American Journal of Botany 95(5): 1–9. 2008.

# LOCAL GENETIC STRUCTURE IN A NORTH AMERICAN EPIPHYTIC LICHEN, *RAMALINA MENZIESII* (RAMALINACEAE)[1]

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Epiphytic lichens possess unique life history traits that can have conflicting effects on genetic structure: symbiotic mutualism between a fungus with its algal or cyanobacterial photobiont, association with a host plant, and ability to reproduce sexually and asexually. Our study species, *Ramalina menziesii*, has small ascospores that can facilitate long-distance gene movement, and it is capable of clonal reproduction. The goals of this study are to test whether different haplotypes were differentially distributed across host plant species, to look for evidence of asexual vs. sexual reproduction, and to assess the local genetic structure of the population. We sampled individuals from multiple trees of three oak species in four lichen subpopulations within a savanna ecosystem. Using DNA sequence data from four fungal nuclear loci, we found no tendency for host specialization. Alleles were randomly distributed across subpopulations. The frequency of multilocus genotypes was consistent with a randomly mating population. Sexual reproduction involving relichenization appeared to be the predominant mode of reproduction of *R. menziesii* at this study site. We found no significant local genetic structure suggesting widespread gene flow at the local scale. The genetic structure of this lichen is comparable to that of widely distributed epiphytic plants.

**Key words:** AMOVA; epiphyte; fungus; genetic structure; lichenized ascomycete; mutualism; *Quercus*; *Ramalina menziesii*; Ramalinaceae; southern California.

Evolutionary biologists frequently assess the genetic structure of populations because the distribution of genetic variation within and among subpopulations indicates historical patterns of gene flow, the opportunity for genetic drift, and the potential for local adaptation. Epiphytic lichens are particularly intriguing organisms for population genetic research because various aspects of their natural history can influence the amount of genetic diversity and its distribution within and among populations in conflicting ways. First, their life form is the result of an obligate mutualism of a lichen-forming fungus with a green algal and/or cyanobacterial photobiont (Galloway, 1992; Richardson, 1999; Purvis, 2000). Second, their loose association with certain host plant species may indicate that lichens require certain conditions for establishment. Within a region, epiphytic lichens tend to be found on a subset of plant species, and lichens generally have tendencies for certain habitats or substrates (Barkman, 1958). When a lichen species grows on more than one host species, differences in the chemistry and structure of host plants could lead to genetic differentiation associated with each host species. Third, the extent of sexual vs. asexual reproduction could differ among sites, and the predominance of one form of reproduction or another will influence the genetic structure of the lichen population (Werth et al., 2006b). Finally, for sexual populations, the small size of their propagules (Barkman, 1958; Bailey, 1976; Galloway and Aptroot, 1995; Snäll et al., 2004) may facilitate gene flow over long distances (Werth et al., 2006a). Long-distance dispersal of lichens has indeed been found: some lichens were shown to colonize newly formed volcanic islands (Bailey, 1976), formerly polluted urban areas where lichens had not been present for decades (Heibel et al., 1999), and disturbed forests (Werth et al., 2006b). Thus, different aspects of lichen life history could either restrict or promote gene flow and, thus, influence the genetic structure of populations.

A few range-wide studies of the genetic structure of the fungal genome of the lichen (the lichenized ascomycete) indicated that haplotypes can be widely distributed (Printzen and Ekman, 2003; Palice and Printzen, 2004). On the other hand, smaller scale studies indicated that lichen dispersal can be restricted. Walser (2004) reported clustering of genotypes on a local scale, others showed isolation by distance (Wagner et al., 2005; Werth et al., 2006b), and additional studies demonstrated genetic differentiation among populations at local (Printzen and Ekman, 2003; Werth et al., 2007) and regional spatial scales (Walser et al., 2005). For vegetative propagules of some lichen species, the maximum measured dispersal distances were in the range of tens of meters only (Armstrong, 1987; Heinken, 1999). Thus, for these species, gene flow via vegetative propagules might be restricted. In sum, available studies provide evidence for both long-distance and restricted dispersal. Clearly, the life history form of epiphytic lichens does not result in one particular pattern of genetic structure.

Most lichen species are capable of reproducing sexually and asexually, and gene movement can occur through either form.

[1] Manuscript received 25 January 2007; revision accepted 14 January 2008.

The authors thank D. Grivet and A. R. Plüss for field assistance and valuable discussions; E. Gonzales, C. Dutech, S. Banke, and G. Segelbacher for feedback on the manuscript; R. J. Dyer, P. E. Smouse, and R. D. Westfall for statistical advice; and D. G. Scofield for writing the R-code for the simulation test to discriminate asexual from sexual propagation. Research was supported by the following sources: a National Geographic Award to V.L.S. and S.W., a postdoctoral fellowship from the Swiss National Foundation (PBBEA-111207) to S.W., and a UCLA Senate research award, UCLA Life Science funds, and a National Science Foundation award (DEB-0089445) to V.L.S. The authors acknowledge the University of California Natural Reserve System Sedgwick Reserve administered by UC Santa Barbara and thank M. Williams, R. Skillin, and B. Huebel for logistical support.

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doi: 10.3732/ajb.2007024

Lichens reproduce asexually when the vegetative body of the lichen (thallus) fragments, and many, but not all lichen species can reproduce in this way. Vegetative reproduction can lead to a clustering of multilocus genotypes (or genets) within and among neighboring trees and to significant spatial structure at short distances (Walser, 2004; Werth et al., 2006b). Because they are larger than sexually produced ascospores, thallus fragments are thought to be dispersed less efficiently than the sexually produced ascospores (Sillett et al., 2000), although vegetative propagules can also be dispersed by wind or birds over longer distances. Some lichen species are obligate outcrossers (heterothallic), whereas others can either self or outcross (homothallic). After sexual reproduction, the dispersed ascospores must find a photobiont to lichenize, which could be a constraint on lichen colonization, even if the spores disperse widely (Werth et al., 2007).

Here, we will examine the local genetic structure of an epiphytic lichen, *Ramalina menziesii*, at a single study site in the center of its distribution range, which includes the western, often coastal, regions of North America from Baja California through Alaska (Rundel, 1974). This species reproduces both sexually through ascospores and vegetatively through thallus fragments. The frequency of thalli with apothecia varies greatly in *R. menziesii* from California, and apothecia-bearing thalli are rare or absent in some localities (S. Werth, unpublished observations). Thus, the genetic structure may be strongly affected by the propagule type—vegetative thallus fragments or sexual ascospores.

In a given locality, *Ramalina menziesii* tends to specialize on a few host plant species, but the host species vary across its range. In California, this lichen is found mainly on canopy branches of three host species, California valley oak (*Quercus lobata*), blue oak (*Q. douglasii*), and coastal live oak (*Q. agrifolia*), and the mutualistic association seems to benefit both the lichen and the host trees. The lichen benefits from the structured space and microclimate created by the oak canopy. *Quercus lobata* and *Q. douglasii* are winter-deciduous white oaks (subgenus *Quercus*, section *Quercus*) that differ slightly in bark texture. *Quercus lobata* is usually found in valleys, while *Q. douglasii* occurs more commonly on the adjacent slopes. *Quercus agrifolia*, an evergreen oak belonging to the black oaks group (subgenus *Erythrobalanus*), does not have strong habitat preferences. This species has a very dense canopy and smoother bark textures than the white oak species. In Californian inland localities, such as our study area, *R. menziesii* grows mostly during the winter season, and winter rainfall provides most of its water (Matthes-Sears and Nash, 1986). Thus, in these inland localities, the deciduous oaks may be a more beneficial substrate during the growing season of *R. menziesii* because they shed their leaves in winter, allowing ample light for the lichen's photobiont. In contrast, light may be a limiting factor for photosynthesis of *R. menziesii*'s photobionts in the canopy of the evergreen oak *Q. agrifolia* (Knops et al., 1996). Differences in the bark chemistry of the three oak species could be another factor influencing habitat quality for the lichen. It is not known whether the differences in habitat quality among the oaks put selective pressure on lichens, but genetic differences associated with substrate have been shown elsewhere (e.g., Lindblom and Ekman, 2006).

The trees benefit from the input of the lichens: oaks colonized by lichens received an increased deposition of nitrogen, phosphorous, and water from local rainfall and fog dripping (Knops et al., 1996). Sometimes amounting to 78% of total canopy lichen biomass (about 299 kg/ha dry mass, Knops et al., 1996), *R. menziesii* can contribute markedly to nutrient cycling in California oak woodland ecosystems. Thus, the association of *R. menziesii* and oaks can lead to a small mutual benefit of all involved taxa and is not highly specific; we consider this association to be an example of a weak mutualism.

This study evaluates the scale of local genetic structure of *Ramalina menziesii*, taking into account the potential impact of host species on that structure. If this species behaves like other epiphytes (e.g., Trapnell et al., 2004; Korpelainen et al., 2005; Trapnell and Hamrick, 2005), we should observe evidence of long-distance dispersal. Yet, as an obligate mutualistic species, it must encounter both a compatible photobiont and a suitable host species, and these factors may favor restricted dispersal. In addition, *R. menziesii* is capable of reproducing sexually or asexually, and the type of reproduction could influence local genetic structure. Given the importance of lichens to California oak ecosystems, it is valuable to know the extent of gene movement and to identify any tendencies for specialization on host species. Using DNA sequence variation at four fungal genes, we will accomplish these specific objectives: (1) to test whether different haplotypes are differentially distributed across host plant species, (2) to look for evidence of asexual vs. sexual reproduction, and (3) to characterize the local genetic structure by direct examination of haplotype networks and analysis of molecular variance (AMOVA).

## MATERIALS AND METHODS

***Study species***—*Ramalina menziesii* Tayl. (Ramalinaceae), also known as the lace lichen or the fish net lichen, is a common epiphytic lichen distributed from Baja California to Alaska, along the coast and near coastal habitats of western North America (Rundel, 1974). Across its range, this species is associated with different host species. In Baja California, *R. menziesii* is found on shrubs in coastal locations as well as on cacti, shrubs, and trees (Fouquieriaceae, Burseraceae) in fog-influenced chaparral and desert sites. In California, the lichen occurs predominantly on oaks, notably *Quercus agrifolia* in coastal localities and on *Q. douglasii, Q. lobata*, and *Q. garryana* in localities at a larger distance from the coast. In Washington and Oregon inland localities, *R. menziesii* is found mainly on deciduous tree species, notably *Q. garryana*, whereas along the coast, it is found on Sitka spruce (*Picea sitchensis*). In British Columbia and Alaska, the lichen is predominantly found on Sitka spruce in sites next to the ocean and disappears quickly in the forest within tens of meters of the ocean edge. Within a location, this lichen typically is found on a single or restricted number of host species. In some cases, the host may profit from increased nutrient and water availability (Knops et al., 1991, 1996).

***Study site***—Our study site is located at Sedgwick Reserve in the Santa Ynez Valley of Santa Barbara County, which is administered by University of California–Santa Barbara as part of the University of California Natural Reserve System (California, USA, 34°42′N, 120°02′W; 2358 ha; elevation 290–790 m a.s.l.). The majority of the reserve is an oak-savanna ecosystem with three major tree oak species (Fagaceae): valley oak (*Quercus lobata*), blue oak (*Q. douglasii*), and coastal live oak (*Q. agrifolia*). For more details about the study site, see Sork et al. (2002a, b).

***Sampling***—The goals of our design were to detect the scale of genetic structure and to identify patterns of differentiation in four fungal nuclear genes that might be associated with host plant species. To detect the scale of local genetic structure, we sampled thalli of *R. menziesii* from four subpopulations (sites) across a landscape, with a minimum distance of 200 m and a maximum of 1900 m among sites. Within each site, we collected lichen thalli in different parts of the canopy from two trees per each of the three *Quercus* species (*Q. lobata*, *Q. douglasii*, *Q. agrifolia*). We thus collected 18 lichen thalli per site, six per *Quercus* species, for a total of 72 thalli collected across all four sites. For purposes of our analyses, we considered the study site as one population and individual sites as subpopulations. This sampling design is sufficient to detect high

genetic structure at the level of individual tree, subpopulation, or host tree species and to identify where additional sampling would be needed if trends of genetic structure are found at any of these levels. This design would not detect the extent of clonal spread among adjacent thalli or genetic clustering on the same branch of the tree.

***Laboratory analysis***—During sample preparation for DNA extraction, we recorded presence or absence of apothecia for each thallus. We used the GenElute plant extraction kit and the manufacturer's protocol (Sigma, St. Louis, Missouri, USA) to extract total genomic DNA from lobe tips of *R. menziesii*, modified to improve DNA quality by adding 4 μL of RNase A (Qiagen, Hilden, Germany) to each sample prior to the incubation step at 65°C.

Each of our fungal-specific polymerase chain reactions (PCR) contained 1× PCR multiplex kit (Qiagen, Hilden, Germany), 200 nM each of forward and reverse primer, 1 μL genomic DNA, and $H_2O$ to a final volume of 40 μL. To amplify beta-tubulin (*bet*), we used primers Bt3-LM [forward] and Bt10-LM [reverse] ($T_m$ = 56°C) (Myllys et al., 2002); for the unidentified locus (*uid*), we used primers CAL228F and CAL737R ($T_m$ = 56°C) (Carbone and Kohn, 1999); for glyceraldehyde 3-phosphate dehydrogenase (*gpd*), i.e., gapC, we used primers gpd-F-and gpd-R at $T_m$ = 56°C (Johannesson et al., 2000). For translation elongation factor 1-α (*efa*), we used primer set efa-F and efa-R at $T_m$ = 52°C (Johannesson et al., 2000). Each amplification reaction consisted of 15 min at 96°C to activate the hot-start polymerase of the multiplex kit, followed by 35 cycles of 30 s at 95°C, 60 s at $T_m$, 90 s at 72°C; and a final extension of 10 min at 72°C.

Primers and salts were removed using ethanol precipitation. We added 2.5 volumes of 96% ethanol and 0.15 volumes of 3 M sodium acetate ($C_2H_3O_2Na$, pH 5.2) to one volume of PCR product, mixed the resulting solution, and left the tubes at room temperature for 15 min to precipitate the DNA, followed by 15 min centrifugation at 16 000 rpm. After the centrifugation step, the supernatant was discarded and 12.5 volumes of 70% ethanol (room temperature) were added. After a centrifugation step of 8 min at 16 000 rpm, the supernatant was discarded. The resulting DNA pellet was dried in an oven at 65°C for 5 min to remove any remaining ethanol, then 25 μL of sterile $ddH_2O$ was added. The tubes were closed and placed in the oven for 15 min to dissolve the DNA.

The concentration of PCR products was estimated using a low-range-mass ruler DNA ladder (Fermentas, Burlington, Canada). The PCR and ethanol precipitation resulted in a single fragment for each locus that was sequenced directly, with the majority of concentrations larger or equal to 10 ng/μL. Each cycle sequencing reaction contained 2 μL Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, California, USA), 500 nM of one primer, 20 ng purified PCR product, and $H_2O$ to a total volume of 10 μL. The cycle-sequencing products were purified with the QuickStep2 PCR purification system (Edge BioSystems, Gaithersburg, Maryland, USA). Sequences were run on ABI 3730 and 3700 DNA analyzers (Applied Biosystems). Sequences were edited using the program Sequence Scanner, version 1.0 (Applied Biosystems), and all unique DNA sequences were deposited in GenBank (accessions EF377538–EF377582, Appendix 1).

***Data analysis***—The analyses are based on 2595 bp of DNA sequences (*bet*, 811 bp; *efa*, 494 bp; *gpd*, 586 bp; *uid*, 704 bp). Sequence alignment was performed using the program ClustalW (Thompson et al., 1994) as implemented in Mega, version 3.1 (Kumar et al., 2004). Sequences were collapsed into haplotypes using the program Snap (Aylor et al., 2006) as implemented in the program Snap Workbench (Price and Carbone, 2005). To test if loci were independent of each other, we performed exact pairwise tests of linkage disequilibrium (Slatkin, 1994) using the program Arlequin, version 3. 1 (Excoffier et al., 2005).

To compare genetic diversity among host species and subpopulations, we calculated the haplotype diversity *Hd*, the number of haplotypes *H*, and the number of polymorphic sites *S* within each host species and subpopulation using Arlequin. We also summarized these parameters for the entire sample.

To assess whether the genetic data are consistent with sexual or a mix of sexual and asexual reproduction, we conducted a simulation test to compare the observed numbers of four-locus haploid genotypes in our sample of 72 thalli against expected distributions of four-locus genotypes in an identically sized sample drawn from a random-mating population with complete sexual reproduction. Asexual reproduction reduces the number of multilocus genotypes in a sample and increases the frequency of multilocus genotype recurrence, that is, the number of times a multilocus genotype is expected to occur more than once. For each of 1000 simulation iterations, 72 four-locus genotypes were constructed assuming no linkage and observed population allele frequencies. These simulated data were used to construct two sample-size-specific expected distributions: the number of unique genotypes, to which the observed number was compared directly; and the frequency distribution of multilocus genotype recurrence, to which the observed recurrences were compared using a $\chi^2$ goodness-of-fit test. (The R code for conducting the simulation tests is available from D. G. Scofield, Department of Ecology and Evolutionary Biology, University of California–Los Angeles).

To investigate whether any alleles (haplotypes) were tied to host species or subpopulation, we calculated the haplotype frequency across host species and sites. For the most common alleles at each locus, we performed $\chi^2$ tests of heterogeneity. These $\chi^2$ tests enabled us to assess whether the observed frequencies of common alleles differed significantly from the null expectation of equal frequency across strata. If there were any structure owing to host species or subpopulation, the observed allele frequency should deviate markedly from the expectation. We performed a further $\chi^2$ test to evaluate whether host species or subpopulation differed due to their number of private alleles. Alleles were considered private if they were sampled in only one site.

To show the relatedness of haplotypes, we constructed median-joining haplotype networks (Bandelt et al., 1999). To see whether certain lineages were associated with host species or site, we examined the distribution of haplotypes and lineages with respect to host species and site.

To analyze genetic structure, we performed analyses of molecular variance (AMOVA) for each locus (Excoffier et al., 1992) using Arlequin separately for host plant species and subpopulation as strata, pooling all observations with each. In AMOVA, population subdivision is quantified using Φ-statistics, which are indices of population subdivision analogous to the standard *F*-statistics. The Φ-statistics in AMOVA is calculated on the basis of differences among DNA-sequence haplotypes. As a distance measure, we used the Jukes–Cantor distance, a distance measure assuming equal nucleotide substitution rates among sites (Jukes and Cantor, 1969). The overall Φ-statistics across all loci, $\Phi_t$, was calculated by averaging across all loci. Moreover, we calculated population differentiation as estimated by θ (Weir and Cockerham, 1984). While Φ-statistics take into account the distance among haplotypes, θ-statistics are based on haplotype frequencies only. We computed θ for each locus separately and the overall θ using the program TFPGA (Miller, 1997).

We also performed two types of multivariate analyses to identify any genetic structure or host specialization: discriminant analysis (Legendre and Legendre, 1998) in SAS (SAS Institute, 1996) and principal coordinate analysis in GenAlEx (Peakall and Smouse, 2006).

## RESULTS

Our study indicated a high level of genetic diversity in *R. menziesii* subpopulations at our study site (Table 1). All loci investigated were polymorphic yielding six total haplotypes in beta-tubulin, four haplotypes in glyceraldehyde 3-phosphate dehydrogenase (*gpd*), 23 haplotypes in translation elongation factor 1-α, and 12 in the unidentified locus (*uid*) (Table 1; see Appendix 1, A–D). All measures of diversity were similar across sites (Table 1). We observed that *uid* and *gpd* were significantly linked at a Bonferroni-corrected probability level of α = 0.05 (Table 2), but none of the loci were linked significantly at α = 0.01. A BlastX search (Altschul et al., 1997) showed that the unidentified locus, *uid*, had a high similarity to the protein glycine dehydrogenase of the ascomycete *Neosartorya fischeri* (accession XP_001262429).

Field observations indicated the presence of sexual reproduction with a high frequency of fertile thalli: 35 of 72 collected thalli (48.6%) bore apothecia. Genetic analyses yielded 49 observed four-locus genotypes, with 40 unique four-locus genotypes and 32 individuals having genotypes that occurred more than once (Fig. 1). Our simulation tests indicated that neither the observed number of four-locus genotypes ($P$ = 0.471) nor the frequency of recurring four-locus genotypes ($\chi^2_2$ = 0.55, $P >> 0.10$) were statistically different from those expected in a completely sexual population, after pooling some classes because of small sample sizes. However, it is notable that two

TABLE 1. Diversity statistics for four subpopulations of *Ramalina menziesii* (sites 1, 2, 3, and 4) situated in a Californian oak savanna summarized by site and overall. The table shows number of samples *N*, number of haplotypes *H*, nucleotide diversity $\pi$ (±SD), number of polymorphic sites *S*, and haplotype diversity *Hd* (±SD). The investigated fungal nuclear loci were beta-tubulin (*bet*), translation elongation factor 1-alpha (*efa*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*), and an unidentified locus similar to glycine dehydrogenase (*uid*).

| Statistic | Site 1 | Site 2 | Site 3 | Site 4 | Total |
|---|---|---|---|---|---|
| Sample size (*N*) | 18 | 18 | 18 | 18 | 72 |
| Number of haplotypes (*H*) | | | | | |
| *bet* | 4 | 3 | 4 | 3 | 6 |
| *efa* | 12 | 11 | 9 | 8 | 23 |
| *gpd* | 3 | 3 | 3 | 4 | 4 |
| *uid* | 5 | 5 | 4 | 5 | 12 |
| Nucleotide diversity ($\pi$) | | | | | |
| *bet* | 0.00666 ± 0.00167 | 0.00653 ± 0.00164 | 0.00660 ± 0.00166 | 0.00662 ± 0.00166 | 0.00641 ± 0.00147 |
| *efa* | 0.00710 ± 0.00178 | 0.00724 ± 0.00182 | 0.00660 ± 0.00166 | 0.00449 ± 0.00113 | 0.00630 ± 0.00144 |
| *gpd* | 0.00076 ± 0.00019 | 0.00170 ± 0.00043 | 0.00170 ± 0.00043 | 0.00213 ± 0.00053 | 0.00154 ± 0.00035 |
| *uid* | 0.00209 ± 0.00052 | 0.00331 ± 0.00083 | 0.00142 ± 0.00036 | 0.00459 ± 0.00115 | 0.00292 ± 0.00067 |
| Number of polymorphic sites (*S*) | | | | | |
| *bet* | 11 | 11 | 12 | 11 | 13 |
| *efa* | 20 | 16 | 13 | 12 | 26 |
| *gpd* | 4 | 4 | 4 | 4 | 4 |
| *uid* | 9 | 8 | 6 | 9 | 15 |
| Haplotype diversity (*Hd*) | | | | | |
| *bet* | 0.627 ± 0.073 | 0.543 ± 0.086 | 0.673 ± 0.069 | 0.569 ± 0.071 | 0.593 ± 0.033 |
| *efa* | 0.922 ± 0.051 | 0.856 ± 0.079 | 0.797 ± 0.090 | 0.791 ± 0.087 | 0.828 ± 0.040 |
| *gpd* | 0.307 ± 0.132 | 0.392 ± 0.133 | 0.307 ± 0.132 | 0.399 ± 0.138 | 0.339 ± 0.067 |
| *uid* | 0.484 ± 0.138 | 0.601 ± 0.113 | 0.471 ± 0.130 | 0.601 ± 0.113 | 0.528 ± 0.064 |

four-locus genotypes were each observed seven times, a higher frequency than expected for random mating (see Fig. 1). This finding might reflect a low level of asexual reproduction in our population and/or a confinement of asexual reproduction to a few genotypes. Our sample size is too low to adequately test either of these possibilities. Considered together, the data and our test results are consistent with a predominantly sexually reproducing population.

To evaluate any tendency for population subdivision by host species, we examined the distribution of alleles across hosts (Table 3). We did not find a significant association of common ($\chi^2_6 = 2.28$; $P > 0.05$) or private alleles ($\chi^2_2 = 0.22$; $P > 0.05$) with host species. Likewise, the distribution of alleles across sites showed little evidence of population subdivision (Table 3). We found no significant association of common ($\chi^2_9 = 3.12$; $P > 0.05$) or private alleles ($\chi^2_3 = 2.19$; $P > 0.05$) with site.

In a visual examination of haplotype networks for which we indicate the sample site of the haplotypes, common haplotypes were evenly dispersed among the sites and clades showed no apparent association with spatial location (Fig. 2). An analysis of the median-joining haplotype networks based on DNA sequence data showed no association of haplotypes or clades with host species in any of the four genes (data not shown).

TABLE 2. Summary of exact tests of pairwise linkage disequilibrium of three fungal, low-copy nuclear genes and an unidentified fungal locus. The table gives the probability of a significant association between loci; the significant linkage is in boldface.

| Locus | *uid* | *gpd* | *efa* |
|---|---|---|---|
| *bet* | 0.714 | 0.274 | 0.570 |
| *uid* | | **0.005** | 0.118 |
| *gpd* | | | 0.608 |

*Abbreviations:* beta-tubulin (*bet*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*), translation elongation factor 1-$\alpha$ (*efa*), unidentified fungal locus (*uid*).

Our analysis of the genetic structure of *R. menziesii* across host species, which was conducted on each gene separately using two kinds of genetic structure models, indicated no significant variation (Table 4). Similarly, we also found no significant genetic differentiation among sites for any of the four genes individually in AMOVA (Table 4). This shallow genetic structure was consistent with the median-joining haplotype networks (Fig. 2). The overall differentiation among sites was not significant regardless of the model used ($\Phi_t = -0.0347$; $\theta_t = -0.0227$). The negative values of the estimates are not significantly different from zero. It is not unusual to calculate slightly negative variance components by chance when there is an absence of genetic structure (Arlequin homepage FAQ; http://lgb.unige.ch/arlequin/).

To ensure that we were not overlooking host-specificity or subtle genetic structure, we used multivariate models to analyze the data, which can provide very sensitive measures of genetic structure (Westfall and Conkle, 1992; Kremer and Zanetto, 1997). Both discriminant analysis (Legendre and Legendre, 1998) and principal coordinate analysis did not identify any genetic structure or genetic differentiation associated with host species (results not shown).

## DISCUSSION

The most dramatic finding of our study is the complete lack of genetic structure in the fungal genome of *Ramalina menziesii* within our oak–savanna study site. The distribution of alleles is essentially random across sites, a pattern that is particularly conspicuous for the most abundant alleles. The haplotype networks illustrate that single-locus haplotypes and clades have no geographic clustering. These patterns and the AMOVA model results suggest that local gene movement is widespread at our study site.

The extensive gene flow suggested by our study is similar to the findings of a study on the old-forest lichen *Lobaria pulmonaria*,

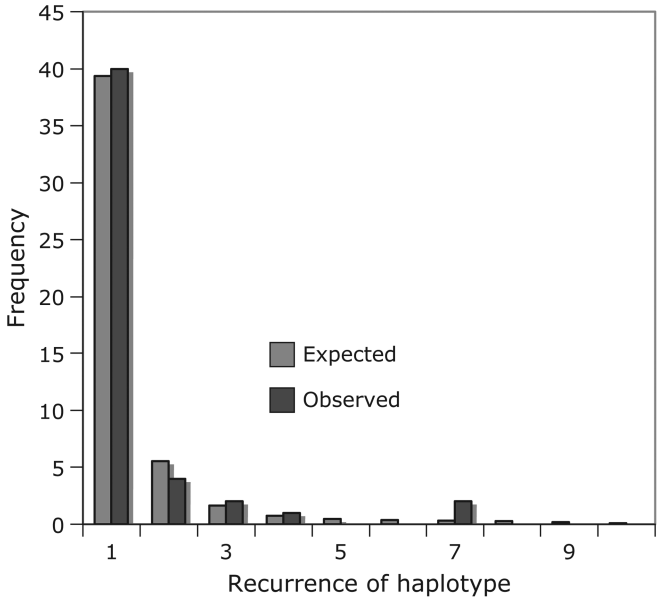


Fig. 1. Frequency of unique and recurrent four-locus genotypes based on the alleles at four fungal genes of the epiphytic lichen *Ramalina menziesii*. Expected values were based on simulations of the expectation for a randomly mating population across the entire population using the sum of the single locus allele frequencies across four sites as shown in Table 3. Observed values were based on the observed 49 four-locus genotypes for those same four loci and the number of times they were sampled once or recurrently.

a lichen dispersing frequently with small clonal propagules and rarely sexually, where high gene flow was found between two forested regions in a sylvopastoral landscape that had been spatially isolated for more than a millennium (Werth et al., 2007). Moreover, direct dispersal estimates from the same landscape documented high levels of long-distance dispersal in *L. pulmonaria* (Werth et al., 2006a). However, in contrast to *R. menziesii*, the presence of long-distance dispersal did not result in a lack of genetic structure: *L. pulmonaria* had significant fine-scale genetic structure, probably due to spatial clustering of clones (Wagner et al., 2005; Werth et al., 2006b), and significant genetic differentiation among populations ($\Phi_{ST}$ = 0.462) and among host trees ($\Phi_{ST}$ = 0.817) (Werth et al., 2007). Johannesson et al. (2001) investigated one Kamchatkan and six Fennoscandian populations of the saprophytic ascomycete *Daldinia loculata* using two of the genes that we included in this study: translation elongation factor 1-α (*efa*), and glyceraldehyde 3-phosphate dehydrogenase (*gpd*). They found $F_{ST}$ = 0.11 in *efa* (*R. menziesii*: $\Phi_{ST}$ = –0.022) and $F_{ST}$ = 0.00 in *gpd* (*R. menziesii*, $\Phi_{ST}$ = –0.042). They used either RFLP of PCR fragments or an alternative fluorescence-based method of genotyping PCR fragments, which may underestimate the true polymorphism in a sample (Weising et al., 2005). Nonetheless, their saprophytic fungus had some amount of genetic structure. Printzen and Ekman (2003) found no significant genetic differentiation among three populations in the epigaeic lichen *Cladonia subcervicornis* collected from islands of western Norway based on DNA sequences of a mitochondrial gene ($F_{ST}$ = –0.013–0.025). However, a fourth population of *C. subcervicornis* collected from another island was significantly differentiated from all other populations ($F_{ST}$ = 0.241–0.312). All of these populations were about 10–15 km apart (Printzen and Ekman, 2003), and thus the spatial scale of this study was considerably larger than that of our study (2 km). In a population genetic study of seven subpopulations of the lichen *Xanthoria parietina* in an island in Norway using the intergenic spacer (IGS) and the complete internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA, Lindblom and Ekman (2006) found $\Phi_{ST}$ = 0.041 and $\Phi_{ST}$ = 0.040, respectively, when removing genetic differentiation due to bark and rock substrates. Given the findings of other lichen studies, we did not

TABLE 3. Haplotype counts of three low-copy nuclear genes and an unidentified locus in the epiphytic lichen *Ramalina menziesii* on each of four sites and three host tree species (Qagr, *Quercus agrifolia*; Qdou, *Q. douglasii*; Qlob, *Q. lobata*) within a southern Californian oak savanna. See Table 1 for description of abbreviations. The most abundant haplotype of each locus is in boldface.

| | | Comparison by site | | | | Comparison by host species | | |
|---|---|---|---|---|---|---|---|---|
| Locus | Haplotype | Site 1 | Site 2 | Site 3 | Site 4 | Qagr | Qdou | Qlob |
| *bet* | *bet1* | 9 | 6 | 8 | 7 | 12 | 9 | 9 |
| | *bet2* | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| | *bet3* | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| | *bet4* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | ***bet5*** | **7** | **11** | **8** | **10** | **10** | **14** | **12** |
| | *bet6* | 1 | 0 | 2 | 0 | 1 | 1 | 1 |
| *efa* | *efa1* | 1 | 1 | 1 | 1 | 2 | 0 | 2 |
| | *efa2* | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| | *efa3* | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| | *efa4* | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| | *efa5* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | *efa6* | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| | *efa7* | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| | *efa8* | 1 | 2 | 1 | 1 | 1 | 2 | 2 |
| | *efa9* | 0 | 0 | 1 | 2 | 1 | 1 | 1 |
| | *efa10* | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| | *efa11* | 1 | 0 | 0 | 1 | 0 | 1 | 1 |
| | *efa12* | 2 | 0 | 0 | 0 | 1 | 1 | 0 |
| | *efa13* | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| | *efa14* | 2 | 1 | 3 | 3 | 1 | 3 | 5 |
| | *efa15* | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| | *efa16* | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | *efa17* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | *efa18* | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| | ***efa19*** | **4** | **7** | **8** | **8** | **10** | **10** | **7** |
| | *efa20* | 2 | 0 | 1 | 0 | 2 | 1 | 0 |
| | *efa21* | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| | *efa22* | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | *efa23* | 0 | 1 | 1 | 0 | 0 | 2 | 0 |
| *gpd* | ***gpd1*** | **15** | **14** | **15** | **14** | **17** | **20** | **21** |
| | *gpd2* | 2 | 2 | 1 | 2 | 4 | 2 | 1 |
| | *gpd3* | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | *gpd4* | 1 | 2 | 2 | 1 | 2 | 2 | 2 |
| *uid* | ***uid1*** | **13** | **11** | **13** | **11** | **16** | **14** | **18** |
| | *uid2* | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | *uid3* | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| | *uid4* | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| | *uid5* | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| | *uid6* | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| | *uid7* | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| | *uid8* | 2 | 4 | 3 | 4 | 4 | 5 | 4 |
| | *uid9* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | *uid10* | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| | *uid11* | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| | *uid12* | 1 | 0 | 0 | 0 | 0 | 0 | 1 |

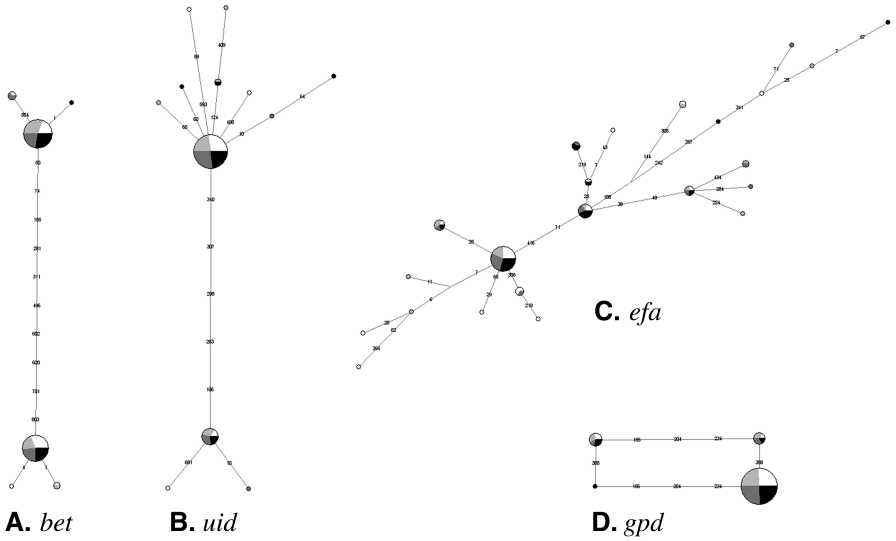


Fig. 2. Median-joining haplotype network for four fungal loci of the epiphytic lichen *Ramalina menziesii*: (A) beta-tubulin (*bet*), (B) unidentified fungal locus (*uid*), (C) translation elongation factor 1-α (*efa*), and (D) glyceraldehyde 3-phosphate dehydrogenase (*gpd*). Each circle corresponds to a DNA sequence haplotype, and the size of a circle is proportional to the haplotype's frequency. The numbers show the mutated position in the sequence alignment. The shading indicates the site where a particular haplotype was sampled: white, site 1; light gray, site 2; dark gray, site 3; black, site 4.

anticipate observing essentially such a lack of genetic structure in *R. menziesii*.

High gene flow has been reported in a variety of epiphytic species, including bryophytes, ferns, and orchids (Ranker, 1992; Akiyama, 1994; Trapnell and Hamrick, 2004; Korpelainen et al., 2005; Trapnell and Hamrick, 2005). In contrast, rare species of bryophytes, ferns, and orchids with small population sizes often have high genetic structure, presumably due to restricted gene movement (Akiyama, 1994; Ranker, 1994; Tremblay and Ackerman, 2001). Thus, the low genetic structure of *R. menziesii* is consistent with that of other widely distributed epiphytic species.

The local genetic structure of *R. menziesii* does not indicate host-plant specialization. At our study site, *R. menziesii* is much more abundant on *Quercus lobata* and *Q. douglasii* over *Q. agrifolia*, which may correspond to the differences in canopy microclimates among host species. However, that tendency does not influence the genetic structure, as measured by the four genes used in this study. Contrary to our results, genetic differentiation due to substrate has been shown in the ubiquitous lichen *Xanthoria parietina* on either bark or rock substrates (Lindblom and Ekman, 2006). When comparing spatially separated subpopulations on one substrate vs. the other, these authors found $\Phi_{ST} = 0.253$ for the IGS locus and $\Phi_{ST} = 0.273$ for the ITS locus. In our study, the host species were intermingled within each subpopulation. In *R. menziesii*, the widespread gene movement is likely to swamp out host plant specialization, even if there were any tendency for selection to differ among the environments associated with each host species.

TABLE 4. Results of AMOVA for each of four fungal nuclear loci of *Ramalina menziesii*. θ is an *F*-statistic based on haplotype frequencies (software TFPGA), whereas Φ is based on the genetic distance among DNA-sequence haplotypes (software Arlequin).

| Source | Locus | Φ | θ |
|---|---|---|---|
| Among hosts | *bet* | –0.0240 | –0.0116 |
| | *efa* | –0.0048 | –0.0074 |
| | *gpd* | –0.0060 | –0.0060 |
| | *uid* | –0.0170 | –0.0204 |
| Among sites | *bet* | –0.0219 | –0.0147 |
| | *efa* | –0.0212 | –0.0145 |
| | *gpd* | –0.0475 | –0.0475 |
| | *uid* | –0.0287 | –0.0287 |

*Abbreviations:* beta-tubulin (*bet*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*), translation elongation factor 1-α (*efa*), unidentified fungal locus (*uid*).

The genetic markers we used yielded high genetic diversity in *R. menziesii* at our oak–savanna landscape. It is difficult to conclude whether this level of diversity is typical or unusual for lichenized ascomycetes or other ascomycete taxa because few comparable data are available. In a local scale study of the saprophytic fungus *D. loculata*, of 33 samples, Johannesson et al. (2001) found four haplotypes in *efa* and 16 in *gpd*. In comparison, of 72 samples, we observed 23 *efa* haplotypes and four *gpd* haplotypes in *R. menziesii* reported here. The haplotype diversity of these two genes differs between the two studies, but the overall diversities are not too dissimilar. Lindblom and Ekman (2006) used two ribosomal DNA loci and their genetic diversity statistics are slightly higher than those of this study. The average haplotype diversity values for the two loci in their study were 0.700 and 0.750, respectively, while our average values range from 0.339 to 0.828 for the four nuclear genes. Their average nucleotide diversity values are 0.0046 and 0.0035, respectively, while the average subpopulation values for *R. menziesii* range from 0.0015 to 0.0064. Because the two studies do not have the same type of genes or the same sample size per population, comparisons are difficult, but *R. menziesii* seems to have the same order of magnitude of diversity. In general, the study by Lindblom and Ekman (2006) shows that genetic diversity of sequence variants can be quite high for lichen-forming fungi and that the results for our study are not unusual.

Given the lack of statistical significance in genetic structure, we considered whether this finding is spurious. Normally,

sample sizes of 18 individuals per site would be sufficient to detect low levels of genetic structure. However, the unexpectedly high allelic diversity at one of the loci (*efa*) would call for more individuals per populations to detect low levels of genetic structure. Nonetheless, the distribution of alleles among sites and the haplotype networks all indicate that the high frequency alleles are distributed evenly among sites in all loci, as one would expect for an unstructured local population. Thus, the biological interpretation of our study would not change even if we increased the sample sizes per subpopulation. It is likely that we would have detected genetic structure if we had sampled at a larger scale (i.e., among geographic locations >2 km apart). In future studies that sample populations of *R. menziesii* across a larger region, the spatial scale at which genetic differentiation occurs could be determined. Here, our focus was on the extent of genetic structure within a locality.

In our study, the frequency of unique and recurrent four-locus genotypes is consistent with a randomly mating population, suggesting that sexual reproduction predominates within our population. On the one hand, the presence of two four-locus genotypes with high recurrence (each were observed seven times) suggests the occurrence of asexual reproduction via thallus fragmentation. On the other, these recurrent genotypes were composed of high frequency alleles, making it likely that they resulted from sexual reproduction instead. This finding motivated us to look for other evidence of asexual reproduction in our study by examining the algal genome. In lichens, if a recurrent fungal multilocus genotype is associated with different photobiont genotypes, it would demonstrate that that the lichen was derived by sexual propagation and relichenization (i.e., the incorporation of an algal photobiont by a germinating fungal spore to form a lichen thallus). To test this possibility, we sequenced the photobiont's ITS region, a part of the nuclear ribosomal DNA cluster (S. Werth and V. L. Sork, unpublished data) and found that the two most frequent recurrent fungal four-locus genotypes with seven occurrences were each associated with six and five ITS haplotypes of the photobiont *Trebouxia decolorans* Ahmadjian (S. Werth and V. L. Sork, unpublished data). Thus, the photobiont sequence data provide compelling evidence for the predominance of sexual recombination in the lichen fungus, followed by incorporation of new algae via relichenization. Our algal sequence data also suggest that clonal reproduction is not frequent, but may occur occasionally in *R. menziesii* at the spatial scale of our sampling. Had we sampled at a smaller spatial scale (e.g., within branches or among neighboring branches), we may have observed far more clonality. The overall pattern of unrestricted gene movement of the fungus *R. menziesii* at our study area appears to apply at least to its sexually produced ascospores.

In conclusion, the genetic structure of *R. menziesii* within a single landscape suggests that this lichen is capable of extensive gene flow via widespread dispersal of sexually produced propagules, with the possibility of movement by some asexually produced propagules as well. Populations with more asexual reproduction may show more genetic structure. For instance, some coastal populations of *R. menziesii* had few fertile individuals, if any (S. Werth, unpublished data), suggesting that thallus fragmentation could be their predominant mode of reproduction. This widely distributed and common mutualistic species has low levels of local genetic structure and high effective gene movement that is comparable to other epiphytic species.

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APPENDIX 1. Haplotype maps for the four genes found in *Ramalina menziesii* indicating position, the site number, the consensus sequence (consensus), the type of polymorphism (v, transversion; t, transition), and the GenBank accession number (accession).

(A) Haplotype map for the locus beta-tubulin (*bet*), showing the position of DNA sequence polymorphisms in the 811-bp alignment.

| | | *bet* | |
|---|---|---|---|
| Position | | 123456678<br>57581900200<br>1404511642013 | |
| Site number | | 1111<br>1234567890123 | |
| Consensus | | TGTGGTTTGTTTG | |
| Site type | | vtttvttttttvv | |
| Character type | | i-iiiiii-iiii | |
| Haplotype | | | Accession |
| *bet1* | (29) | ..C..C.C.CCG. | EF377538 |
| *bet2* | ( 2) | A.C..C.C.CCG. | EF377539 |
| *bet3* | ( 1) | A..AC.C.....C | EF377540 |
| *bet4* | ( 1) | .AC..C.C.CCG. | EF377541 |
| *bet5* | (36) | ...AC.C.....C | EF377542 |
| *bet6* | ( 3) | ...AC.C.A...C | EF377543 |

(B) Haplotype map for the locus translation elongation factor 1-α (*efa*), showing the position of DNA sequence polymorphisms in the 494-bp alignment.

| | | *efa* | |
|---|---|---|---|
| Position | | 11222222333344<br>12234466674811246800491 3<br>67159838257148894274561464 | |
| Site no. | | 11111111112222222<br>12345678901234567890123456 | |
| Consensus | | GCCACCCCGCCTTCGCGAGATTCCGC | |
| Site type | | ttvtttvvvvvtttvvvtvvvvtvtt | |
| Character type | | ii-i-i-i---i-i---ii--ii-i- | |
| Haplotype | | | Accession |
| *efa1* | ( 4) | .....T.A..... | EF377548 |
| *efa2* | ( 1) | AT......T..C..........AA. | EF377549 |
| *efa3* | ( 1) | AT.G.......C..........A. | EF377550 |
| *efa4* | ( 1) | AT.........C..........A. | EF377551 |
| *efa5* | ( 1) | .T.G..G..... | EF377552 |
| *efa6* | ( 1) | .TG........C..........A. | EF377553 |
| *efa7* | ( 1) | .T.G......G..T...GT...T... | EF377554 |
| *efa8* | ( 5) | ...G.......C..........A. | EF377555 |
| *efa9* | ( 3) | ...G..........G..... | EF377556 |
| *efa10* | ( 1) | ...G.........T...GT...T... | EF377557 |
| *efa11* | ( 2) | ...G..... | EF377558 |
| *efa12* | ( 1) | ....T....G.C..........A. | EF377559 |
| *efa13* | ( 1) | ..........C..C......G..A. | EF377560 |
| *efa14* | ( 9) | ..... | EF377561 |
| *efa15* | ( 2) | ............CT......A..... | EF377562 |
| *efa16* | ( 1) | ............T...GT..... | EF377563 |
| *efa17* | ( 1) | ............T...GT...T... | EF377564 |
| *efa18* | ( 1) | ..........C.T...GT...T... | EF377565 |
| *efa19* | (28) | ..........C..........A. | EF377566 |
| *efa20* | ( 3) | ..........C.........G..A. | EF377567 |
| *efa21* | ( 1) | .....T.A........T..... | EF377568 |
| *efa22* | ( 1) | .....T.A..........C..... | EF377569 |
| *efa23* | ( 2) | .....T.A.....T | EF377570 |

(C) Haplotype map for the fungal, low-copy, nuclear gene glyceraldehyde 3-phosphate dehydrogenase (*gpd*), showing the position of DNA sequence polymorphisms in the 586-bp alignment (position).

| | | *gpd* | |
|---|---|---|---|
| Position | | 1223<br>5038<br>6448 | |
| Site no. | | 1234 | |
| Consensus | | TCTT | |
| Site type | | tvtt | |
| Character type | | iiii | |
| Haplotype | | | Accession |
| *gpd1* | (58) | C.CC | EF377544 |
| *gpd2* | ( 7) | .A.. | EF377545 |
| *gpd3* | ( 1) | .A.C | EF377546 |
| *gpd4* | ( 6) | C.C. | EF377547 |

(D) Haplotype map for the unidentified fungal locus (*uid*), showing the position of DNA sequence polymorphisms in the 704-bp alignment.

| | | *uid* | |
|---|---|---|---|
| Position | | 1122334456<br>156660289040998<br>090456438709831 | |
| Site no. | | 111111<br>123456789012345 | |
| Consensus | | CCGCTGAAAAAGCGT | |
| Site type | | vtvvvttttvtvttv | |
| Character type | | i----iiiiii---- | |
| Haplotype | | | Accession |
| *uid1* | (48) | ..... | EF377571 |
| *uid2* | ( 1) | G..... | EF377572 |
| *uid3* | ( 1) | G..G..... | EF377573 |
| *uid4* | ( 1) | G....A.GGTG.... | EF377574 |
| *uid5* | ( 1) | .T.....A. | EF377575 |
| *uid6* | ( 1) | ..T..... | EF377576 |
| *uid7* | ( 1) | ....A..... | EF377577 |
| *uid8* | (13) | .....A.GGTG.... | EF377578 |
| *uid9* | ( 1) | .....A.GGTG...G | EF377579 |
| *uid10* | ( 2) | ......G..... | EF377580 |
| *uid11* | ( 1) | ......G....C... | EF377581 |
| *uid12* | ( 1) | ............T.. | EF377582 |