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Chemical variation of *Usnea longissima* Ach. in the central Oregon Coast Range

Lori J. Kayes[1], Katherine Van Wormer[2], David Kofranek[3], Kathleen Sale[4], and Bruce McCune[5]

[1] Department of Forest Science, Oregon State University, Corvallis, OR, USA, [2] Department of Chemical Engineering, Oregon State University, Corvallis, OR, USA, [3] Botanical Consultant, 843 Snell Street, Eugene OR, USA, [4] Oregon Institute of Technology, Klamath Falls, OR, USA, [5] Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR, USA

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Corresponding author: Lori Kayes, lori.kayes@oregonstate.edu. Accepted for publication April 29, 2008.

Abstract: The lichenized ascomycete, *Usnea longissima*, occurs in cool forests with coastal climates in Europe, Asia and North America and is threatened throughout its range. The Pacific Northwest region of North America is considered a stronghold of this species. While many studies have focused on the ecology of *U. longissima,* knowledge of the secondary chemistry of *U. longissima* is sparse for the region. The objectives of this study were to analyze collections of *U. longissima* for chemotypic variation using thin-layer chromatography and to examine ecological and geographic patterns of chemotype variation within the central Oregon Coast Range. Three major chemotypes were found within the study area with some

additional chemotypic variation documented throughout the global range of *U. longissima*. The most common chemotype (chemotype 1: usnic, barbatic and norbarbatic acids) accounted for 70.9% of samples, with the other 2 chemotypes accounting for 24% (chemotype 2: usnic and diffractaic acids) and 4.2% (chemotype 3: usnic and a minor unknown acid) of samples. Chemotype 2 generally occurred farther west and at higher elevations than chemotype 1. Different chemotypes may deserve protection to ensure the persistence and genetic variation of *U. longissima* throughout its current range.

Key words: 4-*O*-demethylbarbatic acid, barbatic acid, diffractaic acid, *Usnea, Usnea longissima,* chemotypes, thin-layer chromatography, TLC, Coast Range, Oregon

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Introduction: Variation in the secondary chemistry of *Usnea longissima* Ach. in North America is poorly known. *Usnea longissima* is a pendulous epiphytic lichen threatened with declining and rare populations in the northern hemisphere. It occurs in coastal forests and high humidity inland areas, such as stream sides and north-facing slopes (Esseen et al. 1981). *Usnea longissima* lacks substrate specificity, having been reported on *Picea sitchensis* (Bong.) Carr., *Tsuga heterophylla* (Raf.) Sarg., *Alnus rubra* Bong., *Malus fusca* Raf., *Quercus garryana* Dougl., *Pseudotsuga menziesii* (Mirb.) Franco. and *Acer macrophyllum* Pursh. in the Pacific Northwest (PNW) (McCune and Geiser 1997, Halonen et al. 1998, Keon 2002). Increased pollution levels, loss of habitat due to timber harvest, and changing land use have all contributed to the decline of this species resulting in patchy distributions worldwide (Tønsberg et al. 1996, Doell and Wright 2000). Areas of local *U. longissima* abundance are often surrounded by areas with few or no occurrences, even within continuous forest stands (Esseen et al. 1981).

The secondary chemistry for *U. longissima* has been described as "complex" in Fennoscandia and British Colombia (Halonen 2000). *Usnea longissima* in British Columbia was documented as having evernic, diffractaic, barbatic, and 4-O-demethylbarbatic acids sometimes present. Additionally, atranorin, fumarprotocetraric, constictic and salazinic acids were documented as accessory substances. In Fennoscandia, diffractaic acid was present in all specimens of *U. longissima* but none of the other secondary compounds documented in British Columbia specimens was present. In India, one chemotype was reported, with barbatic, usnic and placodiolic acids (Mallavadhani et al. 2004). In Japan, *Usnea longissima* has at least four chemotypes, the most common containing usnic acid, barbatic acid, 4-O-demethylbarbatic acid, squamatic acid, atranorin and unidentified fatty acids (Ohmura 2001).

In the Pacific Northwest, *U. longissima* chemotype variation is of interest due to the abundance of this species, its apparent sensitivity to land use practices, and the general lack of knowledge regarding its chemotypes within the United States. In Europe, *U. longissima* is nearly extirpated from its historic range with the exception of Scandinavia, its population center (Esseen et al. 1981, Gauslaa 1997). In Asia, the species occurs along the north Pacific coast and in montane, conifer forests of the Himalayas. In North America, *U. longissima* occurs on the east coast from Canada to New England and on the west coast from southeast Alaska to northern California. The Pacific Northwest contains the most abundant populations of *U. longissima* in North America (Halonen 2000, Brodo et al. 2001). In the United States, *Usnea longissima* was listed in the US Department of Agriculture Forest Service Record of Decision amendments (USDA 1994) under the Northwest Forest Plan (NWFP) as rare or uncommon with status undetermined within the range of the Northern Spotted Owl. The Oregon Natural Heritage

Information Center (2007) has assigned *U. longissima* a global and state rank of not rare and apparently secure throughout its range but with long-term conservation concerns. The general rarity of this lichen and listing under the NWFP has increased interest in *U. longissima* in the Pacific Northwest.

Variation in chemotypes implies genetic diversity, an important characteristic for survival of a population (Meffe et al. 1997), particularly as the reproduction of this species appears to be primarily asexual. Like many alectorioid lichens, *U. longissima* reproduces primarily by large thallus fragments (Esseen 1985, Gauslaa 1997, Peterson and McCune 2001), with Oregon and Washington populations being non-isidiate and rarely sorediate (McCune and Geiser 1997). Two populations in the PNW have been documented bearing apothecia (McCune and Geiser 1997, Keon 2002, Tønsberg 2002). Ecological studies of *U. longissima* have concentrated on distributional patterns, correlations of environmental variables and the effects of logging practices on distributions (Peck and McCune 1997, Rolstad and Rolstad 1999, Keon 2001). While other studies have found that most lichens have constant secondary chemistry, some species have distinct chemotypes that are related to ecological or geographical distributions (McCune 1987, Culberson et al. 1988, 1990). There are several hypotheses about the functions of secondary chemicals (Lawrey 1986, Purvis 2000), however no studies specifically address the ecological and geographic correlations of chemotypes of *U. longissima.*

In this study, we focused on chemical variation of *U. longissima* within the central Coast Range of Oregon. The objectives of this study are to analyze samples of *U. longissima* for chemotypic variation using thin-layer chromatography (TLC) and to examine basic ecological and geographical patterns associated with chemotypic variation. Specifically we were interested in differences in elevation, stand type, distance to streams, distance to other populations of *U. longissima*, and distance to urban areas. Additionally, we analyzed a limited number of herbarium samples with TLC from across the global range of *U. longissima* for comparison to the Oregon Coast Range chemotypes.

Materials and Methods

Study Site

An area encompassing Benton, Lane, and Lincoln Counties in Oregon was selected as representative of the central Coast Range (43.88° N and 44.72° N to 123.133° W to 124.072°W, Figure 1). The total study area encompasses 8500 km² from the Willamette River to the Pacific Coast. The elevation ranges from 68 m at the Willamette River to 1,249 m at the top of Mary's Peak, the highest point in the Oregon Coast Range. The climate, moderated by the Pacific Ocean, is relatively mild, characterized by cool moist winters and warm dry summers. The average high January temperatures range from 4.1°C to 9.7 °C and average high July temperatures range from 18.3 to 28.9°C. The average annual precipitation in the area ranges from around 108 cm to 230 cm per year with some areas of the Coast Range averaging up to 450 cm per year (Loy et al. 2001). The topography is predominately a network of relatively low ridges, slopes, draws and numerous watercourses. The watercourses vary in size from streams to rivers, many of which are easily accessible due to the frequency of roads parallel to them. *Pseudotsuga menziesii* and *T. heterophylla* dominate the forests with plentiful hardwoods such as *Acer macrophyllum, Alnus rubra* and *Q. garryana* at lower elevations. At the highest elevation, dominance shifts to *Abies procera* Rehder.

Selection of Lichen Material

A total of 139 specimens from OSC (Oregon State University Herbarium), current collections, and the personal herbarium of B. McCune were examined. Many collections were from sites of

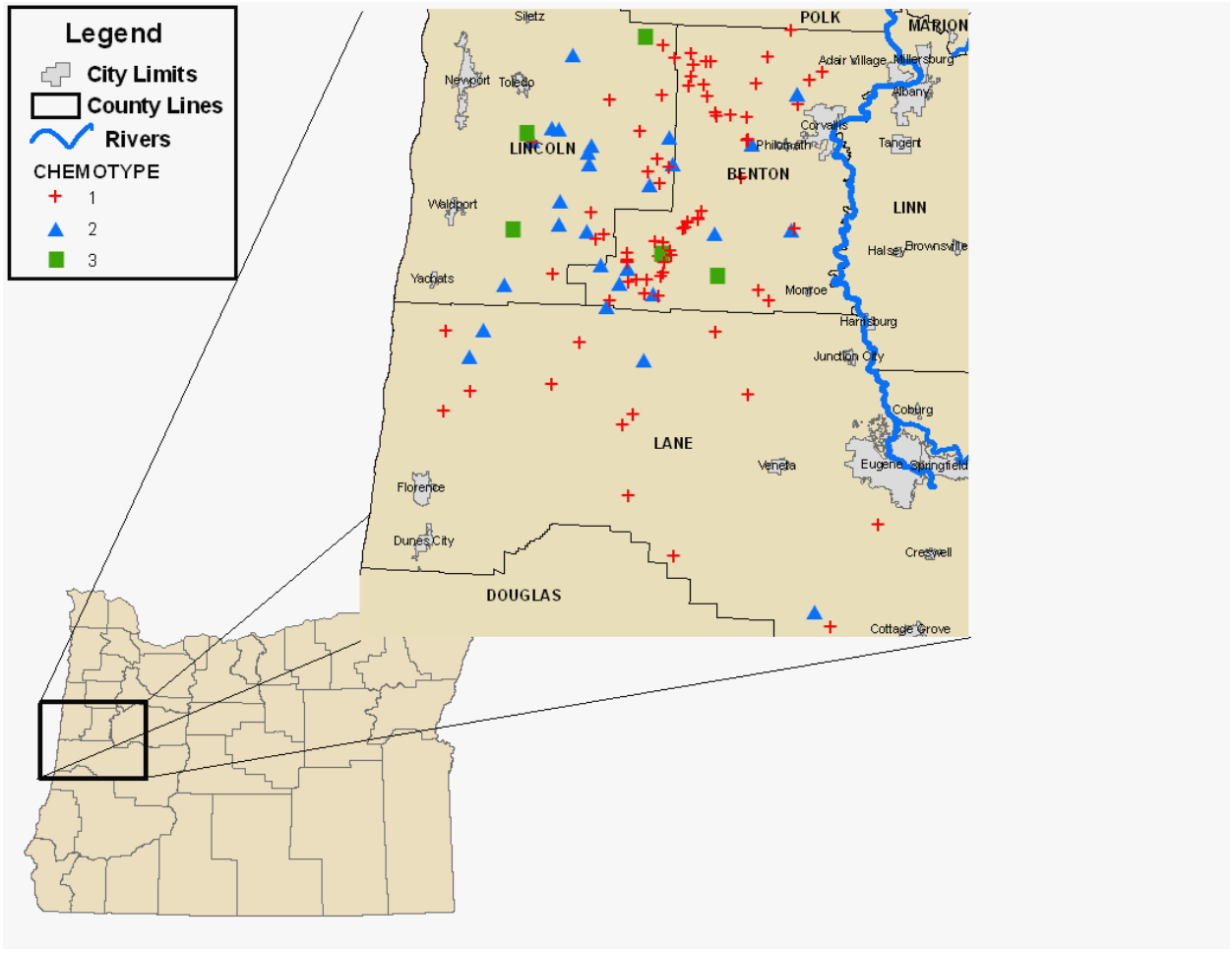

Figure 1. Geographical distribution of *Usnea longissima* used for TLC analysis showing chemotype locations.

previous ecological studies of *U. longissima* (Keon 2001). An Oregon State University Lichenology class under the direction of B. McCune made additional collections of *U. longissima* in April 2005. Collection by the class used travel routes plotted to represent a variety of topographic features and maximize extent of area sampled within Benton County. Collections were made only from abundant and accessible populations of *U. longissima*. At each site, the collector recorded *U. longissima* presence or absence, directions, GPS coordinates, elevation, current land use, topography and stand type (non-forest or open, young, mixed or mature forest). Keon's (2001) sites were selected from U.S. Forest Service and Bureau of Land Management databases, collection data of other researchers and herbarium records. He selected additional sites by arbitrarily traveling selected roads and trails in the study area. Abundant data was collected at the Keon sites but only elevation, stand type and topographic position were utilized. No additional data, except collection location was available from OSC specimens, therefore these were not used in the habitat analysis. Forty six *U. longissima* samples were acquired by the lichenology class and 70 samples were collected from Keon's sites (Table 1) resulting in 116 sites tested with TLC from the central Oregon Coast Range. Additionally, 23 samples from OSC and B. McCune personal herbarium were tested, including North

American collections from Alaska, California, Maine, New Hampshire, Nova Scotia, Oregon, and Washington and international collections from China, Japan, Nepal and Russia. Collection sites were mapped with ArcGIS 9.2 (ESRI 2006). All base map layers were downloaded from the Oregon Geospatial Data Clearinghouse (2005).

Chromatography

We used TLC methods of Culberson (1972). Samples were run on Merck silica gel 60 F_{254} aluminum-backed plates, using solvent B' (Culberson and Johnson 1982) to differentiate between barbatic and diffractaic acids. We ran one specimen from each of the dominant three chemotypes in solvent C to help identify closely aligned spots under solvent B'. Control extracts of lichen species with known chemistry were analyzed to aid in chemical identification. Controls included *Parmotrema hypotropum* (Nyl.) Hale for atranorin and norstictic acid, *Cladonia amaurocraea* (Flörke) Schaerer for barbatic acid, and *Hypogymnia imshaugii* Krog for diffractaic acid. Rf values were compared to those in the literature (Table 2).

Data Analysis

All data analysis was run using SPlus version 6.0 (Insightful Corp, Seattle, WA, USA). Analysis of variance (ANOVA) was used to compare distance to stream, distance to presence of other *U. longissima* populations, elevation, and distance to urban areas among the different chemotypes. The model assumes that the variance among groups is constant and that the data are normally distributed. The assumptions were assessed using standard residual versus predicted value plots and normal percentage plots. When the assumptions were not met (distance to stream and distance to urban) a log transformation was applied. After log transformation all model assumptions were met. Significance levels were set at $p = 0.05$ and only significant differences were reported. The Tukey-Kramer procedure was used when making multiple comparisons. Chi-square test for independence was used to examine differences in stand type.

Results: As in previous studies, the four most frequent primary substances found were usnic acid, barbatic acid, diffractaic acid and 4-*O*-demethylbarbatic acid (norbarbatic acid). These substances were in various combinations that resulted in three primary chemotypes (Figure 2). Eighty-three of our samples (70.9%) contained primarily usnic, barbatic, and norbarbatic acids (chemotype 1). Chemotype 2, containing usnic and diffractaic acids, occurred in 24% of samples. Chemotype 3, usnic and a minor unknown with Rf classes A2, B3, C2, nearly colorless with acid spray and heat, accounted for 4.2% of samples. An additional chemotype (4), a New Hampshire specimen from 1892, was similar to chemotype 1 but lacks norbarbatic acid.

Within the 116 Oregon samples, chemotypes were widely distributed across the central Oregon Coast Range (Figure 1). Based on visual examination of maps, chemotype 2 appears to occur more in the western portion of the study area, although considerable overlap between chemotype 1 and 2 distributions exists. Chemotype 3 showed no geographical pattern, but the sample size was small. Chemotypes 1 and 2 were present in the herbarium specimens from around the world (Table 1). In the herbarium samples, chemotype 1 was found in two California counties (Humboldt and Del Norte), Alaska, Washington, Japan, Nepal, and Russia. Chemotype 2 was found in New York, Nova Scotia, Russia and China. The three specimens with chemotype 3 were from Oregon (Benton and Douglas Counties) and Washington State.

Average elevation differed between chemotype 2 and the other two chemotypes ($F=26.19$ $p < 0.001$; Figure 3). The difference between average elevations was estimated to be between 138 m to 254 m (95% confidence interval (CI)) lower for chemotype 1 than chemotype 2. The difference

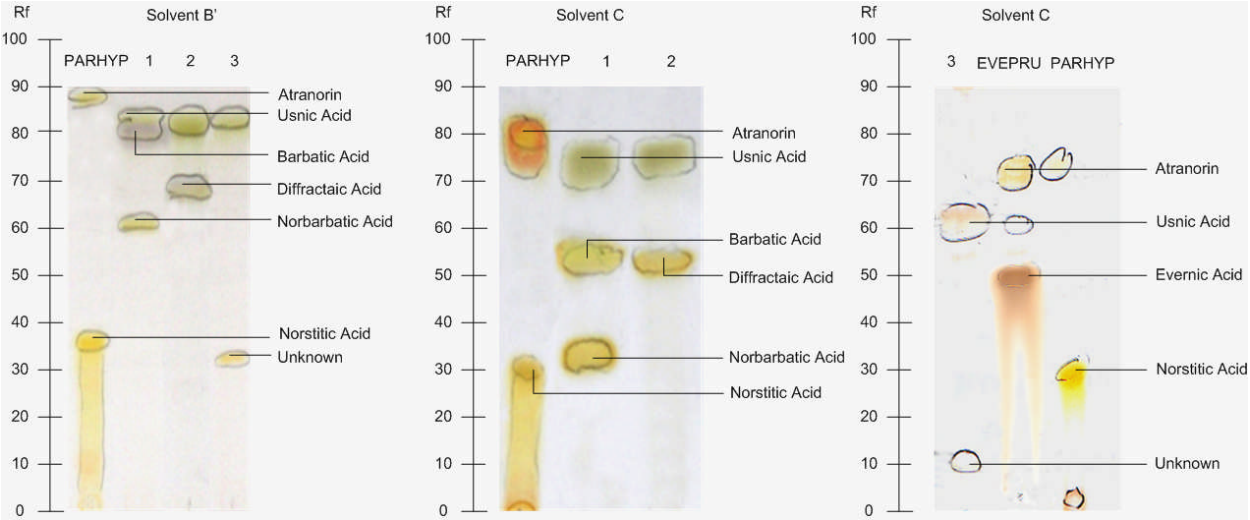

Figure 2. TLC results showing chemotypes 1, 2 and 3 run in B' and C solvent system. *Parmotrema hypotropum* (PARHYP) and *Evernia prunastri* (EVEPRU) were used as controls for known acids. Unknown is a minor unknown with Rf classes A2, B3, C2, nearly colorless with acid spray and heat.

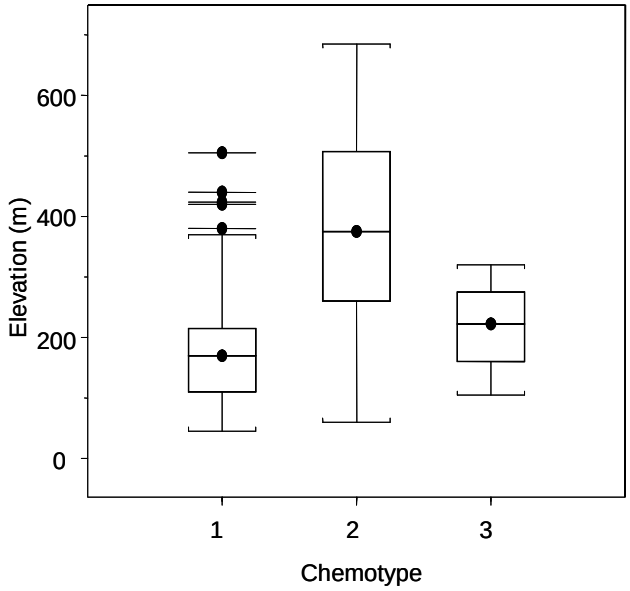

Figure 3. Elevation variance between three chemotypes. The boxes represent the median and the first quartiles, the line and whisker represent the second quartiles and the outlying dots represent points that are beyond the second quartile.

Table 1. Collection localities of specimens used on TLC plates summarized by different chemotypes. *One specimen from each locality was tested.

Chemotype	OSU Class	Keon	OSC Herbarium	Herbarium Collection Localities *	Total
1	42	41	11	Russia; Nepal; Japan; USA: Alaska, Washington, California (Del Norte, Humboldt Cos.), Oregon (Linn, Polk, Marion, Coos Cos.)	94
2	4	24	8	China; Canada: Nova Scotia; Russia; USA: Maine, New York, Oregon (Benton, Yamhill, Tillamook Cos.)	36
3	0	5	3	USA: Oregon (Benton, Douglas Cos.), Washington	8
4	0	0	1	New Hampshire (1982)	1
Total	46	70	23		139

Table 2. Calculated and literature Rf values for lichen substances identified in *Usnea longissima*.

	Solvent B'		Solvent C	
Compound	Calculated	Literature	Calculated	Literature
usnic acid	84	73	73	73
barbatic acid	80	72	54	57
diffractaic acid	67	61	52	55
4-*O*-demethylbarbatic acid	62	58	33	34

between average elevations was estimated to be between 6 m and 313 m (95% CI) higher in chemotype 2 than chemotype 3. The elevations for chemotypes 1 and 3 were not significantly different.

There is evidence that stand type and chemotype are independent (p=0.09) for chemotypes 1 and 2 based on chi-square test for independence. Chemotypes 1 and 2 appeared to be the most frequent in mixed-aged stands (Table 3). Forty percent of chemotype 1 sites occurred in mixed aged stands, 26% in young stands (<50 yrs old) and 20% in mature stands (50 to 150 yrs old). Sixty percent of chemotype 2 sites occurred in mixed aged stands. However, it appears that

Table 3. Frequency of different chemotypes by stand type.

	Chemotype			
Stand Type	**1**	**2**	**3**	**Total**
Non-Forest	1	0	0	1
Open Forest	9	0	0	9
Young	21	4	0	25
Mixed	32	17	1	50
Mature	16	7	3	26
No data	2	0	1	3
Total	81	28	5	114

stand type and chemotype are not independent (p=0.04) for chemotype 3 which had very few sites but occurred 75% of the time in mature stands. Distance to stream, distance to other *U. longissima* populations and distance to urban areas did not differ among chemotypes.

Discussion: *Usnea longissima* has moderate chemical variation in the central Oregon Coast Range, as well as, across its global range. Chemotypes in the Oregon Coast Range appear to differ from those in British Columbia in their relative dominance and chemical make-up. The chemicals that we found in our samples were a subset of the secondary chemical reported from British Columbia (Halonen et al. 1998, Halonen 2000). However, the most common British Columbia chemotype, with evernic and usnic acid, was not found in our study. Chemotype 1 was not reported from British Columbia except with atranorin and salazinic acid also present (Halonen et al. 1998). Chemotype 1 is similar to the dominant chemotype recorded in Japan (Ohmura 2001) but lacks atranorin and squamatic acids. Chemotype 2, with diffractaic and usnic acids, was the main chemotype reported from eastern Fennoscandian (Halonen 2000). However, this chemotype was less common than the barbatic acid chemotype in British Columbia. Chemotype 3 has a minor unknown acid with Rf classes A2, B3, C2 that does not match any of the documented acids found in *U. longissima* around the world. Chemotype 4, found from New Hampshire, was more common than chemotype 2 in British Columbia. We did not find this chemotype in the central Oregon Coast Range.

Subtle habitat differences occur between the three chemotypes in the Oregon Coast Range. Based on our data, chemotype 1 generally occurs at lower elevations and further inland than chemotype 2. This habitat differentiation may be due to more tolerance of disturbance by chemotype 1, as evidenced by more people further inland, or affinity for lower precipitation and/or higher temperatures than the other chemotypes. Presence of chemotypes 1 and 2 in young to mixed aged stands may have resulted from land use patterns in the Coast Range. Historically, this area has been heavily logged and most of the landscape is dominated by young to mid age stands. If chemotype 3 is associated with mature stands, it may have been more abundant in pre-settlement forests.
While our results indicate differences in habitat, we cannot determine the cause of distributional differences between the chemotypes. Further study using more comprehensive data (i.e. humidity, microclimate, precipitation, substrate, etc) and/or a more diverse study area would provide further insight into the chemotypic distribution patterns of *U. longissima*. To

preserve chemotypic variation, it is necessary to understand habitat preferences among chemotypes. While the adaptive significance of chemotypes is unknown (Halonen 2000), preserving chemotypic variation of *Usnea longissima* in the Pacific Northwest may help maintain genetic diversity of this sensitive species (Fahselt 1996).

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