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Reproductive mode and genetic variation suggest a North American origin of European *Letharia vulpina*

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Abstract

Our data on the intercontinental population biology of *Letharia vulpina* show an unexpected shift from a recombining North American population with unique haplotypes to genetically depauperate Swedish and Italian populations, each with many representatives of a single repeated haplotype. Analysis of eight loci in 47 individuals supported recombination in North American populations and showed almost no variation among European populations. We infer that a genetic bottleneck caused by limited long-distance dispersal accounts for the lack of genetic variation found in marginal populations. This lack of variation in the European populations makes it impossible to use population genetic means to distinguish clonal reproduction from self-fertilization or even outcrossing, but phenotype indicates that reproduction in the marginal populations is by clonal spread, via soredia and isidioid soredia.

Keywords: biogeography, clonal reproduction, lichenized fungi, long-distance dispersal, population genetics, recombination, sexual reproduction

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Introduction

Fungi may use recombination and clonality in part of their range, and be exclusively clonal in other parts, particularly when their spread can be associated with agriculture (Carbone *et al*. 1999; Taylor *et al*. 2000) or human disease (Kasuga *et al*. 1999; Fisher *et al*. 2001). To see if this observation could be extended to fungi that have not been dispersed by human activity, we examined the effective reproductive mode among disjunct populations of a lichenized fungus with a mixed reproductive strategy. The lichen *Letharia vulpina* (L.) Hue occurs in western North America, continental Europe (Fennoscandia and central Europe to Spain and Italy), northern Africa, Cyprus and the Caucasus (Ahlner 1948; Gams 1955). The unusually bright yellow colour, due to vulpinic acid, makes the species recognizable to nonlichenologists, and may explain why it was one of the few fungi, including lichens, treated by Linnaeus (1742).

The genus *Letharia* was earlier thought to comprise a 'species pair' (Poelt 1970; 1972): *L. vulpina* and *L. columbiana* (Nutt.) J. W. Thomson (1969); the former species recognized by abundant soredia (small ecorticate propagules that contain both fungal and algal cells) and/or isidioid soredia (fused soredia that resemble isidia) and the latter species recognized by abundant ascomata (sexual reproductive structures that make meiospores). A recent study, based on the congruence of six gene genealogies, found that the genus is composed of at least six such phylogenetic species, two that produce abundant soredia and/or isidioid soredia, and four species that produce abundant ascomata (Kroken & Taylor 2001a). All six *Letharia* species occur in western North America, but *L. vulpina* is the only species that has been found in Europe and northern Africa.

L. vulpina is relatively abundant where it occurs in the coastal ranges of western North America. In contrast, *L. vulpina* is now a rare species in several European countries and is red-listed in the Fennoscandian countries: Norway (Tønsberg *et al*. 1996), Finland (Vitikainen *et al*. 1997) and Sweden (Gärdenfors 2000). In Sweden, several populations have been found that consist of several hundred individual thalli (Degelius 1946; Gams 1955). It appears that short-range

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dispersal is effective in maintaining these populations. However, effective dispersal over longer distances is rare but obviously occurs, because these populations are separated by uncolonized areas with a suitable substrate of live branches and snags of conifers (Ahlner 1948).

In *L. vulpina*, all individuals produce abundant soredia and/or isidioid soredia, but ascomata are rare in western North America and exceedingly rare in Europe. From Sweden, Ahlner (1948) reported six localities with *L. vulpina* producing ascomata in Dalarna from 1895 to 1941 and one in Härjedalen in 1916. The last finding of ascomata in Sweden was from Transtrand parish in 1941, but more recent findings have been reported from continental Europe (Rondon 1971; P. Pfeiffer, personal communication).

The objectives of this study were (i) to characterize the mode of reproduction in *L. vulpina* and (ii) to infer the origin of this species based on the spatial distribution of the genetic variation found. To characterize genetic variation, we analysed DNA polymorphisms at eight loci in a total of 47 *L. vulpina* individual thalli from one Californian, one Italian and three Swedish populations. To characterize the effective reproductive mode of *L. vulpina* in these populations, we used population genetic approaches based on the logic that variable loci distributed throughout the genome remain associated during clonal reproduction, but not during recombination. We found abundant variation and recombination in North American *L. vulpina*, but very little variation in European *L. vulpina*. Although the lack of European variation made population genetic analysis of reproductive mode moot there, the lack of meiospores on the European individuals suggested that reproduction is clonal.

Materials and methods

Field sampling

Forty-seven lichen thalli of the species *Letharia vulpina* were sampled from North America and Europe. The location of the populations and the number of samples characterized from each site were: Middle Ridge trail, Henry Coe State Park, California (n = 7; 37 N, 121 W; 600 m), Rogen, Sweden (n = 10; 62 N, 12 E; 750 m), Enviken, Sweden (n = 10; 60 N, 16 E; 300 m), Öland, Sweden (n = 8; 57 N, 17 E; 10 m) and W. Innerprags (between Pragser Wildsee and Grünwaldalm), Italy (n = 12; 46 N, 12 E; 1900 m). Voucher specimens of all individual lichen thalli are deposited in the University of California herbarium (UC) in Berkeley, California, USA.

Laboratory procedures

DNA was isolated from the lichen thalli by using the procedures of Kroken & Taylor (2001a). The eight loci studied in this project include sequences of chitin synthase I (*chs*), an intron at position 287 in the SSUrDNA, as well as six other anonymous loci named *CT*, *CS*, *BA*, 4, *12* and *13* (Kroken & Taylor 2001a). All loci for each characterized lichen thallus were (polymerase chain reaction) PCR amplified and sequenced from both strands.

Data analysis

Sequences were manually edited and aligned in Sequence Navigator (ABI). Overall nucleotide diversity (Π) based on single nucleotide polymorphisms (SNPs) for California and Europe was calculated according to Nei (1987).

To distinguish recombined and clonal population structures, we tried to reject the null hypothesis of recombination using the Index of Association (I_A, Maynard Smith *et al*. 1993) and the Phylogenetic Tree Length Permutation Test (PTLPT, Burt *et al*. 1996) and we attempted to reject the null hypothesis of clonality using Incongruence Length Difference (ILD, Farris *et al*. 1995), also known as the Partition Homogeneity Test (PHT, Huelsenbeck *et al*. 1996). The I_A was performed as described in Burt *et al*. (1996), and the PTLPT and the ILD/PHT were performed in PAUP 4.0b4a for Macintosh (Swofford 1999). The latter two tests were designed to detect incongruity among characters used for phylogenetic analyses, and have been applied to detect incongruity caused by recombination in population genetic analyses (Burt *et al*. 1996; Taylor *et al*. 1999). Prior to the population genetic analyses, the gene genealogy for each locus was checked to guarantee the absence of any homoplastic sites, so that incongruity detected in the combined data set could be attributed to recombination, and not to nucleotide reversals (Burt *et al*. 1996; Barker & Lutzoni 2000).

The I_A is a measure of the rescaled variance of the genetic distance for all pairs of multilocus haplotypes. For a recombining population, the distances are normally distributed around a mean and the variance is low, whereas for a clonal population the distances are not normally distributed and the variance is high. The I_A is determined by comparing the variance of the observed data set to the distribution of variances for 10 000 artificially recombined data sets, where the alleles at each locus are resampled without replacement. The probability represents the number of resampled data sets with variances as high as or higher than that of the observed data set. A score of $P = 0.05$ is taken to indicate significant linkage disequalibrium in the data set, rejecting a null hypothesis of recombination (Taylor *et al*. 1999).

The PTLPT test was used to compare the length of the most parsimonious tree(s) based on the combined, observed data sets to a distribution of tree lengths based on 100 000 artificially recombined data sets, in which the alleles of each informative site had been sampled with replacement. The probability represents the percentage of trees from the resampled data sets that are as short as or shorter than the observed tree. Similar to the I_A, a score of

Table 1 Single nucleotide polymorphisms (SNPs) for eight loci in the lichenized fungus *L. vulpina*. Haplotypes and loci are specified in the material and methods section

Haplotype/Locus	intron	chs	CT	12	4	CS	13	BA
Henry Coe, USA 1 (37 N)	GG	TCAGAT	CTACCCCGATTCG	–GAGCC	TT	ATCCACTGTTTG	CTAC	G
Henry Coe, USA 2	GG	GTTATC	CTACTCCGATTCG	TAGATG	TT	GTTGGTCTGCCT	CTAC	G
Henry Coe, USA 3	GG	GTAGTC	CTACCCCGATTCG	TAGATG	TT	ATCCACTGTTTG	CTAC	G
Henry Coe, USA 4	CA	TCAGAT	TCCGCTTAGCCTA	TAGATG	CA	ATCCACTGTTTG	CTAC	A
Henry Coe, USA 5	GG	TCAGAT	CTACTCCGATTCG	TAGATG	TT	ATCCACTGTTTG	CTAC	G
Henry Coe, USA 6	GG	TCAGAT	CTACCCCGATTCG	TAGATG	TT	ATCCACTGTTTG	CTAC	G
Henry Coe, USA 7	GG	GTTATC	CTACTCCGATTCG	–GAGCC	TT	GTTGGTCTGCCT	CTAC	G
Tyrol, Italy 1–12 (46 N)	GG	GTAGTC	CTACCCCGATTCG	TAGATG	TT	GCTGGTCTGCCT	CTAC	G
Öland, Sweden 1–8 (57 N)	GG	GTAGTC	CTACCCCGATTCG	TAGATG	CA	GCTGGTCTGCCT	CTAC	G
Enviken, Sweden 1–10 (60 N)	GG	GTAGTC	CTACCCCGATTCG	TAGATG	CA	GCTGGTCTGCCT	TGGT	G
Rogen, Sweden 1–10 (62 N)	GG	GTAGTC	CTACCCCGATTCG	TAGATG	CA	GCTGGTCTGCCT	CTAC	G

$P = 0.05$ is taken to indicate significant structure in the data set, rejecting a null hypothesis of panmixia (Burt *et al*. 1996).

The ILD/PHT was designed to assess congruity among data sets. In our case, each locus was considered to be one data set and we assumed that the several loci would have congruent trees under clonality, and incongruent ones under recombination. The test compares the sum of the most parsimonious tree lengths for the observed data to the distribution of sums of tree lengths of 10 000 data sets for which the parsimony informative sites have been swapped among data sets. If the loci are congruent, swapping the informative nucleotide positions among loci will have little or no effect (as the loci are effectively linked), and the artificial data sets will produce sums of tree lengths similar to those for the original data set. However, if the loci are incongruent, swapping sites among loci will introduce homoplasy among loci and result in longer trees (note that in the ILD/PHT, shorter observed trees are consistent with recombination, whereas in the PTLPT test, shorter observed trees are consistent with clonality). The probability represents the percentage of trees from the resampled data sets that are as long as or longer than the observed tree. A score of $P = 0.001$ is taken to indicate significant conflict among loci, rejecting of the null hypothesis of clonality (Cunningham 1997; Taylor *et al*. 1999).

Results

Genetic diversity

To examine the genetic diversity and reproductive mode of *Letharia vulpina* in North American and European populations, we characterized 46 variable nucleotide positions among *c*. 2400 nucleotides of DNA sequence spread over eight loci among 47 individual lichen thalli from one Californian, one Italian and three Swedish populations (Table 1). Ten haplotypes were discovered among the 47 thalli. All seven individuals sampled from one population in Henry Coe State Park, California had different haplotypes. Allelic differences were found in seven of the eight loci, and nucleotide diversity (Π, Nei 1987) based on SNPs was calculated to be 0.35 on a scale of 0–1. In contrast, the 40 European thalli had just three haplotypes that differed at only two loci. Here, nucleotide diversity based on SNPs was 0.05. Each of the four local populations was composed of a single haplotype. The two Swedish populations sampled in Rogen ($n = 10$) and Öland ($n = 8$) shared the same haplotype and the third Swedish population, Enviken ($n = 10$), was identical at all loci except locus *13*, which was fixed for an alternative allele. Similarly, the Italian population ($n = 12$) was identical to the populations from Rogen and Öland except for locus *4*, which was also fixed for another allele. The relationships of these populations are depicted in the phylogram in Fig. 1, in which the four European populations constitute one small clade compared to the genetically diverse North American population.

Reproductive mode

Three tests were used to distinguish clonality from recombination within the Californian population. The I_A could not reject the null hypothesis of recombination ($P = 0.446$), and nor could the PTLPT ($P = 0.822$). Similarly, the ILD/PHT showed that the individual data sets from seven loci were incongruent ($P = 0.0001$), rejecting the null hypothesis of clonality. Thus, all tests are consistent with the hypothesis of recombination in the California population, despite the fact that ascomata are rare (only one of the 40 individuals collected from this site produced a few small ascomata, Fig. 2). This result complements the findings of recombination and outcrossing in *L.* '*lupina*', another phylogenetically recognized *Letharia* species that is also sorediate and rarely produces ascomata (Kroken & Taylor 2001b). Based on the morphological and genetic observations, the reproductive mode fits a model of

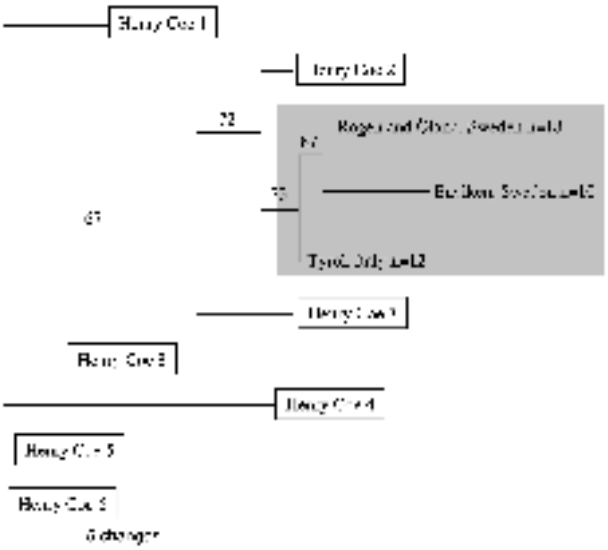

Fig. 1 Unrooted maximum parsimony tree based on nucleotide sequences from all eight loci. Starting tree obtained via stepwise addition with tree-bisection–reconnection as branch-swapping algorithm. Bootstrap values are based on 1000 replicates. Tree length is 62 steps. Consistency index = 0.79. Homoplasy index = 0.21. The European clade is shown in a grey box.

recombined genotypes from sexual reproduction that form clonal runs via soredia.

In contrast, due to lack of known genetic variation, it is impossible to use population genetic methods to determine the reproductive mode of *L. vulpina* in Europe. Clonal reproduction, selfing and outcrossing would all produce the same distribution of haplotypes, given that there was so little variation. However, judging by the lack of ascomata and the presence of soredia and/or isidioid soredia on the Swedish and Italian specimens, it seems likely that the mode of reproduction is clonal.

Discussion

Reproductive modes of lichenized fungi

Letharia vulpina is effectively recombining and outcrossing in genetically diverse North American populations, whereas it is effectively clonal in genetically depauperate European populations. These inferences provide a model for lichen reproductive behaviour in addition to those proposed for the lichens *Lobaria pulmonaria* (L.) Hoffm (Zoller *et al*. 1999) and *Graphis scripta* (L.) Ach. (Murtagh *et al*. 2000).

Similar to *L. vulpina* in Fennoscandia, *L. pulmonaria* is a threatened lichen in large parts of Europe. A study of six local populations in Switzerland suggests a reduction of genetic diversity associated with the decline of this species, as only two variable loci were found among the eight sequenced loci (Zoller *et al*. 1999). If the populations of *L. pulmonaria* found in Africa, Asia and North America are part of the same phylogenetic species, they may be found to be more genetically diverse than the European populations. Also similar to *L. vulpina*, *L. pulmonaria* reproduces sexually and clonally, and sexuality appears to be lost in populations with a limited number of haplotypes. Haplotypes of *L. pulmonaria* that did not produce ascomata in populations with only 1–2 haplotypes present

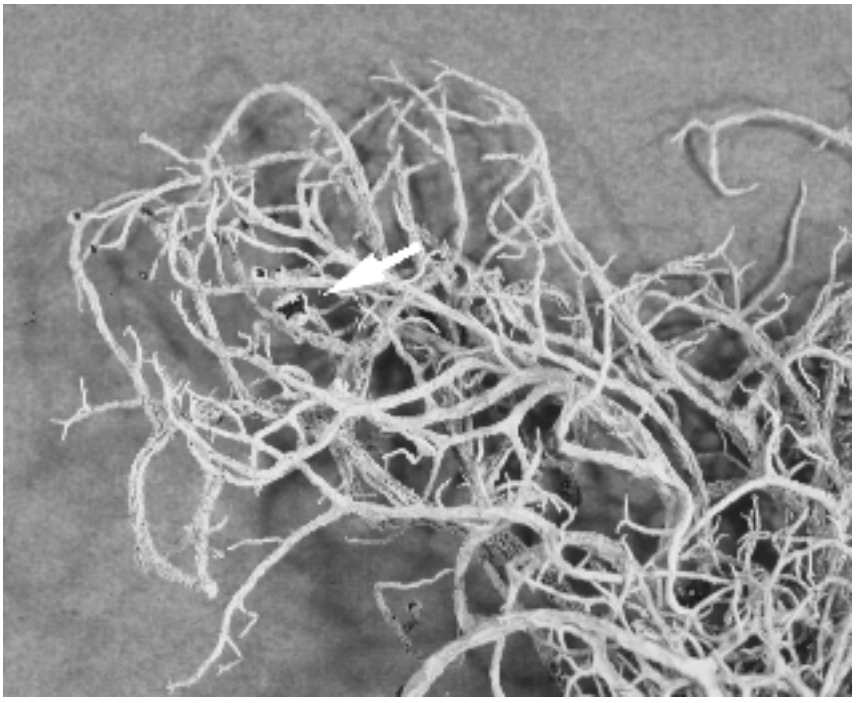

Fig. 2 Mature thallus of *Letharia vulpina*. This is an example of a rarely encountered individual that has small fungal sexual reproductive structures, ascomata (*c*. 1 mm in diameter). One of the ascomata is pointed out by the arrow.

did produce ascomata in populations with 4–5 haplotypes present, suggesting that *L. pulmonaria* is also outcrossing (Zoller *et al*. 1999).

In contrast, Murtagh *et al*. (2000) report that *G. scripta* is reproducing by selfing in Wales (UK), where random amplified polymorphic DNA marker (RAPD) loci shown to be variable among different thalli were claimed to be homozgyous in ascomata. However, phylogenetic species had not been determined beforehand. Based on our experience with *Letharia* species (Kroken & Taylor 2001a) and nonlichenized fungi (Burt *et al*. 1996; Geiser *et al*. 1998; Kasuga *et al*. 1999; reviewed in Taylor *et al*. 1999), each morphologically recognized species is likely to contain two or more genetically isolated species whose distributions are often sympatric. Should this also be the case for *G. scripta*, many of the RAPD loci could be variable among genetically isolated species, but fixed within species (Grube & Kroken 2000). Without evidence that the polymorphisms are intraspecific, they cannot be used to distinguish between selfing and outcrossing.

Long-distance dispersal

Our study provides an example of long-distance dispersal (migrationist biogeography), which has been invoked to explain distributions of many species of lichenized fungi that occur in ecologically similar but geographically disjunct habitats (Galloway 1996). Such explanations have been questioned for lack of evidence, and for alternative hypotheses such as vicariance biogeography (e.g. isolation by continental drift). We infer that *L. vulpina* originated in western North America and migrated to Europe based on the fact that they are both members of the same phylogenetic species, the relatively low genetic variation among European individuals ($\Pi = 0.05$) compared to those of North America ($\Pi = 0.35$), and that the other five of the six *Letharia* species have been found only in western North America, the centre of diversity for the genus. Given the almost complete lack of genetic variation among European isolates, there cannot have been many migration events from North America to Europe.

This global population structure for *L. vulpina* fits well into Darwin's (1859) model of occasional dispersal as discussed in the *Origin of Species* to explain the occurrence of geographically disjunct populations of the same species. Fungi associated with agriculture and forestry show such disjunct populations regularly, presumably due to dispersal by humans e.g. *Phytophthora infestans* (Mont.) de Barry (Goodwin *et al*. 1994), *Sclerotinia sclerotiorum* (Lib.) de Barry (Kohli & Kohn 1996). The same may be true for the human pathogenic fungus *Coccidioides immitis*, which may have been carried from North America to South America by human migration (Fisher *et al*. 2001). *L. vulpina*, however, is now the only fungus shown to have dramatic shifts of its genetic structure without dispersal by humans, either recent or historical.

The long-distance dispersal of *L. vulpina* could have occurred via fungal meiospores, or via soredia and/or isidioid soredia. However, a germinating meiospore has the problem of finding a suitable alga to re-establish the lichen symbiosis. *L. vulpina* is specific to a single lineage of the green algal morphospecies *Trebouxia jamesii* Hildreth & Ahmadjian (Kroken & Taylor 2000). Relichenization must occur in local populations, judging from the observation of recombination. However, a lack of compatible algae may limit the effective long-distance dispersal of meiospores. The algal component of *L. vulpina* has an identical internal transcribed spacer (ITS) sequence in North America and Europe (Kroken & Taylor 2000), indicating that intercontinental transfer was more likely to have occurred via lightweight soredia, which contain both the fungal and algal partners.

Applications and future directions

The results of this study have implications for conservation practices, as *L. vulpina* is threatened in many European countries. We did not detect any gene flow between the central Swedish population Enviken and the other two Swedish populations, nor between Swedish populations and the Italian population. Our data are consistent with local fixation for different alleles of the only two loci that showed variation in the European material. The genetic data presented in this study fit well into previous speculations that dispersal within Europe is restricted in current times, based on the lack of correlation between available substrates and the local distribution of *L. vulpina* (Degelius 1946). Our data suggest that local populations in Europe may not differ from each other in their importance as genetic reservoirs. However, critical attention should be given to any population that is found to contain individuals producing ascomata, as these are potentially recombining populations that may harbour more genetic diversity than the putatively clonal populations that we observed (Zoller *et al*. 1999). Populations that are more genetically diverse may also be encountered among potential glacial refugia in locations to the south of Fennoscandia, the Alps and the Pyrenees, such as the Canary Islands, Cyprus and the Caucasus and Atlas mountains.

The study of genetic variation in such refugia will provide further information about the species' natural history in Europe and northern Africa. The most parsimonious explanation for the genetic variation among the populations characterized in this report is long-distance dispersal from North America to Europe. However, further data could support an alternative hypothesis of postglaciation re-establishment of Europe from Mediterranean and African refugia rather than from North American populations.

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This work was conducted in the Taylor lab, Department of Plant Biology, UC Berkeley in line with earlier work with regard to speciation, recombination and population genetics of ascomycete fungi. Nils Högberg works with population biology of fungi and experimental biology of saprotrophs. Scott Kroken studied the speciation and effective reproductive mode in the lichen genus *Letharia*, and is currently using a comparative genomic approach to investigate the evolution of secondary metabolism of ascomycete fungi at TMRI. John Taylor studies the evolutionary relationships of fungi from ancient events (phylogenetics) to recent events (population genetics). Göran Thor's research interest is within the taxonomy, dispersal including establishment of lichenized fungi and its application in conservation biology.
