Fungal taxonomy / Taxonomie fongique

Genetic diversity and population structure of *Valdensinia heterodoxa*, a potential biocontrol agent for salal in coastal British Columbia

Jennifer E. Wilkin, Simon F. Shamoun, Carol Ritland, Kermit Ritland, and Yousry A. El-Kassaby

Abstract: *Valdensinia heterodoxa* is an ascomycete fungus currently being considered as a potential biocontrol agent against salal (*Gaultheria shallon*). To assess its effectiveness and risks as a biocontrol agent, we investigated its population structure in three geographically separate populations on Vancouver Island and in coastal mainland, British Columbia. *Valdensinia heterodoxa* was cultured from infected salal leaves, and single-spore cultures were obtained prior to DNA isolation. Amplified fragment length polymorphisms (AFLPs) were used to generate individual DNA fingerprints for each *V. heterodoxa* isolate. We found low genetic diversity, with just 30 of the 214 loci being polymorphic. There were many shared haplotypes within each population, and analysis of pairwise kinship coefficients showed that as spatial distance increased, genetic similarity decreased. Analysis of molecular variance among populations revealed significant genetic differentiation, with an F_{ST} of 0.182, suggesting limited gene flow. The findings suggest that with low diversity and high population differentiation, the effectiveness of *V. heterodoxa* as a biocontrol agent to manage salal in forested areas may be limited to localized salal populations or to use in combination with other control methods.

Key words: Valdensinia heterodoxa, amplified fragment length polymorphism, genetic diversity, population structure, biocontrol, *Gaultheria shallon*, salal.

Résumé : Présentement, le champignon ascomycète *Valdensinia heterodoxa* est considéré un possible agent de lutte biologique contre la gaulthérie shallon (*Gaultheria shallon*). Afin d'évaluer son efficacité et les risques liés à son utilisation en lutte biologique, nous avons étudié la structure de sa population chez trois populations géographiquement éloignées, sur l'île de Vancouver et sur le littoral continental de la Colombie-Britannique. Le *V. heterodoxa* fut isolé de feuilles de gaulthérie shallon infectées, et des cultures monosporées furent obtenues avant de procéder à l'isolement de l'ADN. Le polymorphisme de taille des fragments amplifiés fut utilisé pour générer des empreintes d'ADN individuelles pour chaque isolat de *V. heterodoxa*. Nous avons trouvé peu de diversité génétique, seulement 30% des 214 loci étant polymorphes. Chaque population comprenait plusieurs haplotypes communs, et l'analyse par paire des coefficients de parenté montra que la similarité génétique décroît avec l'augmentation de l'éloignement géographique. L'analyse de la variance moléculaire parmi les populations révéla une différentiation génétique significative, avec un F_{ST} de 0,182, ce qui suggère un flux génétique limité. À cause d'une faible diversité et d'une forte différentiation des populations, les résultats portent à croire que l'efficacité du *V. heterodoxa* comme agent de lutte biologique contre la gaulthérie shallon en régions forestières serait restreinte aux populations locales ou à l'utilisation en combinaison avec d'autres méthodes de lutte.

Mots clés : Valdensinia heterodoxa, polymorphisme de taille des fragments amplifiés, diversité génétique, structure des populations, lutte biologique, *Gaultheria shallon*, gaulthérie shallon.

Accepted 2 August 2005.

J.E. Wilkin, C. Ritland, K. Ritland, and Y.A. El-Kassaby. Department of Forest Sciences, University of British Columbia, 2424 Main Mall, Vancouver, BC V6T 1Z4, Canada.
S.F. Shamoun.[1] Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, 506 West Burnside Road, Victoria, BC V8Z 1M5, Canada.

[1]Corresponding author: (e-mail: sshamoun@pfc.cfs.nrcan.gc.ca).

Introduction

Valdensinia heterodoxa Peyronel (Sclerotiniaceae; teleomorph *Valdensia heterodoxa*[2]) is a parasitic fungus that causes necrotic leaf spot and defoliation of its hosts. It infects at least 33 plant species in Europe and 17 plant species in North America (Aamlid 2000; Redhead 1979). The most common hosts are ericaceous shrubs that grow under moist to wet conditions in low to subalpine conditions. *Gaultheria shallon* Pursh., commonly known as salal, is a densely growing perennial evergreen ericaceous shrub native to the Pacific Northwest, occurring from California to Alaska (Fraser et al. 1993). In British Columbia, the vigorous growth of salal in harvested regeneration sites has become a problem because it competes with commercially valuable conifers for light and soil resources, including nutrients and moisture, and slows their early establishment.

Much research has focused on trying to determine a reliable, cost-effective method to control salal (Prescott 1999). Biological control may provide an alternative to traditional control methods such as herbicide treatment, burning, manual brushing, or other means of scarification. A natural pathogen to salal in British Columbia, *V. heterodoxa*, has been identified as a potential biocontrol agent for salal in managed forests (Shamoun et al. 2000). To control salal, large amounts of an indigenous pathogen (mycoherbicide) would be applied to the target plant, causing disease onset and suppression of the host (Wall et al. 1992). Much work has been done to investigate the biology, conidia discharge, inoculum production, and delivery techniques for *V. heterodoxa* (Shamoun and Vogelgsang 2002; Vogelgsang and Shamoun 2002, 2004).

This study investigates the patterns of genetic diversity in natural populations of *Valdensinia heterodoxa* (the anamorphic state of the pathogen), in the context of biocontrol, to help predict the effects of releasing the agent into the environment. The anamorphic state of the pathogen consists of multicelled, star-shaped, asexual macroconidia, termed stauroconidia (Redhead and Perrin 1972a, 1972b). Norvell and Redhead (1994) have suggested that dispersal of *V. heterodoxa* occurs primarily through these asexual stauroconidia. Spore discharge is facilitated by moisture, which causes the appendages to swell and form a tear-dropped shape that presses against the substrate (i.e., leaf), propelling the propagule to a height of 20 cm. Germination occurs radially through short appressoria (swollen hyphae) that adhere to the host and promote infection (Redhead and Perrin 1972a; Norvell and Redhead 1994). Infecting conidia often remain attached to the center of brown, concentrically ringed necrotic leaf spots; mature infections are characterized by sclerotia on the host veins, followed by leaf death and defoliation.

The teleomorph (*Valdensia heterodoxa*[2]) is characterized by small, stalked, brown apothecia that arise from sclerotized veins of fallen leaves and that release ascospores, which, while rarely observed in nature, are thought to produce the initial inoculum in the spring (Norvell and Redhead 1994). Secondary, single-celled spores reported by Redhead and Perrin (1972a) in the laboratory were originally thought to be asexual phialospores. However, further research placed *V. heterodoxa* in the Sclerotiniaceae (Holst-Jensen et al. 1997, 2004), suggesting that these spores are spermatia, i.e., haploid spores that are part of the sexual cycle (Drayton and Groves 1952). The type of life cycle proposed for *V. heterodoxa* by Norvell and Redhead (1994) follows the epidemic model, which lies between the extremes of strict clonality and random mating (Maynard Smith et al. 1993). Annual sexual cycles provide frequent recombination, followed by an asexual epidemic resulting in a few successful individuals reproducing clonally and occurring at high frequencies in the population.

In addition to the type of reproductive cycle, the levels of genetic variability, local differentiation and adaptation to hosts, and gene flow among populations can potentially affect the dynamics of both host and natural-pathogen populations. For proposed biocontrol agents, Canadian federal regulations require information based upon the most up-to-date approaches and methodology (Health Canada 2001). Thus, the objectives of this research were to: (1) investigate the level of genetic diversity in *V. heterodoxa* populations of coastal British Columbia, (2) determine the genetic population structure and assess the evolutionary forces affecting these populations, and (3) discuss the implications of these results in relation to the potential use of *V. heterodoxa* as a biocontrol agent for salal in managed coastal British Columbia forests.

Materials and methods

Site description and sampling strategy

Salal leaves infected with *V. heterodoxa* were collected from Mesachie Lake (ML) and Port Hardy (PH) on Vancouver Island and from Deak's Peak (DP) on coastal mainland, British Columbia, during the spring of 2002 (Fig. 1). A "population" consisted of samples collected from one site.

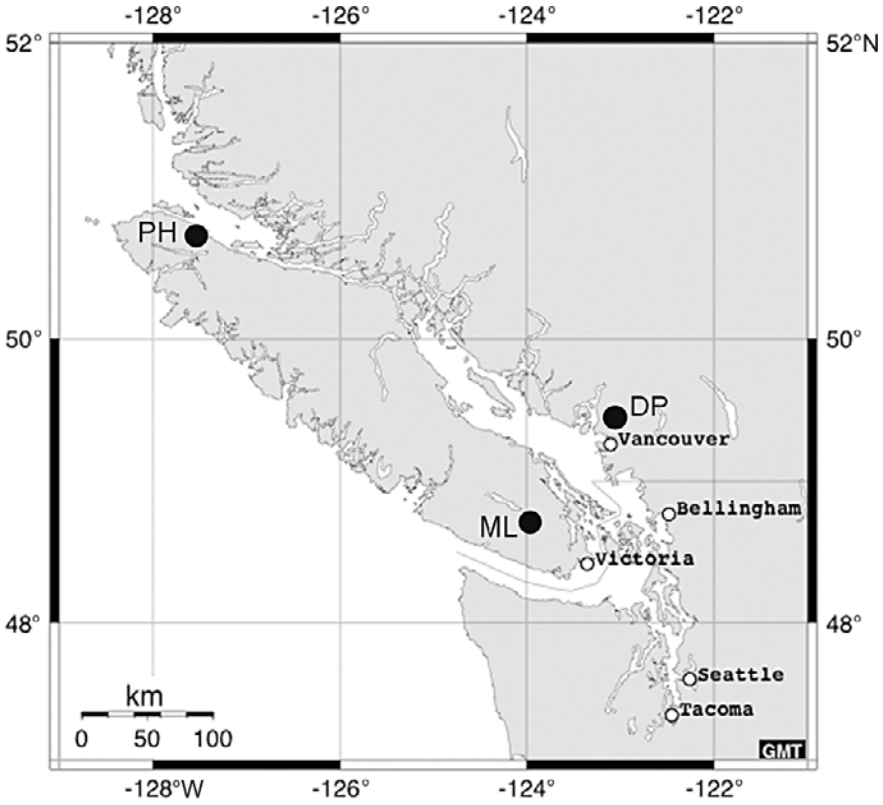
Because diseased plants displayed a patchy distribution in natural populations, samples were collected according to this natural distribution. Moving along a transect, two to three infected salal leaves were collected from each of five salal stems within a 1-m radius in infection centres (patches). To ensure that different individuals were sampled, salal stems were collected at least 20 cm apart, a distance greater than the documented vertical spore-dispersal distance for *V. heterodoxa* (Redhead and Perrin 1972a). For each population, 10 patches of infected salal were sampled, and within each patch, five different salal stems were sampled, resulting in 50 samples per site.

Isolation procedure

Diseased tissues (0.33 cm^2) were surface sterilized in 95% ethanol for 45 s., followed by 10% bleach for 45 s, and then transferred through three successive rinses of sterile distilled water for 1 min each. Leaf pieces were placed on potato dextrose agar (PDA, Difco®, Becton Dickinson, Md., USA) and incubated at 20 °C in the dark. Plugs from the growing edge of the colony were transferred to salal PDA (SPDA: PDA, 39 g/L; salal extract, 15 g/L. The salal

[2] The abbreviated form *V. heterodoxa* will subsequently refer to the anamorph (*Valdensinia heterodoxa*), while the teleomorph will be referred to by its full name.

Fig. 1. Map of Vancouver Island and mainland British Columbia showing locations of the three *Valdensinia heterodoxa* populations sampled for this study, including Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP). GMT, Greenwich meridian time.



extract consisted of 15 g of salal leaves pureed in blender with deionized water (dH_2O) and filtered through cheesecloth; the final volume adjusted to 1 L with dH_2O]. Cultures were incubated at 20 °C in the dark until sufficient *V. heterodoxa* growth was reached.

Once putative *V. heterodoxa* cultures, displaying typical discolouration of the medium and slow-growing white mycelium, were obtained, sporulation was induced by transferring them to weak-oatmeal agar (oatmeal agar, 15 g/L; agar, 12 g/L; Sigma–Aldrich, Mo., USA) (Vogelgsang and Shamoun 2002). Single star-shaped spores were isolated under a dissecting microscope and transferred to SPDA plates. Hyphal tips were then transferred to individual plates. From each diseased leaf sample that sporulated, three single-spore isolates were sampled for this study, each considered to be separate genetic individuals. While 50 samples of infected leaves collected from field populations were expected to yield 150 *V. heterodoxa* individuals per population, only 60 single-spore individuals were collected from ML, 36 from PH, and 52 from DP because not all cultures sporulated.

DNA isolation

Fungal cultures were initially incubated in liquid media to generate mycelium for DNA isolation. However, with *V. heterodoxa*, this resulted in high polysaccharide contamination and DNA that was unsuitable for fingerprinting. Subsequently, cultures were grown on cellophane laid over agar plates, adapted from Arroyo Garcia et al. (2002) and Nielsen et al. (2001). Semi-permeable cellophane (BioRad, Hercules, Calif., USA) was autoclaved and placed overtop SPDA plates, using 500 µL of sterile dH_2O to adhere the cellophane to the plates by surface tension. Plugs of the single-spore isolates were inoculated onto the cellophane plates and incubated in the dark at 20 °C for 5 days. The resulting mycelial layer was very thin, dry, and papery. Mycelium (10–40 mg) was transferred to a 1.5-mL Eppendorf tube and immediately frozen in liquid nitrogen prior to storage at –80 °C.

DNA was isolated according to a modified Zolan and Pukkila (1986) miniprep cetyltrimethylamonium bromide (CTAB) protocol that was previously applied for sclerotineaceous fungi (Holst-Jensen et al. 1997). Optimizing this protocol for *V. heterodoxa* resulted in high-quality-DNA recovery and low contamination. Using a small amount of starting material (20 mg of mycelia), and liquid nitrogen to lightly crush the mycelium once, was critical to the success of this protocol. Any further grinding resulted in polysaccharide contamination from which the DNA could not be separated. An isolation buffer was added to the ground tissue and consisted of: CTAB, 2.5%; polyvinyl pyrrolidone 40 (PVP-40), 1%; NaCl, 1.4 mol/L; ethylenediaminetetraacetic acid (EDTA), 0.02 mol/L; Tris–HCl, 0.1 mol/L; β-mercaptoethanol, 0.5%; Sigma–Aldrich, Oakville, Ont., Canada. The tissue was then vortexed for 15 s and incubated for 30 min at 60 °C. An equal volume of chloroform – isoamyl alcohol mixture 24:1 was added to the tissue – buffer solution to extract DNA, and the two combined solutions were vortexed until mixed, then centrifuged at room temperature for 5 min at 12 000 r/min (1 r = 2π rad; 15 000*g*) to separate the layers. To reduce polysaccharide

and protein contamination, only the top portion of the aqueous layer was transferred to a new test tube. DNA was precipitated with ice-cold isopropanol, and the supernatant was pipetted from the diffuse pellet, which was often associated with a clear viscous gel (polysaccharides). The pellet was resuspended in 300 µL of TE buffer (Tris–CL, 10 mmol/L, pH 8.0, and EDTA, 1 mmol/L). RNA was digested with RNase A (final concentration of 1 mg/mL, Sigma), followed by a second chloroform extraction. The aqueous phase was collected, and ammonium acetate (5 mol/L) was added to achieve a final concentration of 1.5 mol/L. DNA was precipitated by adding 2× volume of ice-cold 95% ethanol. The resulting pellets were rinsed in ice-cold 70% ethanol. The supernatant was poured off, leaving a small pellet that was dried and then resuspended overnight in 40 µL TE at 4 °C. The concentration and quality of samples was determined by electrophoresis on 2% agarose gels, with *Hin*dIII-digested λ DNA as the molecular weight marker.

Amplified fragment length polymorphism analysis

A list of primers was generated from a literature search (e.g., Cilliers et al. 2000; Gonzalez et al. 1998; Majer et al. 1996) and tested on *V. heterodoxa*; only primers producing clear banding patterns and polymorphisms were selected for use. To ensure reproducibility, each *V. heterodoxa* sample was analyzed twice and only highly repeatable loci were included in the analysis. For *V. heterodoxa* samples, 5 µL of total DNA was used in each amplified fragment length polymorphism (AFLP) reaction. The DNA was digested with *Eco*RI and *Mse*I, and AFLP was performed following the protocol developed by C. Ritland (Genetic Data Centre, University of British Columbia). The final polymerase chain reaction (PCR) amplification was performed with +2 *Eco*RI/+3 *Mse*I primers. Four different primer combinations were employed as follows, where the first primer refers to *Eco*RI and the second refers to *Mse*I: AC/CAC, AC/CAG, AG/CAC, and AG/CAG. The PCR-generated fragments were separated by electrophoresis with a LI-COR 4200 autosequencer (LI-COR Inc., Lincoln, Nebr., USA). Gels were analyzed with SAGA-MX for AFLP (LI-COR Inc.), and bands were scored as present or absent. Raw AFLP data is available upon request from the corresponding author.

Data analysis

Valdensinia heterodoxa AFLP fingerprints were analyzed with Arlequin, version 2.0 (Schneider et al. 2000), and SPAGeDi, version 1.1 (Hardy and Vekemans 2002). To compare fingerprints between gels and among populations, it was assumed that comigrating fragments of the same size were identical in sequence. Pearson correlations from principal components analysis (PCA) in Statistical analysis system, version 8.0 (SAS Institute Inc. 1999), was used to select only those loci that contributed to the observed variation. Allele frequencies were then calculated for each locus as the frequency of the observed allele over all samples. Average gene diversity (the probability that two randomly chosen individuals have different alleles), equivalent to expected heterozygosity, was determined after correcting for sample size (Schneider et al. 2000). Genotype diversity, or number of genetically distinct individuals in a population (McDonald 1997), was equivalent to haplotype diversity in this species and is the number of unique haplotypes relative to the number of individuals in each population.

Distribution of haplotypes and linkage disequilibrium were analyzed to assess the level of clonality. Analysis of linkage disequilibrium between pairs of loci was performed in Arlequin for each population separately and for pooled samples. The number of pairwise comparisons was calculated as $n(n-1)/2$, where n is the number of loci. To establish a clonal population structure, the null hypothesis of random association of loci must be rejected for the majority of loci pairs (Kohli and Kohn 1998), relying on a modification of Fisher's exact probability test with Bonferroni's correction for multiple comparisons. A clonal population structure may be falsely detected if recombination is masked by recurring haplotypes. Therefore, clone-corrected data sets, where each haplotype was represented only once, were used to account for this.

The genetic structure of the *V. heterodoxa* populations was investigated with the analysis of molecular variance (AMOVA), using Arlequin (Excoffier et al. 1992). Comparisons were made among the three populations, and nested analyses were used to examine the genetic structure imposed by the patchy distribution of *V. heterodoxa*. Population differentiation was estimated from F_{ST}, the correlation coefficients of alleles for genes within populations relative to all populations, to determine the amount of genetic variability among populations (Weir and Cockerham 1984; Hardy and Vekemans 2002). S refers to subpopulations (in this case, the three populations in the study), and T refers to total population (all three populations combined). Pairwise population F_{ST} values were also compared.

Correlations between genetic variation and geographic distance, matrices of population pairwise F_{ST}, Nei's D (Nei 1972), and spatial distance were estimated using SPAGeDi. The Mantel option in Arlequin was then selected to test for isolation by distance. Within populations, individual pairwise spatial and genetic distances, calculated as pairwise kinship coefficients according to Ritland (1996), were determined in SPAGeDi. Pearson correlations were used to test whether genetic similarity within populations was related to spatial distance.

Results

Genetic diversity

The four primer pairs studied yielded 214 scorable loci, of which 65 were polymorphic, resulting in low overall diversity of 30.4% (Table 1). Figure 2 shows a typical *V. heterodoxa* AFLP gel with many strong, reproducible, monomorphic loci. The percentages of polymorphic loci in each population were 25.2 (54/214) in ML, 23.8 (51/214) in PH, and 24.7 (53/214) in DP, when based on all 214 loci, compared with 83.1 (54/65) in ML, 78.5 (51/65) in PH, and 81.5 (53/65) in DP, when based only on polymorphic loci.

To determine the extent of genetic diversity within each population, PCA was performed on the 65 polymorphic loci for each population, and a matrix of Pearson correlations between the principal components and loci was generated. Loci with high loadings (correlations > 0.55) on principal components with eigenvalues > 1 contributed the most to the variation described by the principal components and

Table 1. Summary of the repeatable and polymorphic loci of *Valdensinia heterodoxa* from three populations of British Columbia, Canada.

	Number of loci[a]		
Primer pair	Total	Polymorphic	% Polymorphic loci
AC/CAC	54	13	24.1
AC/CAG	49	15	30.6
AG/CAC	61	23	37.7
AG/CAG	50	14	28.0
Total	214	65	30.4

[a]Only loci with allele frequencies greater than 0.05, or less than 0.95, were included in the analysis to reduce statistical bias introduced by very rare or very frequent loci.

were selected for analyses. The Pearson correlations from PCA reduced the 65 loci to 39 in ML, 40 in PH, and 42 in DP, explaining 92.8%, 90.0%, and 87.1% of the variation, respectively. Different alleles were used for within-population analyses to avoid underestimating the level of gene diversity by including less informative loci. Figure 3 shows the distribution of allele frequencies within each population. The average gene diversity (H_e) was low to moderate within each population, ranging from 0.314 ± 0.160 (ML) and 0.338 ± 0.171 (DP) to 0.410 ± 0.208 (PH); this was expected because PH also had the highest haplotype diversity.

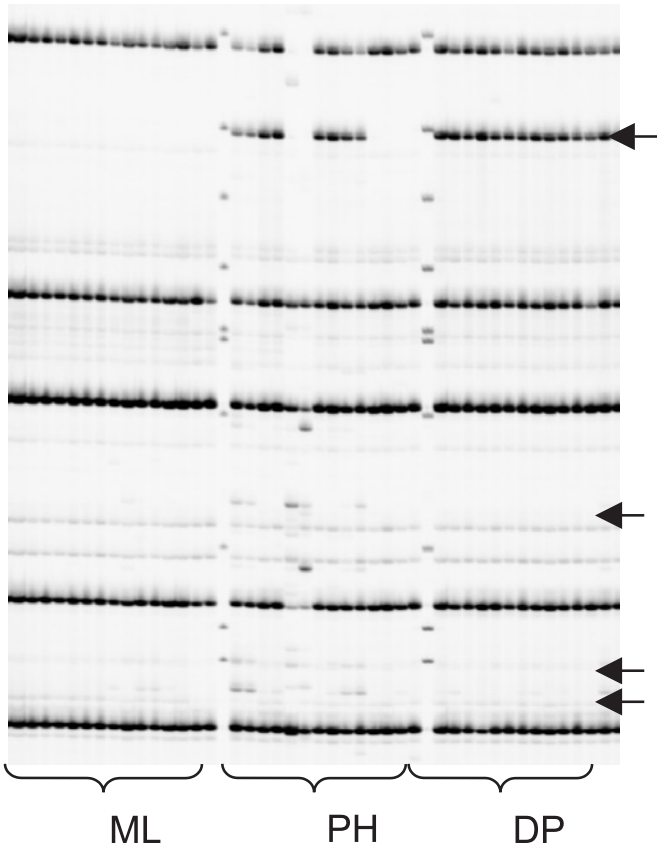
To compare the amount of genetic diversity among populations, PCA was performed again with all populations combined, and the 65 polymorphic loci resulted in 15 principal components with eigenvalues > 1, explaining 83.6% of the variation. Pearson correlations between the 15 principal components and the 65 polymorphic loci showed that 32 of these loci had loadings greater than 0.55, and therefore, contributed the most to the variation observed in the principal components. These 32 loci were incorporated in all population-level analyses unless otherwise specified.

Allele frequencies varied considerably among loci, although most loci had allele frequencies < 0.3, and relatively few loci had frequencies > 0.7, particularly for DP. PH had the most alleles with moderate frequencies (0.4–0.6). The average gene diversity over all loci was low, where ML had the lowest (0.231 ± 0.121), PH had the highest (0.288 ± 0.150), and DP was intermediate (0.263 ± 0.137).

Low haplotype diversity, or high similarity, was observed within and among populations. The removal of loci increased the level of similarity recorded and decreased sensitivity: more shared haplotypes were detected when the number of loci was reduced from 65 to 32 (Table 2). With 32 loci, ML had the lowest haplotype diversity (46.7%), while PH, although having a number of haplotypes comparable with those noted in other populations, had the highest haplotype diversity (80.6%) (Table 2). The difference between the observed and expected haplotype diversities was significant (χ^2 test, $p < 0.05$). With 65 loci, no haplotypes were shared among populations; however, when 32 loci were analyzed, haplotype 50 occurred numerous times in both PH and DP, and haplotype 11 was found repeatedly in all three populations (Table 3).

Haplotype diversity can be further assessed by comparing the number of shared *V. heterodoxa* haplotypes isolated from each salal stem collected. For example, in 8 of the 21 salal stems sampled from ML, all three *V. heterodoxa* individuals collected from the same salal stem had identical haplotypes, while only 4 stems shared no common *V. heterodoxa* haplotypes. The average percentages of shared haplotypes for *V. heterodoxa* individuals from the same salal stem were 68.3 for ML, 53.0 for DP, and 19.5 for PH.

Fig. 2. Section of a typical amplified fragment length polymorphism (AFLP) gel emphasizing the strong, monomorphic loci observed in all three populations of *Valdensinia heterodoxa* at Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada. Lanes between populations are markers (50- to 700-bp molecular size). Arrows indicate polymorphic loci.



Population structure

In all three populations, geographically proximal individuals had high kinship coefficients (Fig. 4), and kinship declined with distance. A steep drop-off in correlations past 1.5 m, particularly in DP, showed that individuals were more genetically similar within patches than among patches.

The distribution of haplotypes within a population can indicate the degree of population structure and further clarify the relationship between genetic similarity and geographic distance. For each population, individuals were plotted on X and Y coordinates, according to their locations within each population, and each unique haplotype was given a number (Table 3). Haplotypes were usually shared among isolates from the same salal stem and within sampling patches (see Fig. 5 as an example). In ML, all individuals in the last patch of the population shared haplotype 28, compared with

Fig. 3. Distribution of allele frequencies for within-population analyses of three populations of *Valdensinia heterodoxa* from British Columbia, Canada. The number of loci used for each population were: Mesachie Lake (ML), 39; Port Hardy (PH), 40; and Deak's Peak (DP), 42.

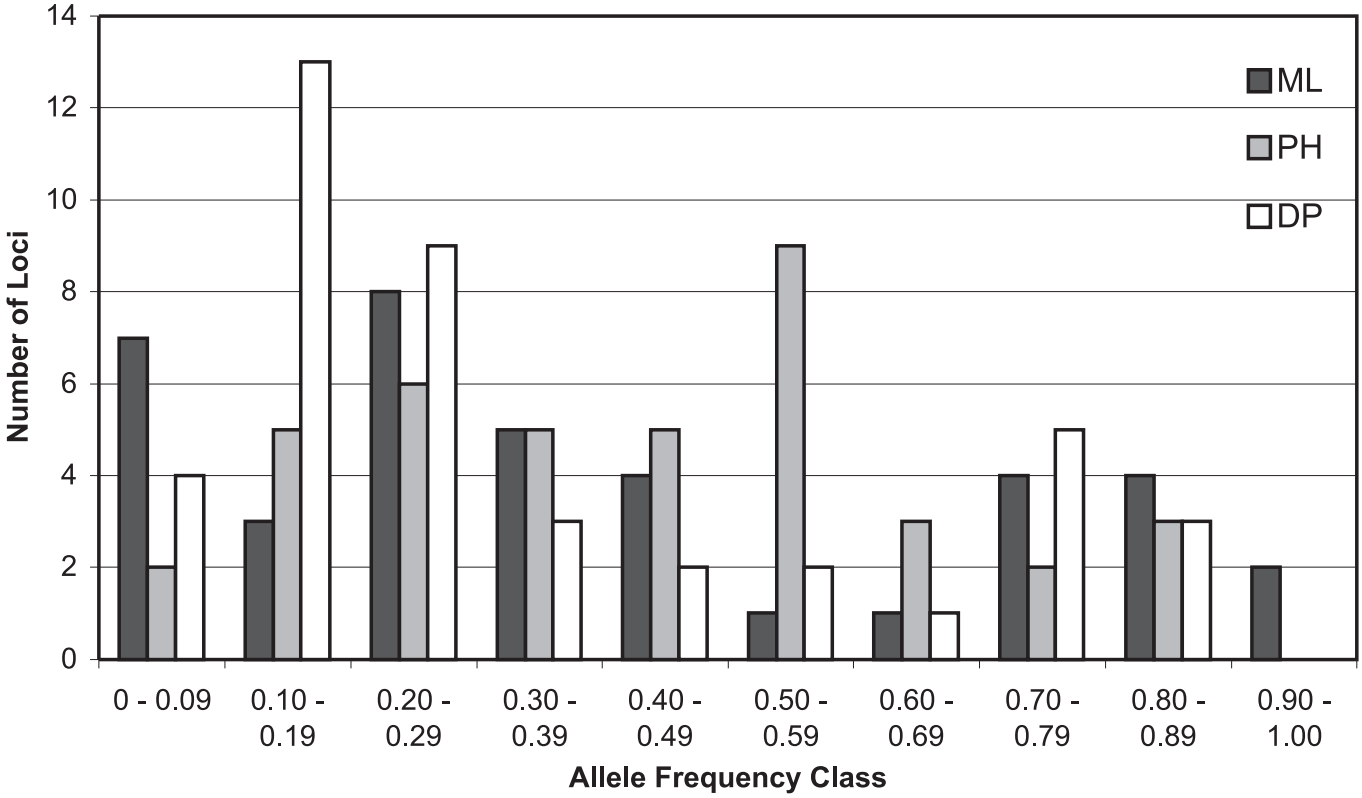


Table 2. Comparison of the number of unique haplotypes, and of haplotype diversity, for 65 and 32 AFLP loci, in three populations of *Valdensinia heterodoxa* from Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada.

		Haplotype diversity (%)	
Population, *n*	Number of unique haplotypes	Observed	Expected
65 Loci			
ML, 60	38	63.3	97.6±0.8
PH, 36	33	91.7	99.5±0.8
DP, 52	41	78.8	98.9±0.6
Total, 148	112	75.7	99.5±0.2
32 Loci			
ML, 60	28	46.7	95.3±1.3
PH, 36	29	80.6	98.6±1.1
DP, 52	32	61.5	96.1±1.4
Total, 148	86[a]	58.1	98.7±0.3

Note: AFLP, amplified fragment length polymorphism.
[a]The sum of unique haplotypes over all three populations is 89, but taking into account the two haplotypes shared between populations, the actual total number of haplotypes is 86.

individuals in the middle patch, which had four different haplotypes (Fig. 5).

To assess the level of population structure and genetic differentiation among populations, PCA was performed, and ordination of the first two principal components, which accounted for 25.8% of the variance, revealed somewhat isolated clusters from each population as well as a cluster comprising mainly individuals from populations PH and DP (Fig. 6). As well, significant variation was detected among populations, with an F_{ST} value of 0.182 (AMOVA, $p < 0.0001$), where 81.8% of the variation was within populations (Table 4). An additional nested AMOVA was conducted to assess the genetic structure imposed by the patchy distribution of *V. heterodoxa* (Table 4). Samples from 27 patches were obtained over all three populations. When AMOVA was repeated to include this patchy distribution, only 11.7% of the variation was due to differences among populations, 50.3% of the variation occurred among groups within populations, and 38.0% of the variation was within groups ($p < 0.0001$, Table 4). While there were significant differences among populations, most of the variation was due to differences among groups within populations.

Population pairwise F_{ST} and Nei's D showed that there were significant differences among all populations ($p < 0.0001$). The greatest differences were between ML and PH ($F_{ST} = 0.266$, $D = 0.103$) whereas PH and DP were the most similar ($F_{ST} = 0.116$, $D = 0.063$, Table 5). When comparing spatial distances, ML and DP were the closest (1.152°), followed by ML and PH (3.842°), and PH and DP were the furthest (4.412°, Table 5). However, ML and PH are both on Vancouver Island and are separated from DP by the Straight of Georgia, suggesting that populations are more differentiated north to south than east to west. The re-

Table 3. List of haplotypes and their frequencies, based on 32 loci, observed in each of three populations of *Valdensinia heterodoxa* from Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada.

Mesachie Lake		Port Hardy		Deak's Peak	
Haplotype No.	Frequency	Haplotype No.	Frequency	Haplotype No.	Frequency
1	3	11[a]	2	11[a]	5
2	2	29	1	50[a]	7
3	1	30	1	57	1
4	1	31	1	58	2
5	1	32	2	59	1
6	2	33	1	60	1
7	1	34	1	61	1
8	2	35	1	62	1
9	3	36	2	63	3
10	1	37	1	64	1
11[a]	4	38	1	65	1
12	1	39	1	66	1
13	2	40	1	67	1
14	3	41	1	68	1
15	1	42	3	69	1
16	3	43	3	70	1
17	5	44	1	71	2
18	1	45	1	72	1
19	1	46	1	73	1
20	1	47	1	74	1
21	6	48	1	75	1
22	1	49	1	76	1
23	1	50[a]	1	77	1
24	1	51	1	78	1
25	1	52	1	79	1
26	1	53	1	80	2
27	1	54	1	81	1
28	9	55	1	82	1
		56	1	83	6
				84	1
				85	1
				86	1
Total number of haplotypes					
28		29		32	
Total frequency					
60		36		52	

[a]Haplotype occuring in more than one population.

sults of a Mantel test comparing spatial distances to pairwise F_{ST} values over 1000 permutations were not significant ($p = 0.678$).

Linkage disequilibrium

Linkage disequilibrium characterizes the extent to which alleles are correlated among loci. In clonal populations compared with nonclonal populations, high linkage disequilibrium is expected since recombination reduces linkage disequilibrium in nonclonal populations. Table 6 shows the number of pairwise associations observed in each of the three populations. Less than 20% of the comparisons were significant (i.e., linked); ML had the fewest unlinked loci (Table 6). Tests on the pooled data set showed that the majority of loci were linked across all populations (Table 6). The number of significant associations between pairs of loci was greater when all samples were used than in the clone-corrected subset.

Discussion

Evidence for clonality

The abundance of identical haplotypes detected within populations of *V. heterodoxa* is a strong indication of clonal population structure (Taylor et al. 1999). From the AFLP fingerprints generated in this study, 36 haplotypes were identified. Relatively few loci had distinctive haplotypes, so that if these differences arose through mutation, the actual number of haplotypes would be even less. It has been suggested that mutation rate is elevated in predominantly clonal

Fig. 4. Scattered plots and Pearson correlations (r) of pairwise kinship coefficients and geographic distance for three populations of *Valdensinia heterodoxa* from British Columbia, Canada: (a) Mesachie Lake. (b) Port Hardy. (c) Deak's Peak. All correlations were significant ($p < 0.0001$).

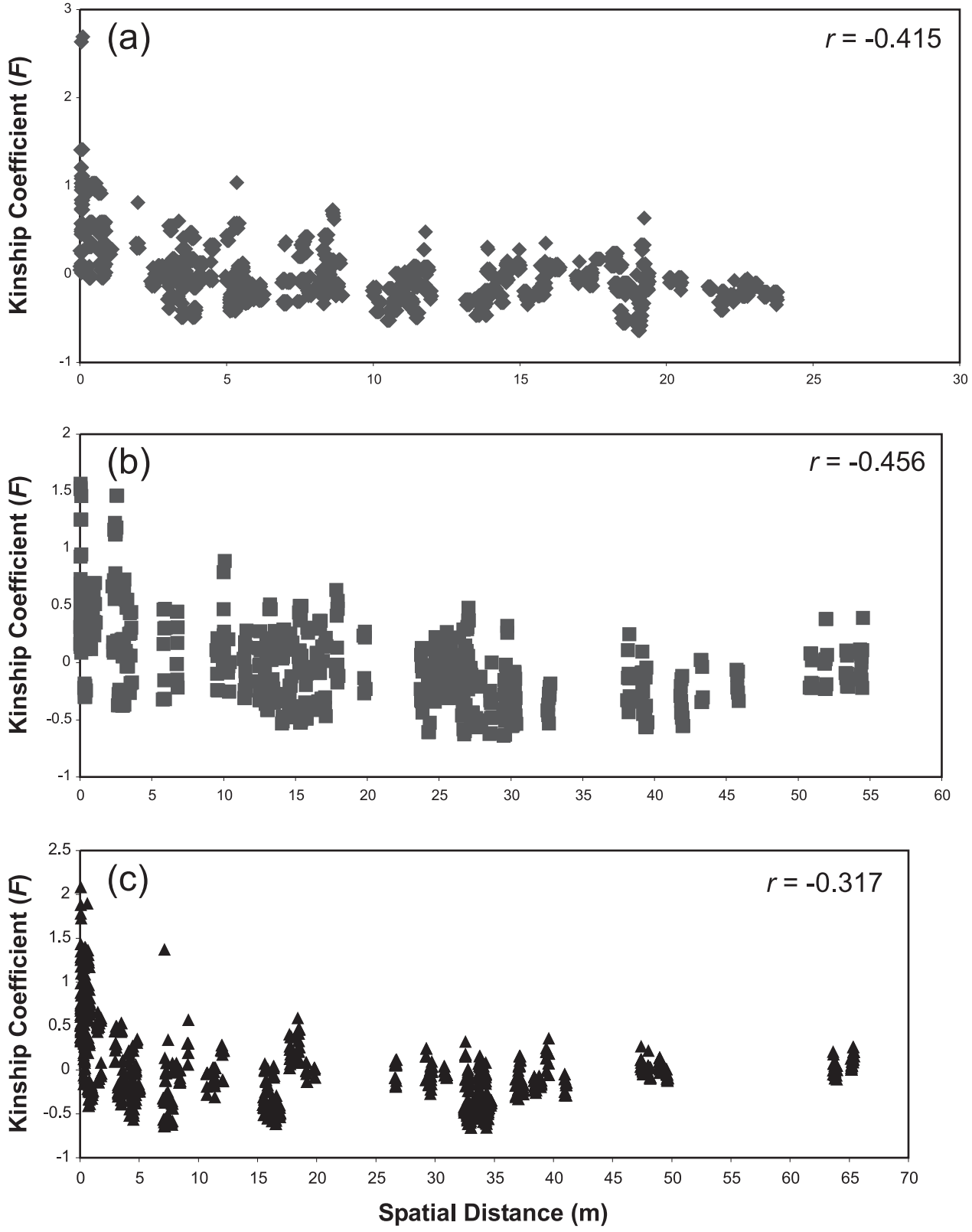


Fig. 5. Map of *Valdensinia heterodoxa* population from Mesachie Lake, British Columbia, Canada, showing the location of each haplotype with a distinctive symbol. Clusters of haplotypes reflect naturally occurring patches. Haplotypes were based on the 32 loci used to analyze all populations. Numerical labels refer to identification numbers of haplotypes, and their repetition reflects the frequency of each haplotype (see Table 3).

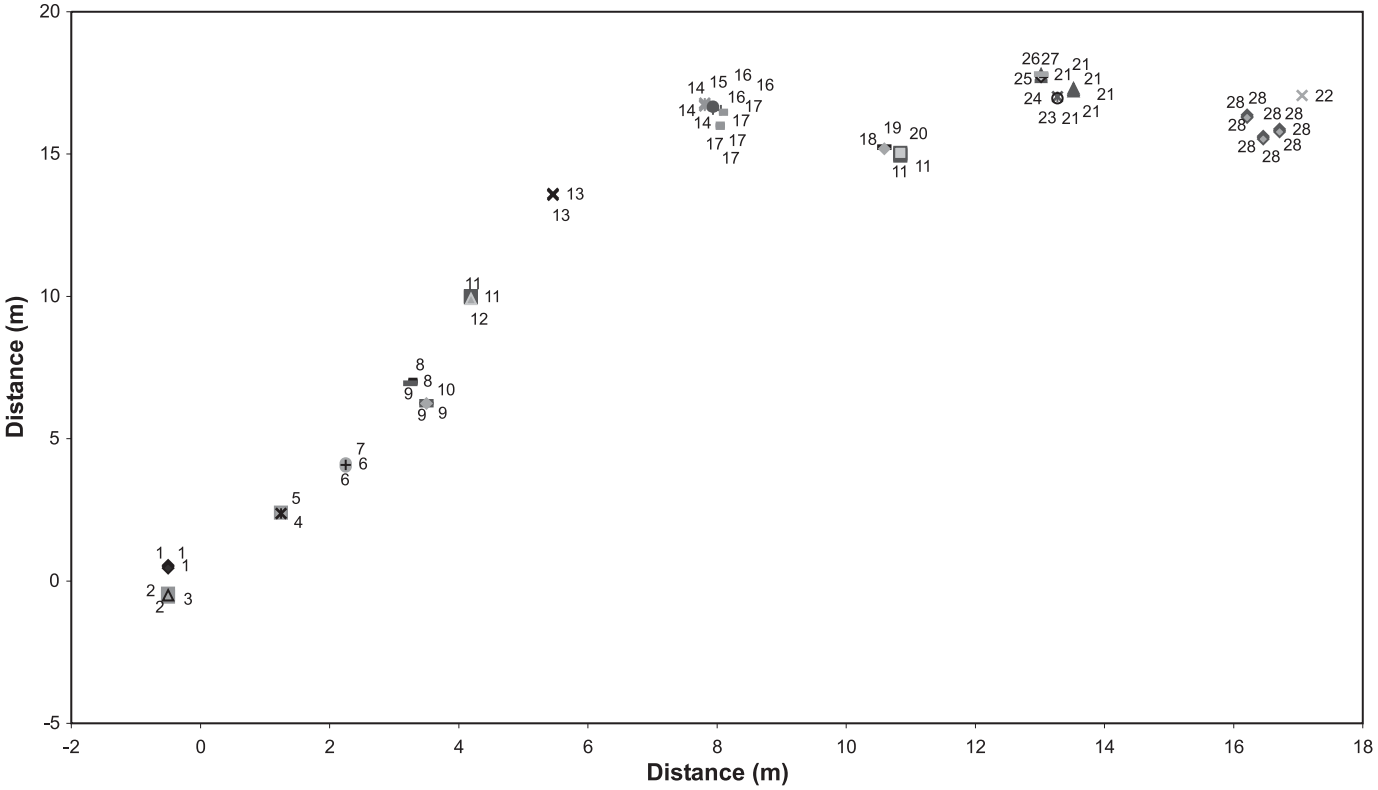
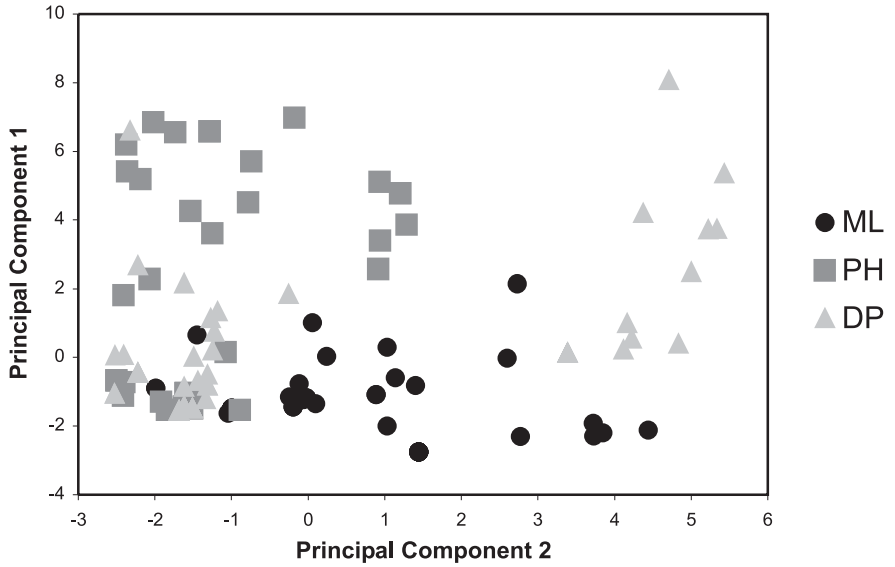


Fig. 6. Ordination of the first two principal components explaining 38.2% of the variation observed on 32 polymorphic loci over all three populations of *Valdensinia heterodoxa* from Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada.



populations (Milgroom 1996). Also, analysis of linkage disequilibrium showed that, on average, 15% of loci pairs had nonrandom associations of alleles. Given that the a-priori probability of linkage between two loci is low, this also indicates that clonality is strong in these populations. When populations were corrected for asexual replicates (i.e., re-

Table 4. Analysis of molecular variance in three populations of *Valdensinia heterodoxa* from Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada.

Source of variation	df	Sum of squares	Variance components	% Variation
Population level				
Among populations	2	96.108	0.910	18.2
Within populations	145	593.622	4.094	81.8
Total	147	689.730	5.004	100
Nested level[a]				
Among populations	2	96.108	0.587	11.7
Among groups within populations	24	363.724	2.518	50.3
Within groups	121	229.898	1.900	38.0
Total		689.730	5.004	100

Note: All $p < 0.0001$ based on 1023 permutations.
[a]For the nested analysis, populations were divided into groups based on patches observed in natural populations.

Table 5. Matrix of population pairwise genetic distances (F_{ST} above diagonal, Nei's D above diagonal in parentheses, and spatial distance[a] below diagonal) for three populations of *Valdensinia heterodoxa* from Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada.

Population	Mesachie Lake	Port Hardy	Deak's Peak
Mesachie Lake	—	0.266 (0.103)	0.161 (0.089)
Port Hardy	3.842°	—	0.116 (0.063)
Deak's Peak	1.152°	4.412°	—

[a]Spatial distance was measured as latitude and longitude in degrees.

Table 6. Association between pairs of loci[a] within three populations of *Valdensinia heterodoxa* from Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP), British Columbia, Canada.

Sample	No. of isolates	No. of pairwise comparisons	% Significant comparisons[b]
Mesachie Lake			
All	60	741	12.7
Clone-corrected	32	741	7.4
Port Hardy			
All	36	780	13.7
Clone-corrected	32	780	10.3
Deak's Peak			
All	52	861	18.1
Clone-corrected	37	861	14.3
Pooled	148	496	46.4

[a]Analysis based on 39 loci for Mesachie Lake, 40 for Port Hardy, 42 for Deak's Peak, and 32 for the pooled population.
[b]The corrected p values were 0.000 067 5, 0.000 064 1, and 0.000 058 1 for Mesachie Lake, Port Hardy, and Deak's Peak, respectively.

peated haplotypes were removed), the level of linkage disequilibrium was only slightly reduced.

Other factors may cause apparent nonrandom associations within a population (Milgroom 1996). Population subdivision is the major one, being caused by localized genetic isolation arising from ecological or biological barriers (Maynard Smith et al. 1993; Taylor et al. 1999). However, this study involved fine-scale sampling over a small area unlikely to evolve local differentiation. Also, while growth of *V. heterodoxa* is dependent on a host, salal was ubiquitous, so any structuring was not due to barriers imposed by host discontinuity (Wilkin et al. 2005; see also Wilkin (2004) for comparison of genetic diversity between *V. heterodoxa* and salal). However, this does not preclude the effects of host resistance or susceptibility on the distribution of *V. heterodoxa*. As well, researchers in Sweden have observed a positive correlation between the amount of nitrogen, specifically glutamine, and the level of *Valdensia heterodoxa* infections. They proposed that nutrient composition affects the distribution and abundance of *Valdensia heterodoxa* infections on *Vaccinium myrtillus* L. (Strengbom et al. 2002, 2003).

While *V. heterodoxa* populations show a clonal population structure, indicating that reproduction is mainly by asexual spores, other patterns of variation are consistent with some recombination and, hence, sexual reproduction. The small number of differences that separate haplotypes could be due to mutation; however, the lack of linkage disequilibrium between the majority of loci pairs indicates the presence of significant recombination. This is also evident from observation, in natural populations, of sexual structures (Redhead 1974) suggesting that meiosis occurs, indicating that the potential for recombination exists. Thus, in addition to asexual reproduction, genetic exchange must also occur to generate both the lack of linkage disequilibrium and the extent of unique haplotypes observed. Recombination resulting even from rare sexual events can be sufficient to generate diversity in populations (Burt et al. 1996). While unexplored mechanisms may contribute to the variation noted among haplotypes within *V. heterodoxa* populations, the most likely is that sexual reproduction is relatively frequent. This supports the lifecycle proposed by Redhead (1974), which predicted annual sexual reproduction, and suggests that *V. heterodoxa* populations do follow an epidemic population model.

Population structure and gene flow

The PCA found groups of differentiated individuals in each population and also indicated that individuals from PH

and DP were more similar to each other than to individuals from ML. F_{ST} values were moderate relative to other fungi for both pooled (0.182) and hierarchical (0.117) AMOVA. For example, in an AFLP study on the haploid ascomycete *Epichloe festucae* Leuchtmann et al., an F_{ST} of 0.197 between two populations 41 km apart was reported (Arroyo Garcia et al. 2002).

Genetic drift, mutation, selection, and a predominantly clonal mating system all contribute to local adaptation and differentiation among fungal populations. Gene flow disrupts local adaptation and promotes genetic exchange among populations (Slatkin 1987). As well, if *V. heterodoxa* was applied as a biocontrol agent, gene flow could facilitate the spread of the biocontrol among isolated populations. Gene flow can be estimated directly from the number and breeding success of migrants, and also from the geographic distribution of variation among populations (Slatkin 1987). However, this can be difficult with fungal populations because they use multiple modes of reproduction and have alternative methods for genetic exchange (Anderson and Kohn 1998). Ideally, populations should be monitored over time to determine the average values for gene flow, which may be more evolutionarily relevant (McDermott and McDonald 1993), but this is not usually feasible. While a direct measure of gene flow may be obtained by observing segregation in sibling ascospores derived from sexual reproduction, *V. heterodoxa* apothecia are rarely seen (Redhead 1974). Therefore, the results from the present study provide only an indication of gene flow, and given the observed similarities among all three populations studied, there was likely considerable historic gene flow among populations. The higher F_{ST} value recorded between ML and PH may indicate less gene flow between these two populations. As well, *V. heterodoxa* populations in the field were discontinuous, and not all suitable habitats were colonized, which may indicate local adaptation or restricted gene flow. To obtain more definitive evidence of direct gene flow, genetic markers that could identify individuals or private alleles found in only one population, such as microsatellites, should be used and monitored over time to determine if the private alleles or individuals appear in other populations.

The age of *V. heterodoxa* populations in British Columbia is unknown. However, the species was not documented in British Columbia until 1970 (Redhead and Perrin 1972a), so populations may be relatively young, which could explain the high degree of monomorphism and the moderate degree of differentiation. However, if monitoring over time did reveal limited gene flow, depending on the level of recombination, the populations could become more differentiated. This was also hypothesized by Linde et al. (2002) who suggested that, because of relatively recent colonization (centuries ago), populations of *Mycosphaerella graminicola* (Fuckel) J. Schröt. have not yet reached an equilibrium between gene flow and drift, such that the source and temporal scale of the observed gene flow could not be determined. It would be interesting to compare *V. heterodoxa* from British Columbia to isolates from other parts of Canada to assess the level of differentiation among these regions and investigate the age of these populations.

The dispersal mechanisms in *V. heterodoxa* may also indicate a reduced potential for gene flow. Since reproduction in *V. heterodoxa* is asexual for most of its life cycle (Redhead and Perrin 1972a), the predominant dispersal mode would be via the large stauroconidia, which, because of their limited dispersal, would lead to localized structure within populations. The dispersal distance of the sexually produced ascospores is unknown, but since apothecia have been observed growing only on fallen leaves, it was predicted that they are discharged onto new leaves and provide the initial inoculum at the beginning of the season (Norvell and Redhead 1994). If this is the case, their dispersal could also be limited to localized areas. The secondary spores reported by Redhead and Perrin (1972a), likely spermatia, may have the greatest potential for migration and gene flow in *V. heterodoxa*. Although they have been recorded only in the laboratory, and despite the fact that their contribution in natural populations is unknown, their small size suggests that they could travel over great distances (Redhead and Perrin 1972a).

The expected relationship of genetic distance increasing with spatial distance was observed within each of the three *V. heterodoxa* populations. The patchy distribution of *V. heterodoxa* in the field reflects that individuals within the same patch (less than 1.5 m apart) were more genetically similar than individuals in different patches, and that groups from the same section of the population were more similar to each other than to more distant groups. Among populations, a Mantel test was not significant ($p = 0.7$), suggesting that the differences between populations could not be explained by geographic distance. However, it may be that geographic separation between populations was not great enough to result in a significant correlation, although it could also be a function of the low power of the Mantel test. Qualitative comparison of the matrices suggests that there is some relationship between genetic and spatial distance, particularly when the geographic barriers are considered (Table 5). For example, the strong genetic differentiation between ML and PH reflects their latitudinal separation, though they are both on Vancouver Island.

Biocontrol implications

In considering the release of a biocontrol agent into the environment, the potential implications are usually not of too much concern when the control strategies are directed against nonindigenous plants. However, salal is native to the area and has many important roles in the ecosystems. For example, salal is food for many birds, animals, and microorganisms (Fraser et al. 1993) and is a traditional staple for First Nations people of costal British Columbia (Pojar and MacKinnon 1994). As a result, it becomes important to consider what may happen to the residual salal populations, particularly since the goal of the mycoherbicide management strategy is not to eradicate entire host populations, but rather to suppress them (Wall et al. 1992).

One major concern is that continued application of highly virulent pathogen strains will select for resistant plant strains, depending on the amount of gene flow and migration among founder populations (Slatkin 1987; McDermott and McDonald 1993). As the host recolonizes an area, it will do so with these resistant genotypes, making it more difficult to achieve control and necessitating the development of more virulent natural strains. If there is high gene

flow among host populations, than it may facilitate the spread of the biocontrol, making it's containment more difficult. As well, gene flow from the biocontrol agent to naturally occurring populations of the pathogen may introduce a rare virulent gene into the environment and alter the balance of extant evolutionary processes, increasing the ability of local pathogen populations to colonize the host (Becker et al. 2005; Hintz et al. 2001; Templeton et al. 1979). *Valdensinia heterodoxa* has many hosts other than salal, including *Vaccinium* species, some of which, such as blueberries, are of considerable economic value in British Columbia. Thus, before this pathogen is considered for application as a biocontrol agent, pathogenicity testing should be performed with various isolates of *V. heterodoxa* on salal collected from different geographic regions to test their efficacy, some of which has been done (Vogelgsang and Shamoun 2004). This testing should also extend to secondary hosts of *V. heterodoxa*, such as *Vaccinium* species.

Analyzing the risks of releasing a mycoherbicide from a population genetics perspective reinforces the importance of investigating population structure and provides a baseline for retrospective comparisons to the actual effects of releasing a mycoherbicide into the environment.

Acknowledgements

This research was supported by Weyerhaeuser Canada Ltd. (to SFS), the Johnson's Family Forestry Biotechnology Fund (to YAE), and a Natural Sciences and Engineering Research Council of Canada Discovery Grant (to KR). We thank J. Krakowski, S. Vogelgsang, S. Zhao, C. Konoff, G. Sumampong, C. Liewlaksaneeyanawin, and J. Beland for their help in the field, in the laboratory, and on initial drafts of this manuscript.

References

Aamlid, D. 2000. Infections of *Valdensinia heterodoxa* and *Pucciniastrum vaccinii* on bilberry (*Vaccinium myrtillus*). Implications for monitoring ground vegetation. For. Pathol. 30: 135–139.

Anderson, J., and Kohn, L. 1998. Genotyping, gene genealogies and genomics bring fungal population genetics above ground. Trends Ecol. Evol. 13: 444–449.

Arroyo Garcia, R., Martinez Zapater, J., Garcia Criado, B., and Zabalgogeazcoa, I. 2002. Genetic structure of natural populations of the grass endophyte *Epichloe festucae* in semiarid grasslands. Mol. Ecol. 11: 355–364.

Becker, E., Shamoun, S., and Hintz, W. 2005. Efficacy and environmental fate of *Chondrostereum purpureum* used as a biological control for red alder (*Alnus rubra*). Biol. Control, 33: 269–277.

Burt, A., Carter, D., Koenig, G., White, T., and Taylor, J. 1996. Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. Proc. Natl. Acad. Sci. USA, 93: 770–773.

Cilliers, A., Herselman, L., and Pretorius, Z. 2000. Genetic variability within and among mycelial compatibility groups of *Sclerotinium rolfsii* in South Africa. Phytopathology, 90: 1026–1031.

Drayton, F., and Groves, J. 1952. *Stromatinia narcissi*, a new, sexually dimorphic discomycete. Mycologia, 44: 119–140.

Excoffier, L., Smouse, P., and Quattro, J. 1992. Ananlysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131: 470–491.

Fraser, L., Turkington, R., and Chanway, C. P. 1993. The biology of Canadian weeds. 102. *Gaultheria shallon* Pursh. Can. J. Plant Sci. 73: 1233–1247.

Gonzalez, M., Rodriguez, R., Zavala, M., Jacobo, J., Hernandez, F., Acosta, J., Matinez, O., and Simpson, J. 1998. Characterization of Mexican isolates of *Colletotrichum lindemuthianum* by using differential cultivars and molecular markers. Phytopathology, 88: 292–299.

Hardy, O., and Vekemans, X. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol. Ecol. Notes, 2: 618–620.

Health Canada. 2001. Guidelines for the registration of microbial pest control agents and products. Pest Management Regulatory Agency, Health Canada, Ottawa, Ont., Canada, Publication No. DIR2001–02.

Hintz, W., Becker, E., and Shamoun, S. 2001. Development of genetic markers for risk assessment of biological control agents. Can. J. Plant. Pathol. 23: 13–18.

Holst-Jensen, A., Kohn, L., and Schumacher, T. 1997. Nuclear rDNA phylogeny of the Sclerotiniaceae. Mycologia, 89: 885–899.

Holst-Jensen, A., Vralstad, T., and Schumacher, T. 2004. *Kohninia linnaeicola*, a new genus and species of the Sclerotiniaceae pathogenic to *Linnaea borealis*. Mycologia, 96: 135–142.

Kohli, Y., and Kohn, L. 1998. Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. Fungal Genet. Biol. 23: 139–149.

Linde, C., Zhan, J., and McDonald, B. 2002. Population structure of *Mycosphaerella graminicola*: from lesions to continents. Phytopathology, 92: 946–955.

Majer, D., Mithen, R., Lewis, B., Vos, P., and Olivier, P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycol. Res. 100: 1107–1111.

Maynard Smith, J., Smith, N., O'Rourke, M., and Spratt, B. 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA, 90: 4384–4388.

McDermott, J., and McDonald, B. 1993. Gene flow in plant pathosystems. Annu. Rev. Phytopathol. 31: 353–373.

McDonald, B. 1997. The population genetics of fungi: tools and techniques. Phytopathology, 87: 448–453.

Milgroom, M. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34: 457–477.

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106: 283–292.

Nielsen, K., Justesen, A., Funck Jensen, D., and Yohalem, D. 2001. Universally primed polymerase chain reaction alleles and internal transcribed spacer restriction fragment length polymorphisms distinguish two subgroups in *Botrytis aclada* distinct from *B. byssoidea*. Phytopathology, 91: 527–533.

Norvell, L., and Redhead, S. 1994. *Valdensinia heterodoxa* (Sclerotiniaceae) in the United States. Can. J. For. Res. 24: 1981–1983.

Pojar, J., and MacKinnon, A. (*Editors*). 1994. Plants of Coastal British Columbia, including Washington, Oregon, and Alaska. Lone Pine Publishing, Vancouver, B.C. Canada.

Prescott, C. 1999. The salal cedar hemlock integrated research program (SCHIRP). Forum, 6(1): 21.

Redhead, S. 1974. *Epistolae mycologicae*. IV. *Valdensinia heterodoxa* Peyr. (Sclerotiniaceae). Syesis, 7: 235–238.

Redhead, S. 1979. Mycological observations: 1, on *Cristulariella*; 2, on *Valdensinia*; 3, on *Neolecta*. Mycologia, 71: 1248–1253.

Redhead, S., and Perrin, P. 1972a. *Asterobolus*: a new parasitic hyphomycete with a novel dispersal mechanism. Can. J. Bot. 50: 409–412.

Redhead, S., and Perrin, P. 1972b. *Asterobolus*: a synonym of *Valdensia*. Can. J. Bot. 50: 2083–2084.

Ritland, K. 1996. Estimators for pairwise relatedness and individual inbreeding coefficients. Genet. Res. 67: 175–185.

SAS Institute Inc. 1999. SAS/STAT user's guide. Version 8. SAS Institute Inc., Cary, N.C.

Schneider, S., Roessli, D., and Excoffier, L. 2000. Arlequin ver. 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Shamoun, S., and Vogelgsang, S. 2002. Culture, formulation and delivery techniques of *Valdensinia heterodoxa*, and its use as a biological control agent of salal (*Gaultheria shallon*). US Application Patent Serial No. 10/155,992.

Shamoun, S., Countess, R., Vogelgsang, S., and Oleskevich, C. 2000. The mycobiota of salal (*Gaultheria shallon*) collected on Vancouver Island and the exploitation of fungal pathogens for biological control. Can. J. Plant Pathol. 22: 192. [Abstr.]

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. Science (Washington, D.C.), 236: 787–792.

Strengbom, J., Nordin, A., Nasholm, T., and Ericson, L. 2002. Parasitic fungus mediates change in nitrogen-exposed boreal forest vegetation. J. Ecol. 90: 61–67.

Strengbom, J., Walheim, M., Nasholm, T., and Ericson, L. 2003. Regional differences in the occurrence of understorey species reflect nitrogen deposition in Swedish forests. Ambio, 32: 91–97.

Taylor, J., Jacobson, D., and Fisher, M. 1999. The evolution of asexual fungi: reproduction, speciation, and classification. Annu. Rev. Phytopathol. 37: 197–246.

Templeton, G., TeBeest, D., and Smith, R., Jr. 1979. Biological weed control with mycoherbicides. Annu. Rev. Phytopathol. 17: 301–310.

Vogelgsang, S., and Shamoun, S. 2002. Growth, sporulation, and conidia discharge of *Valdensinia heterodoxa*, a foliar pathogen of salal, as influenced by temperature and photoperiod *in vitro*. Mycol. Res. 106: 480–490.

Vogelgsang, S., and Shamoun, S. 2004. Evaluation of an inoculum production and delivery technique for *Valdensinia heterodoxa*, a potential biological control agent for salal. Biocontrol Sci. Technol. 14: 747–756.

Wall, R., Prasad, R., and Shamoun, S. 1992. The development and potential role of mycoherbicides for forestry. For. Chron. 68(6): 736–741.

Weir, B., and Cockerham, C. 1984. Estimating F-statistics for the analysis of population structure. Evolution, 38: 1358–1370.

Wilkin, J.E. 2004. Genetic diversity and population structure of the potential biocontrol agent, *Valdensinia heterodoxa*, and its host *Gaultheria shallon* (salal). M.Sc. thesis, Department of Forest Sciences, The University of British Columbia, Vancouver, B.C., Canada.

Wilkin, J., Shamoun, S., Ritland, C., Ritland, K., and El-Kassaby, Y. 2005. Population genetics of *Gaultheria shallon* in British Columbia and the implications for management using biocontrol. Can. J. Bot. 83: 501–509.

Zolan, M., and Pukkila, P. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. 6: 195–200.