Table 2). The low number of recombination events found here further supports a high rate of clonality or self-fertilization.

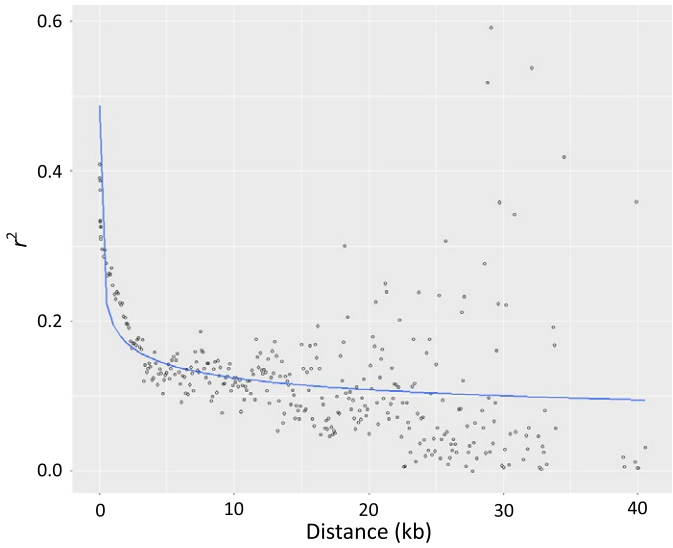

FIGURE 3. Linkage disequilibrium decay plot for *Cetradonia linearis*.

TABLE 1. Site names (mountains), numbers of *Cetradonia linearis* individuals sampled (*n*), average percent single-nucleotide polymorphisms covered for each site/mountain range (% Cov), and average nucleotide diversity (π).

Site	(*n*)	% Cov	π
Smokies	5	58.1	0.338
Nantahalas	12	52.6	0.329
Blacks	6	98.6	0.268
Balsams	4	71.9	0.208
Roan	3	64.4	0.148
All	32	66	0.328

TABLE 2. Recombination events identified by RDP4 for all samples and subsets of samples of *Cetradonia linearis*. "Ambiguous events" are those potentially attributable to other evolutionary processes. SC and FC are sites in the same watershed in Great Smoky Mountains National Park, and PV and SH are sites in the same watershed in the Nantahalas.

Site	All recombination events (*n*)	Recombination events after ambiguous events removed (*n*)
All samples	218	141
Great Smokies	37	28
Balsams	16	16
Balsams + Roan	43	35
Nantahalas	73	50
Blacks	115	87
SC + FC	19	19
PV + SH	14	14

Population structure was first explored through relational analyses. The unrooted NJ tree recovered distinct, mutually exclusive groups that corresponded to distinct mountain ranges (Fig. 2). Sampling sites also largely formed mutually exclusive groups, except PV. The one PV sample that did not cluster with other PV samples formed a group with SH, a site that was only 1.5 km downstream. The InStruct analysis chain with the lowest DIC found four clusters (Appendix S4). These four clusters were evident in the results: one that included all samples from the Great Smoky Mountains, one from the southern Nantahalas, one that included the Balsam Mountains and Roan Mountain, and one that included all samples

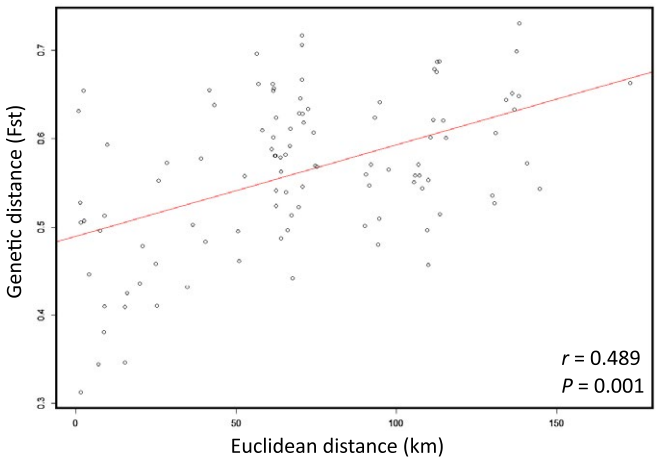

FIGURE 4. Scatterplot of genetic vs. geographic distance for *Cetradonia linearis* and the outcome of a statistically significant partial Mantel test.

from the Black Mountains (Fig. 2). The mean posterior distribution of selfing rates averaged 0.637, with the mean (and variance) for each cluster inferred as follows: 1 = 0.606 (0.0195), 2 = 0.615 (0.016), 3 = 0.650 (0.020), 4 = 0.675 (0.013). Ten clusters were found as the most likely grouping of the samples using DAPC. Most clusters comprised all individuals from single sampling sites. Group 4 was the only one that included samples from multiple sites, for a total of 15 individuals from nine sites that included the Great Smoky Mountains, Balsam Mountains, Nantahala Mountains, and Roan Mountain (Appendix S5). Each of the three sites sampled from the Black Mountains formed their own distinct group, despite their close proximity to each other (1–173 km apart).

We tested the influence of geographic vs. environmental distance on genetic distance using two methods, and both showed geographic distance as a more significant factor correlating with population structure. First, a partial Mantel test showed a significant correlation between genetic distance, measured as pairwise F_{ST}, and geographic distance, measured as pairwise Euclidean distance in kilometers, where $r = 0.489$ and $P < 0.01$ (Fig. 4). There were no correlations between genetic distance and any of the environmental distances (Appendix S6). The second analysis was a Bayesian approach implemented in the program BEDASSLE (Bradburd et al., 2013). Here, the relevant value is the ratio of effect size of each environmental variable vs. the effect size of the geographic distance (αE:αD). The results were similar to the partial Mantel test, and geographic distance far outweighed the effect of environmental distance. Specifically, the results of the first analysis, which included both habitat and BIO10 as environmental variables, estimated the habitat αE:αD = 0.712 (95% CI: 0.544–0.978) and the BIO10 αE:αD = 0.026 (95% CI: 0.020–0.036). This can be interpreted as follows: the effect of 10°C mean temperature of the warmest quarter was equal to the effect of 0.026 km of geographic distance, and the effect of occurrence in different habitats was equal to 0.75 km of geographic distance. In the second analyses, where only habitat was retained as an environmental variable, the habitat αE:αD = 0.309 (95% CI: 0.011–1.408). Hindcasting the SDM of *C. linearis* supported the hypothesis that its refugial range was located predominantly in the southern edge of its current range during the LGM (Fig. 5). The quality of the SDM was high, with an area under the curve of 0.919.

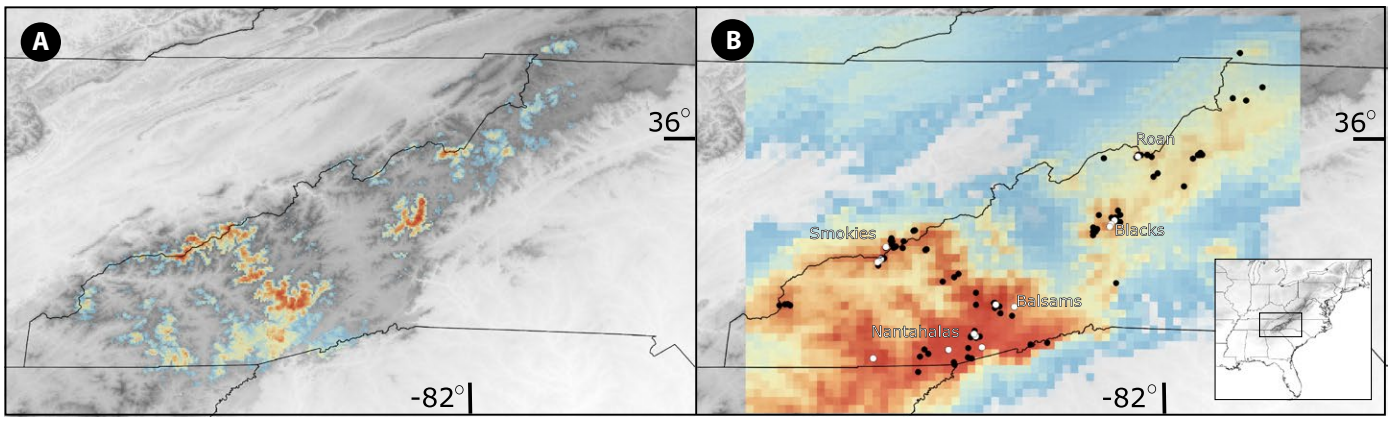

FIGURE 5. Species distribution model of *Cetradonia linearis* (A) in the present and (B) at the Last Glacial Maximum. Probability of *C. linearis* grades from low (blue) to high (red). Inset shows the larger spatial orientation of the study area.

DISCUSSION

An understanding of diversification mechanisms in lichens has been hampered by a lack of population genetic studies in these diverse clades of fungi. This study is the first to report the results of a genomic approach for investigating the population structure of a lichen. Low-coverage whole-genome sequencing of lichen fragments produced large quantities of SNP data (>122,000 SNPs) among individuals within a species, after contaminants were removed by stringent filtering. These results demonstrate that culturing is not required for lichen population genomics. The original hypothesis that the main dispersal strategy of *Cetradonia linearis* is through clonal propagation was not supported, in that no clones were identified across or within sites using a criterion of 95% identity. However, we found evidence that the species infrequently undergoes outcrossing and sexual recombination, based on the high rates of linkage disequilibrium (~122,000 SNPs reduced to ~10,000; Fig. 3), estimated selfing rates >0.5, the putatively unisexual mating system, and low recombination rates (Table 2). The detected recombination rates are comparable to those of other fungi with partially clonal reproduction, such as *Verticillium dahlia* (Milgroom et al., 2014) and *Cryphonectria parasitica* (Dutech et al., 2012). We hypothesize that the reason we did not detect any clones, despite these results suggesting a high rate of clonality or selfing, could be the accumulation of somatic mutations in long-lived clonal lineages. Future detailed demographic studies measuring growth rates and age to reproductive maturity would be required to establish generation times for this species and test this hypothesis.

In contrast to prior work suggesting that fungi are not dispersal-limited, the hypothesis that there are low rates of gene flow among populations was supported by high F_{ST} values (0.312–0.730), significant correlation between genetic and geographic distance (Mantel test, $r = 0.489$, $P < 0.01$), and proportionally higher influence of geographic distance on genetic distance when compared to environmental distance ($\alpha E{:}\alpha D < 1$). There was no evidence for isolation by environment based on the partial Mantel test and BEDASSLE results. However, further studies of other environmental variables, such as average high temperatures of warm months or quarters, may reveal signals of adaptation not recovered here. Additionally, future spore trapping and viability assays would be a useful way to directly measure dispersal potential. The sites with the highest genetic diversity were concentrated in the southern portion of the range of the taxon as predicted, suggesting that these may have acted as refugial areas during the LGM. Our results support the notion that gene flow among fungal populations decreases with distance (populations show strong signs of IBD despite the production of small propagules; O'Malley, 2007), suggesting that genetic drift may represent a more important process in diversification of lichenized fungi than previously appreciated. Our data also indicate that recombination can be low despite the frequent presence of sexual spore-producing structures. This finding highlights the phenomenon that the observed reproductive mode does not necessarily translate directly to the frequency of recombination (Taylor et al., 2015).

Influence of reproductive strategy on population genetic structure in lichens

Our data on *Cetradonia linearis* contribute to a growing understanding of the relationship between fungal reproductive types and genetic diversity and structure. Three species of lichenized fungi were previously examined in detailed population genetic studies including analysis of the mating system (Itten and Honegger, 2010; Singh et al., 2012; Alors et al., 2017). One of these species was investigated with RAPD-PCR and the other two with microsatellites, so comparisons among the studies, and with our study based on genomic data, must be done with consideration of the very different underlying data. Nonetheless, because no genomic studies on lichen population genetic structure have been published to date, a careful comparison of our results with this previous research is useful. The lichen *Xanthoria parietina* was found to be unisexual, having only the MAT1-2 gene present in all individuals investigated, and no observed instances of trichogynes, though it is almost always fertile (Scherrer et al., 2005). The population genetic structure of *X. parietina* based on RAPD-PCR fingerprinting revealed high rates of genotypic diversity within populations, even on a microsite scale, and much lower genetic diversity between populations than within them (Itten and Honegger, 2010), but there was a strong signal of adaptation to specific substrates (Lindblom and Ekman, 2006, 2007). A study of the lichen *Parmelina carporrhizans* found a similar pattern of very high gene flow among most populations sampled, though it is a heterothallic species (Alors et al., 2017). The pattern observed in these two species starkly contrasts with that of *Lobaria pulmonaria*, a heterothallic species that is often observed without sexual reproductive structures, in which apothecia usually

are not produced until individuals are 15–25 yr old (Denison, 2003; Høistad and Gjerde, 2011; Singh et al., 2012). *Lobaria pulmonaria* consistently displays high rates of clonality within populations and sampling sites (Werth et al., 2006; Singh et al., 2012). One way to explain the difference between the population genetic patterns of the two heterothallic species is the ratio of the two alternate MAT idiomorphs: *L. pulmonaria* ratios are often skewed in populations whereas *P. carporrhizans* populations have equal ratios (Singh et al., 2012; Alors et al., 2017). The population genetic structure and biology of *C. linearis* is more similar to those of *X. parietina* and *P. carporrhizans* because (1) it is almost always fertile; (2) no clones have been identified (defined as >95% shared SNPs for the genomic data), even from closely collected colonies; and (3) there is a high level of polymorphism within each population ($\pi = 0.148$–0.338). However, our results indicate that *C. linearis* populations have low rates of gene flow, which contrasts with the pattern of little genetic structure found in both *X. parietina* and *P. carporrhizans*. Furthermore, there is evidence for ecological adaptation in *X. parietina* (Lindblom and Ekman, 2006, 2007), which is lacking in *C. linearis*. Additional studies using genomics to investigate population genetics in lichenized fungi will allow more direct comparisons of genetic structure across diverse fungal clades. These results, along with the high rate of linkage disequilibrium, suggest that while *C. linearis* does not seem to frequently reproduce clonally, there must be some rate of self-fertilization or clonality and dispersal restriction that leads to the genetic isolation of populations. To draw large-scale conclusions about the influence of observed reproductive mode on recombination, further studies tackling a greater breadth of taxonomic sampling throughout lichenized fungi will be required. Specific efforts are needed to target phylogenetic, morphological, ecological, and reproductive diversity to determine what factors most strongly shape recombination.

Biogeographic history

The southern Appalachian mountain range is one of the oldest continuously exposed land masses on earth and has served as a refugium at multiple points in geological history (Braun, 1950). Thus, although it is a relatively small area, the long and complex geological history of the region has shaped similarly strong, complex population genetic patterns in endemic species across multiple domains of life (Manos and Meireles, 2015). The population genetic structure of *C. linearis* is no exception. The southern portion of the current extent of *C. linearis* was likely a refugium during Pleistocene glaciation. The lines of evidence to support this hypothesis include the observation that genetic diversity is higher in southern populations and the location of suitable areas predicted by the hindcast SDM (Fig. 5). Interestingly, the latter model also suggested an expansion of the range to lower-elevation areas (Fig. 5). This finding is consistent with hypotheses that ranges of present-day high-elevation endemics expanded downslope during Pleistocene glaciation (Bruhl, 1997; Crespi et al., 2003; Premoli et al., 2007; Desamore et al., 2010). While this downslope expansion might have been expected to connect populations and diminish the signal of IBD, the data generated for the present study still show a strong geographic signal of increasing structure with distance. Population genetic studies of other high-elevation, southern Appalachian endemics showed similarly strong signals of IBD, including the salamander *Desmognathus wrightii* (Crespi et al., 2003) and the spider *Hypochilus pococki* (Keith and Hedin, 2012). A further parallel between the genetic structures of *D. wrightii* and *C. linearis* is that Roan Mountain populations did not group with populations from the Black Mountains, despite their close geographic proximity (Crespi et al., 2003). Population differentiation was so strong in *Hypochilus pococki* that Keith and Hedin (2012) suggested it may actually comprise multiple cryptic species. The long and complex geological history of the southern Appalachians has resulted in not only high levels of species diversity, but also high genetic diversity within species.

CONCLUSIONS

The results presented here provide strong evidence that the rare, narrowly endemic lichen-forming fungus *Cetradonia linearis* has highly geographically isolated populations over the small area of its distribution (Fig. 2). The population structure of *C. linearis* is congruent with other high-elevation southern Appalachian endemics, suggesting that dispersal among mountain peaks in the region is not frequent for multiple groups of organisms (Crespi et al., 2003; Keith and Hedin, 2012; Fig. 5). We found no evidence of clones in our sampling, but we did find evidence for low rates of recombination, possibly facilitated by a homothallic reproductive system that allows self-fertility (Fig. 3). These results support a growing body of literature suggesting that fungal dispersal can be limited across relatively small spatial scales, despite the production of very small propagules (O'Malley, 2007; Taylor et al., 2015). Although other studies have found that environmental factors strongly influence population structure in fungi (Branco et al., 2015), we found no evidence for isolation by environment (Appendix S6). Future comparative studies are required to fully understand how extrinsic and intrinsic factors shape the population structure and recombination rates of fungi with different ecological requirements, life histories, and reproductive strategies (Grünwald et al., 2016; Peter and Schacherer, 2016). These studies will be facilitated by rapid advancements in population genomics methods, which promise to reshape current perspectives on fungal biology.

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AUTHOR CONTRIBUTIONS

J.L.A. designed this research project, applied for and acquired all required permits and funding for fieldwork, conducted all fieldwork and DNA extractions, analyzed data, and wrote the manuscript.

S.K.M. assisted with portions of the fieldwork, advised on sequencing techniques, and analyzed data. R.S.S. advised on sequencing techniques and analyzed data. S.E.A. advised on research project design, writing and editing, and data analysis.

DATA ACCESSIBILITY

The reference genome and raw reads can be accessed through NCBI BioProject ID PRJNA475321 (http://www.ncbi.nlm.nih.gov/bioproject/475321).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information section at the end of the article.

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