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Chemotaxonomic investigation of hybridization between Larix occidentalis and Larix lyallii; a preliminary study

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CHEMOTAXONOMIC INVESTIGATION OF HYBRIDIZATION BETWEEN *LARIX OCCIDENTALIS* AND *LARIX LYALLII*

A Preliminary Study

by

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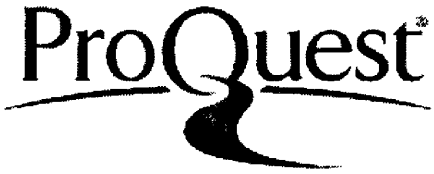

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER I

THE PROBLEM AND ITS SIGNIFICANCE

I. INTRODUCTION

Classical plant taxonomy as conceived by Linnaeus over two centuries ago has undergone considerable evolution. The system which Linnaeus developed was based on floral morphology and was superseded by a more "natural" classification system which reflects the situation as it is felt exists in nature. Currently, in addition to plant morphology, geographical distribution, and chromosome number, taxonomists are using a chemical approach to systematics. Chemical constituents in many cases appear to be genetically controlled and have the advantage over morphological characters in that they can be described in terms of specific structural formulas. The chemical approach to systematics, although still in its infancy, has been used for the elucidation of phylogenetic relationships, and recently biochemical research has proven to be useful in the study of hybrid populations. Alston and Turner (1959, 1962, 1963b) made an extensive biochemical study of hybridization in Baptisia and later concluded that chemical taxonomy offers a great deal of promise in studies related to introgressive and transgressive hybridization.

Although some individuals tend to minimize the significance of interspecific hybridization (Mayr, 1942), others feel that recombination might be considered a third evolutionary process with an importance equal to that of mutation and selection (Stebbins, 1957). If natural

interspecific hybridization does occur, it is of primary interest to plant breeders and foresters alike to determine the effect of recombination on the modification of future generations and on the intrinsic variation found within species.

Until recently hybridization of western larch (Larix occidentalis Nuttall) and subalpine larch (Larix lyallii Parl.) had not been recognized. Ostenfield and Larsen (1930) noted that extensive overlapping of the geographical ranges of the two species exists and stated that hybridization could be occurring. Schoenike (1961) also implied this possibility; however, he stated that no reliable evidence exists that would substantiate this conjecture.

The ability of the two larches to hybridize artificially was positively established by Carlson (1965). Along with success in producing first filial generation (F_1) hybrids, he also located an area in the Bitterroot Mountains of western Montana where both larches as well as several putative hybrids are found concurrently. On the basis of morphological features Carlson suggested that natural hybridization is possible wherever the botanical ranges of the two larches overlap.

II. OBJECTIVE

Recently, the use of biochemical methods in addition to the classical Linnaen methodology have proven to be invaluable for establishing the existence of a hybrid and analyzing populations where hybridization is taking place. Specifically, the objective of this study was to apply chemotaxonomic methods to the problem of determining if the putative hybrids as identified by Carlson are, in fact, natural hybrids.

Although the scope of this study did not encompass the identification of each compound *per se*, it was necessary to develop a technique for isolating the taxonomically significant compounds. Since the study was exploratory in nature and principally concerned with discovering a feasible technique for making distinctions between the species, statistical significance was not established. The number of trees studied was only enough to establish a tentative chemotaxonomic procedure for studying this instance of apparent interspecific hybridization.

CHAPTER II

REVIEW OF THE LITERATURE

I. THE BIOCHEMICAL APPROACH TO TAXONOMY

> Chemotaxonomy or chemosystematics is one of the newest and most exciting developments in a science that traces its history back to Aristotle. Chemical data represent a powerful tool available to taxonomists in their attempt to answer questions about relationships among organisms and in devising natural systems for their classification. It is important, however, to realize that the application of chemical information to taxonomic problems does not demand a new scheme for classifying plants and animals but complements and expands existing approaches to classification systems erected mainly on the basis of morphology (Mabry et al., 1965).

Although the biochemical approach to taxonomy originated over a century ago, only recently has there been any real enthusiasm regarding biochemical systematics. Early pharmacologists were aware of the healing and economic potential of various chemicals found within certain plants. Plants were classified entirely on the basis of the medicinal value of the chemical constituents. Hegenaur (1958), reviewing the work of A. P. de Candolle, noted that certain plants were capable of producing specific medicinal products. For example, Cinchona species reduced fever, the Amentiferae had astringent bark, and the Convolvulaceae were laxative. Perhaps the actual biochemical approach to systematics was started in 1854 when Rochleder recognized the importance of biochemical characters to taxonomy (Florkin, 1962).

The biochemical era in taxonomy, although slow to develop, came into prominence in the early 1960's. Significant progress in enzymology,

genetics, and the development of various chromatographic techniques used in the isolation of specific chemicals made possible the surge in chemical systematics (Turner, 1967a). Many investigators who feel that morphological characters are merely an exemplification of the plant biochemistry are commencing studies demonstrating the utility of this new approach to systematics (Swain, 1963).

Although the ultimate in chemotaxonomy will be the study of comparative enzymology and comparative RNA and DNA, most of the current work is concerned with secondary constituents of the plants such as flavanoids, alkaloids, terpenes, and several water-soluble pigments (Alston et al., 1963; Alston and Turner, 1963). These secondary compounds, formed as end-products of metabolism, are felt to be of very significant taxonomic value (Florkin, 1962; Flück, 1963).

A recurring question regarding chemotaxonomy concerns the effect of environment and season of the year on the presence of various chemical constituents. Mirov (1961), after an extensive biochemical survey of the terpenes in the genus Pinus, concluded that terpentine composition varied very little during the growing season. Blake (1963) also noted that the chemical composition of aspen vegetative buds did not vary significantly from season to season.

Mirov found that the chemical character of the terpentine was a characteristic which was strongly controlled genetically. The oil fraction of terpentine from Pinus pinea L., a native of the Mediterranean region, contains almost entirely l-limonene when grown either in Italy or in California. Clones of western white pine (Pinus monticola, Dougl.), when grown on three diverse sites, were found to show negligible

differences in terpentine levels (Hanover, 1965). Krestinsky and Bazhenova-Kozlovskaia (1932) observed that the terpentine of Pinus sylvestris L. had the same physical properties and chemical composition regardless of the ecological conditions affecting the tree.

II. BIOCHEMICAL ANALYSIS OF HYBRIDIZATION

Documentation of the occurrence of complex hybridization is one of the most successful applications of chemical taxonomy. Perhaps the most thorough chemical analysis of hybridization has been in the genus Baptisia. Leguminoseae (Alston and Turner, 1959; Alston and Simmons, 1962; Alston and Hempel, 1964; McHale and Alston, 1964). Alston and Turner (1959), after studying the morphology of a hybrid swarm from the genus Baptisia species, concluded that trihybridization and a complicated backcross was involved. Two-dimensional chromatograms of the leaf extracts of the three Baptisia species involved revealed that the situation was not as complex as suspected and involved mostly two-way crossing. Subsequent morphological investigation substantiated the chromatographic data.

Chromatographic analysis of the flavanoid compounds in the leaf extracts enabled Hunter (1967) to support an assumption, based on herbarium data of interspecific hybridization in Veronia. Clausen (1961), working with hybridization of Betula papyrifera Marsh. x Betula glandulosa, found results similar to those of Alston and Turner (1959). Although some of the hybrids chromatographically resembled one of the parents, most hybrids contained compounds from both. Certain new compounds not found in either parent were felt to be due to new gene

combinations in the hybrid. Smith (1967) sampled the resin of thirty-four Pinus hybrids and found that the hybrids were intermediate in chemical composition to the parents.

III. HYBRIDIZATION OF LARIX OCCIDENTALIS AND LARIX LYALLII

The occurrence of interspecific hybridization in the genus Larix is widely accepted. Schoenike (1961) noted eight spontaneous hybrids, among which is the best known example of larch hybridization, the Dunkheld Hybrid Larch (L. x eurolepis Henry). The Dunkheld larch, the result of a cross between Japanese larch (L. leptolepis (Sieb. & Zucc.) Gord.) and European larch (L. decidua Miller) has proven to be a tree of exceptional quality and is now commercially cultivated.

Carlson (1964, 1965) established that hybridization of western and subalpine larch is possible. Utilizing subalpine larch as the female parent he found that the species are capable of hybridizing although heterosis was not evident.

Secondly, he studied the variation of several morphological characters within the range of western and subalpine larch. Three characters, the number of epithelial cells surrounding resin canals in the needles, pubesence of year-old twigs, and the texture of the bark within four feet of the tree top, were found by an analysis of variance to be highly significant between the parental species. Carlson concluded that these characters were under a strong genetic influence and were therefore reliable for use in a hybrid index.

Finally, a species overlap area was located in a small drainage of the Bitterroot Mountains about fifteen miles southwest of Missoula,

Montana. Recurring snowslides have removed the spruce-fir zone normally separating the two species and subalpine larch has come into contact with western larch near the bottom of the slope. Both species of larch exist sympatrically on the bottom one-third of the slide and on this area sampling for natural hybrids was undertaken. A subsequent scatter diagram and hybrid index analysis suggested a distinct possibility of natural hybridization in the area although the sample was too small to conclusively establish this fact.

IV. DESCRIPTION AND HABITAT OF THE SPECIES

Range

Western larch and subalpine larch are deciduous conifers and are generally restricted in range to the Upper Columbia River Basin. Subalpine larch, a timberline conifer, is found essentially on remote and relatively severe mountainous sites. It occurs on mountains of both the Rockies and the Cascades of Washington, the Bitterroot Range of Montana and Idaho, and the southern Canadian Rockies, primarily in Alberta (Arno, 1966).

Western larch, definitely not a timberline species, is found on mountainous slopes and valleys of southeastern British Columbia, northwestern Montana west of the Continental Divide, northern Idaho, and northeastern Washington. The species is also abundant along the east slopes of the Cascades of Washington and north central Oregon and in the Wallowa and Blue Mountains of northeastern Oregon and southeastern Washington (Arno, 1966; Sudworth, 1908; USDA, 1965).

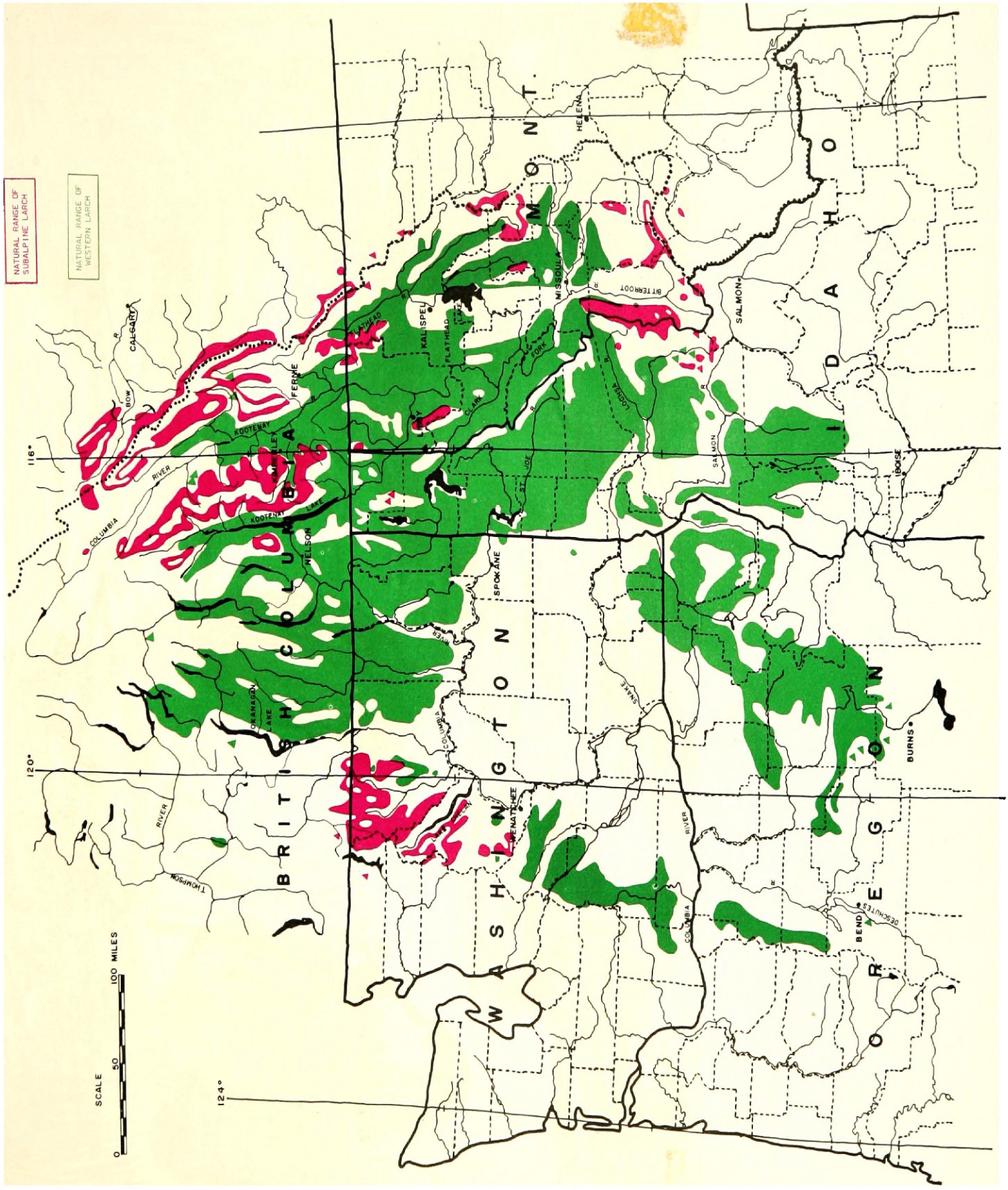

Figure 1. Natural ranges of western and subalpine larch (Knudsen et al., 1968).

Western and Subalpine Larch Differentiation

Typically the two species are separated by 500 to 1000 feet elevation. Subalpine larch is generally restricted to high mountainous sites very near the alpine timberline, while western larch usually is found in the low and mid-elevation forest zones.

Perhaps the most consistent morphological difference between the two species is the character of the current year's twigs. Twigs of subalpine larch are densely pilose while those of western larch are nearly glaborous.

It is also possible to differentiate between the two species on the basis of the bark texture within four feet of the tree top. The bark of western larch is smooth and possesses longitudinal fissures. Subalpine larch bark contains both longitudinal and horizontal fissures, giving it a scaly appearance (Carlson, 1964).

Climate

The geographical range of western larch is delineated in general by climatic factors. The upper limit is marked by low temperatures while the lower boundary is a result of inadequate moisture (USDA, 1968). The zone in which western larch grows is characterized by an average annual precipitation of 28 inches which ranges from 18 to 35 inches. The mean annual air temperature is 43 degrees Fahrenheit and the range is -49 to 107 degrees (Larsen, 1930; USDA, 1965).

Subalpine larch, as formerly noted, is restricted to severe sites. Temperatures ranging from 70 degrees Fahrenheit in the summer to -50 or less in the winter are characteristic of subalpine larch sites.

Precipitation on subalpine larch sites is generally in the form of snow. The snowfall is quite substantial and ranges from 200 to 400 inches per year (Arno, 1966).

Edaphic Factors

Northerly exposures, valley bottoms, benches, and rolling topography are characteristic of typical western larch sites (USDA, 1968). The species occur at altitudes ranging from 2,000 feet mean sea level to 5500 feet in the northern portion of its range and to 7000 feet in the southern portions (Larsen, 1930; USDA, 1965).

Nimlos (1963) noted that western larch is found on two great soil groups, the brown podzolic and the grey wooded. Larsen (1940) considered deep, porous soils of mountain slopes and valleys essential for optimum larch development.

Similar to western larch, subalpine larch is usually found on northerly exposures. Although attaining its best development on high sheltered areas with rather gentle topography, subalpine larch grows on mountain divides or high cirques, often above the limit of arborescent form for its associate species (Arno, 1966). In the southern portion of its range, subalpine larch is found from 7000 to 10,000 feet, although it has been found as low as 5000 feet in parts of the Cascades (Arno, 1966; Ostenfield and Larsen, 1930).

Subalpine larch, in contrast with its lowland counterpart western larch, occurs on poor, rocky soils with adequate moisture. Arno (1966) noted that:

> Alpine larch will grow on fresh piles of coarse talus which have not previously been occupied by vascular plants so long as the site is cool and moisture is not critically short in

supply. Sometimes even the invasion of lichens onto granite talus has not progressed very far by the time alpine larch inhabits it.

Vegetational Classification

Western larch plays a seral or temporary role on all sites. Its expansive distribution is a result only of recurring fires and disturbances (Larsen, 1929). The principal arborescent associates of western larch are Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco var. glauca (Beissn.) Franco), Engelmann spruce (Picea engelmannii Parry), subalpine fir (Abies lasiocarpa (Hook.) Nutt.), lodgepole pine (Pinus contorta Dougl.), white spruce (Picea glauca (Moench) Voss), ponderosa pine (Pinus ponderosa Laws.), western red cedar (Thuja plicata Donn), western hemlock (Tsuga heterophyla (Raf.) Sarg), and western white pine (Pinus monticola Dougl.) (USDA, 1968).

Subalpine larch is generally considered a very shade intolerant species (Baker, 1949). This in part explains its inability to compete effectively with other tree species and why it is restricted to rugged sites near the timberline. On such areas, subalpine larch is considered climax (Daubenmire, 1952). White-bark pine (Pinus albicaulis Engelm.), subalpine fir and Engelmann spruce are the primary associates of subalpine larch; however, at times mountain hemlock (Tsuga mertensiana (Bong.) Carr.) and lodgepole pine are present (Arno, 1966).

From the preceding discussion it is evident that western and subalpine larch are found in separate and distinct environments. Typically the species are separated by a zone of Engelmann spruce and subalpine fir such that an elevational range overlap is usually not possible. An instance where the spruce-fir zone was removed by a snowslide and

subalpine larch was brought from the top of a ridge into contact with western larch is cited by Carlson (1965). The continual snowslides formed environmental niches similar to those found on the ridgetop. These niches allowed subalpine larch to occur on the same site as western larch (Area where hybridization is suspected, Figure 2).

Figure 2. Area where hybridization is suspected. Western and subalpine larch are found together throughout the slide area (solid line) located in the Carlton Creek drainage of the north Bitterroot Mountains. The dotted line indicates where the putative hybrids were found. Lolo Peak, elevation 9075 feet, is in the background.

CHAPTER III

PROCEDURES

I. PLANT TISSUE ANALYZED

Heartwood

Investigation of the heartwood extractives has been of significant value for several taxonomic studies, although various other plant materials have been utilized. Since the heartwood is dead tissue and a virtual storehouse for metabolic end products, it should be less affected by the environment than living tissue and should, therefore, exhibit a more consistent chemical composition. Erdtman (1963) felt that secondary plant substances found in phylogenetically old organs such as bark or wood are generally the most useful plant constituents for systematic studies. For this reason, heartwood was initially selected as the material for analysis in this study.

Care was taken in obtaining heartwood from healthy trees, unaffected by such factors as disease or mechanical damage. Oxidation and decomposition of the heartwood extractives or changes in plant metabolism resulting from exposure to environmental or fungal agents could alter the chemical composition of the wood. Hasegawa and Shirato (1959) observed that the wood from a Prunus species, after sustaining a fungal attack, contained fewer flavanoids than unaffected material. Aside from having fewer flavanoids, the decayed wood contained a preponderance of a compound (a lignan) normally found in neither the wood nor the fungus.

A destructive sampling technique was utilized for obtaining heartwood samples. Falling the trees and subsequently cutting out a portion of the heartwood with a chain saw was necessary for obtaining sufficient heartwood. The extraction procedure necessitated obtaining approximately 1000 grams of ground heartwood. After removal from the tree, the samples were placed in plastic bags and stored at -20 degrees C. until needed for extraction.

Vegetative Buds

While in the process of conducting the heartwood analysis it was found that an inadequate amount of heartwood was present in the putative hybrids. Upon discovering this fact and reassessing the study, it became evident that the procedure was too limiting and efforts were made toward finding a technique that would be applicable to future studies involving statistically significant samples. It was resolved that a new technique must be non-destructive since the number of available hybrids is extremely limited.

Turner (1967a) suggested studying the terpene composition of needles or vegetative buds. This recommendation was founded upon knowledge of the successful use of gas chromatography for analyzing needle terpenes and reevaluating allopatric introgression in the genus Juniperus (von Rudloff et al., 1967).

The terpene content of needle primordia rather than fully developed needles was studied since this phase of the study was conducted after needles had been cast by the deciduous larch. Buds were collected from the lower branches on the north side of each study tree.

II. SELECTION OF STUDY TREES

In order to eliminate certain doubts regarding the genetic tenacity with which the chemical characters are controlled, it was essential to select study trees from diverse and geographically isolated sites. Figures 3 and 4 demonstrate the diversity of sites upon which subalpine larch study trees were found. It also should be noted that western larch study trees were selected from similarly diverse sites. Exogenous factors such as climate and soil are known to affect the quantitative occurrence of different plant substances while the qualitative chemical composition of individuals remains primarily constant (Flück, 1963). If the synthesis of a particular plant constituent is strongly controlled genetically, it should be present regardless of the environment. With current analytical methods the chemicals occurring within a plant should be detectable even if only small amounts are present.

Sampling over much of the range of the species is necessary in order to establish which characters are essentially unchanged by a changing environment. Such a technique allows one to determine the variation of any particular character throughout the population. It is imperative to determine if morphological as well as chemical differences are the result of genetic disparity or simply a consequence of similar genotypes interacting with dissimilar environments.

The possibility of recent genetic interaction between study trees was precluded by selecting sample points that were greater than 50 miles distant from one another (Location of sample trees, Figure 5). Thus, chemical likenesses between trees could be attributed to genetic

PHOTO 1

PHOTO 2

Figure 3. Illustration of subalpine larch site diversity. Photo 1 shows the sheltered cirque in which subalpine larch A grew. Photo 2 shows the contrasting windswept ridge upon which subalpine larch B was found. Both trees, used in the heartwood analysis, were found at about the same elevation.

PHOTO 1

PHOTO 2

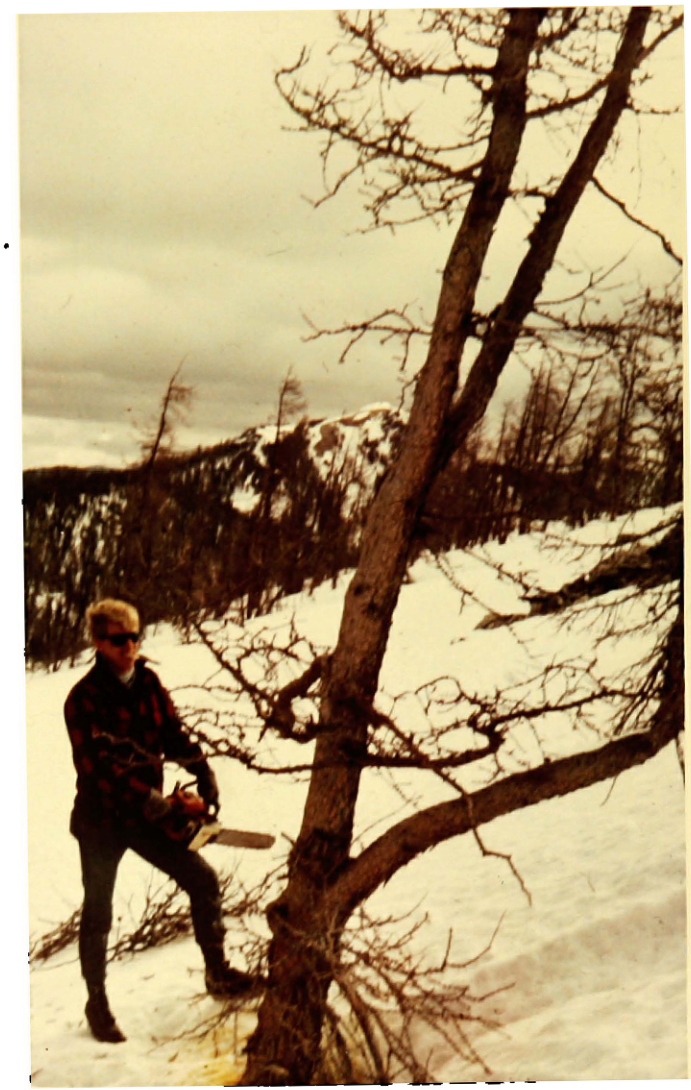

Figure 4. Difference in morphology of the subalpine larch used in the heartwood analysis. Photo 1. Subalpine larch A, a tree of good form found growing in a sheltered cirque, attained a height of 60 feet. Photo 2. Subalpine larch B, similar to subalpine larch A, was slow growing; however, it grew on a harsh, talus ridge and was only 20 feet in height.

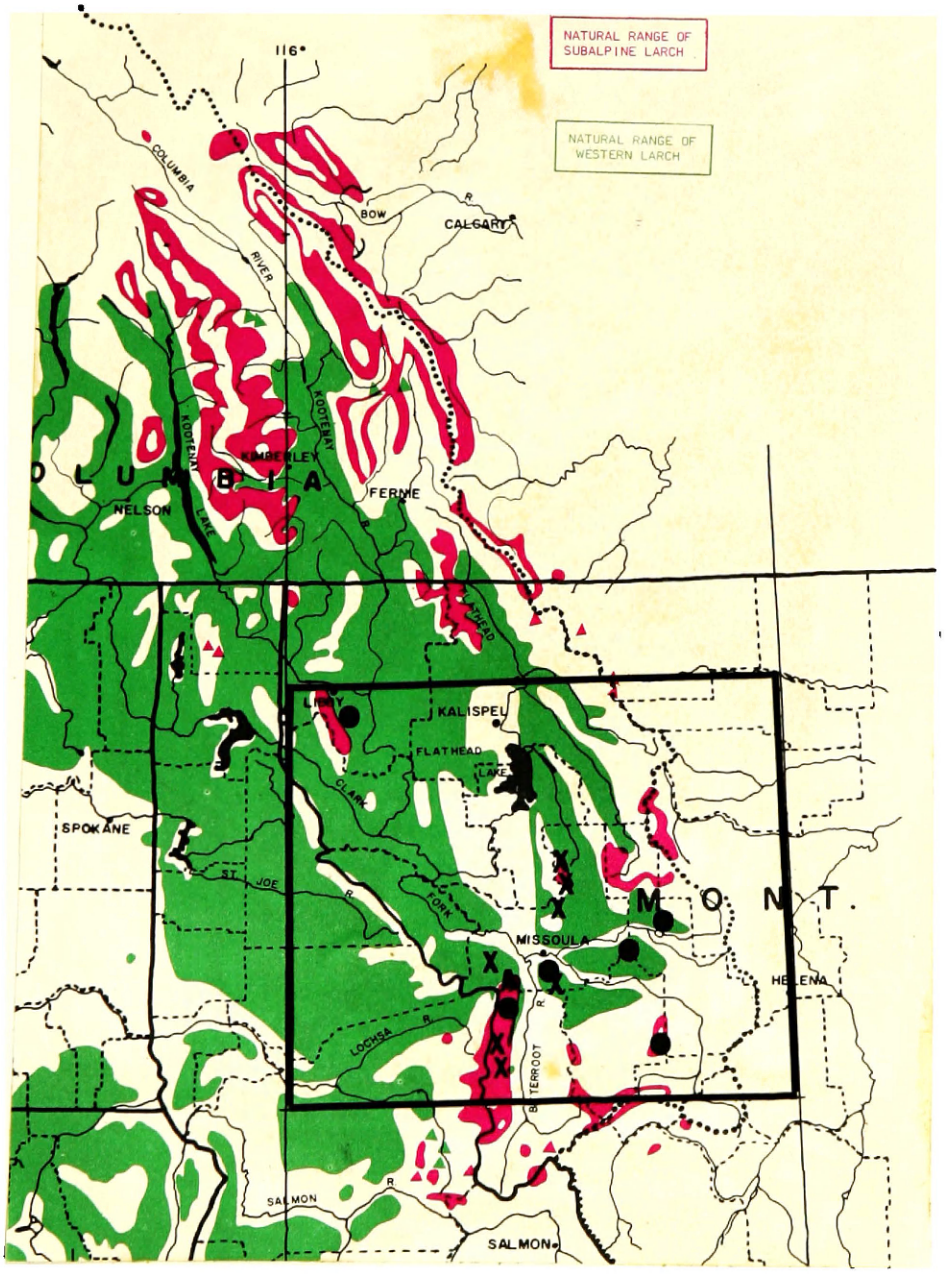

Figure 5. Location of study trees. The location of trees used in the heartwood analysis are indicated by circles. The location of trees used in the vegetative bud analysis are indicated by x-marks. The location of the putative hybrid (15 miles southwest of Missoula, Montana) is noted with a triangle.

similarities within the species and not to genotypes of closely related individuals.

The vegetative buds of a putative hybrid were sampled; however, not enough heartwood was present for an extractive analysis. The hybrid, a tree of intermediate morphological character between western and subalpine larch, was initially located and identified by Carlson (1965).

III. ANALYTICAL PROCEDURES

Chromatography

Isolation of the various compounds was accomplished with the use of thin-layer and gas chromatography. Chromatography is a technique devised in the early 1900's for separating mixtures into their constituents (Stein and Moore, 1951). It is based upon the principle of absorption, defined as the adhesion of a dissolved substance to the surface of a solid body. Significant refinements in the field of chromatography have been made possible due to the contributions of numerous individuals throughout the world. One such refinement, gas chromatography, has such resolving power that closely similar compounds occurring in concentrations of one part per billion are detectable as separate entities.

Thin-layer chromatography. Thin-layer chromatography, a rapid and simple technique, is another such refinement. The separations are performed on an absorbent paper or glass coated with a substance such as silica gel, magnesium carbonate, or certain clays. A small amount of the material to be fractionated is applied to the chromatographic sheet which in turn is placed in a tray containing a solvent. The solvent

flows over the material, moves it up the paper, and the various compounds involved are separated from one another. The distance a particular compound moves up the paper relative to the total distance traveled by the solvent is referred to as the Rf value and is expressed as a per cent. The Rf is primarily a function of the chemical and physical properties of the compounds involved, the absorbent properties of the paper used, and the environment in which the chromatogram is run. Detecting reagents, which react with specific groups of compounds, yield colored products on what would otherwise appear as a colorless chromatogram.

The procedural aspects of thin-layer chromatography are of such simplicity that biologists possessing only a rudimentary background in chemistry are able to establish tentative biochemical differences or similarities between species. Detailed chemical identification necessitates a more extensive knowledge of such procedures as fractional distillation, mass spectral analysis, or elemental analysis (Alston and Turner, 1963).

Gas chromatography. A highly sophisticated chromatographic technique is gas-liquid-chromatography. This sensitive chromatographic technique involves the use of absorbent columns through which flows a carrier gas. Compounds, depending on their chemical and physical nature, are moved through the column by the carrier gas at different rates. Emerging from the column, they are volatilized and with the use of a sensitive ionization detector and an elaborate electronic mechanism, each compound is recorded as a peak on a moving graph. Alston and Turner (1963) stated that crude extracts, with the aid of proper column

systems, can be chromatographed to produce complex chromatograms of use in the study of populations where natural hybridization is occurring. Systematic conclusions are possible even if the various chemical peaks on the chromatogram are not identified, although subsequent and more thorough analyses require their identification.

Heartwood Analysis

Extraction procedure. An extraction procedure employed by Nair and von Rudloff (1959, 1960) for analyzing the heartwood extractives of tamarack (Larix laricina (DuRoi) K. Koch) and subalpine larch was the basis of the procedure used in this study (Procedure for extracting larch heartwood, Figure 6). The procedure was adapted in order that compounds which are difficult to work with and which have little or no taxonomic value relative to the study were excluded from further analysis. Substances such as fatty acids and steroids were discarded and the taxonomically useful phenolic compounds were obtained for the subsequent chromatographic analysis. The heartwood from four trees was analyzed. Two of the trees were western larch and two were subalpine larch.

Isolation technique. The isolation of the heartwood extractives was carried out in an Eastman Chromogram Developing Apparatus. Between 10 and 50 microliters of petroleum ether insoluble material dissolved in benzene (10 mg/ml) was applied to Gelman Instant Thin-layer Chromatography Paper. The material was concentrated in a small spot, one inch from the bottom of the chromatographic sheet with a micro-syringe. Large spots, over 0.1 inch in diameter, resulted in poor separations.

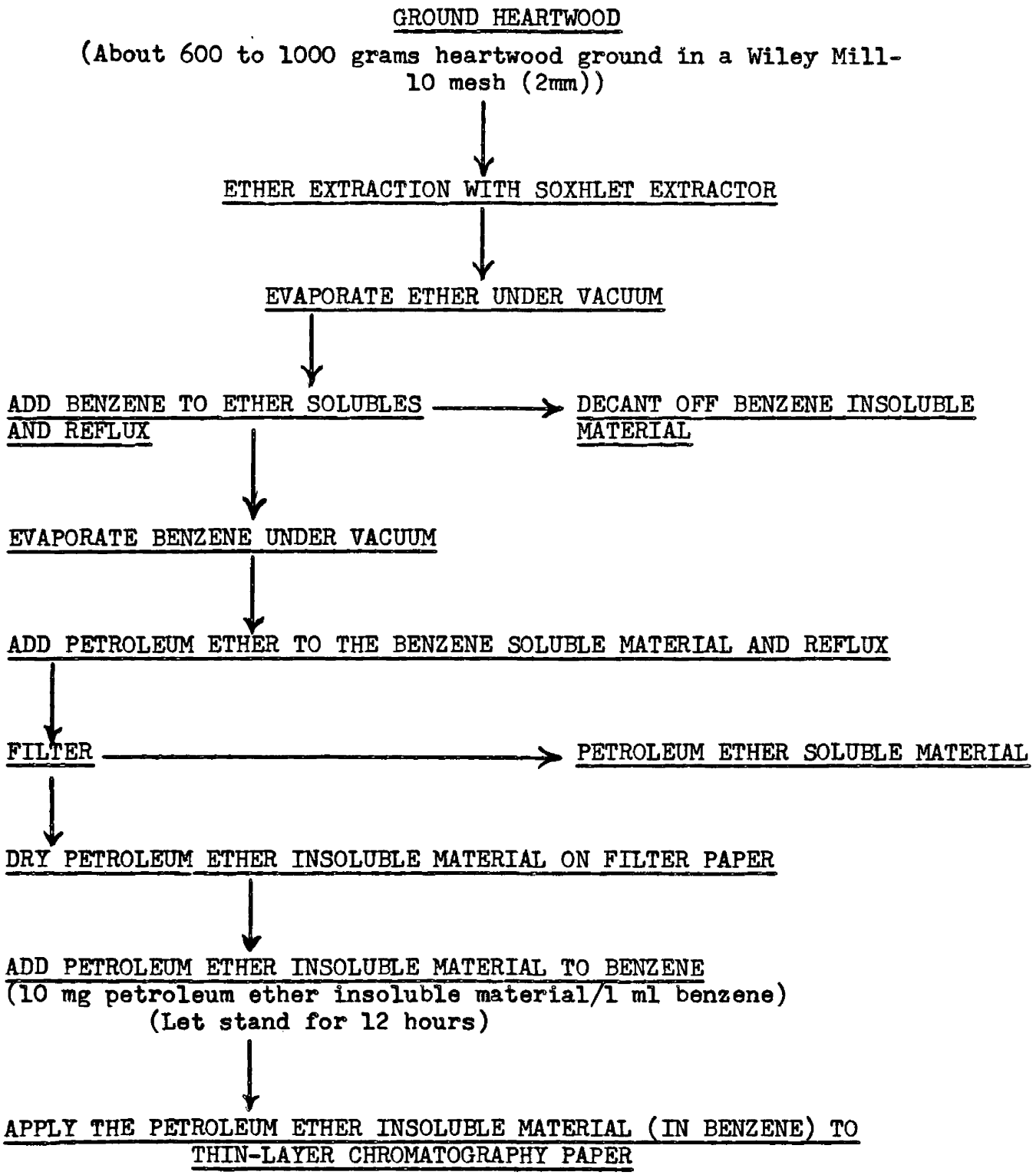

Figure 6. Procedure for extracting larch heartwood.

The developing solvent was a mixture of ether and benzene (1:9). A development time of 17 minutes was required for the solvent front to move within an inch of the top of the chromatographic sheet. Dried chromatograms were analyzed after being viewed in long wave ultra-violet light and then sprayed with either sulfuric acid-ethyl alcohol (1:1) or bis-diazotized benzidine (Table I, Appendix). Drying of reagent-treated chromatograms in a 120 degree C. oven for about 5 minutes was necessary for the colors to become visible.

Spectral analysis was used to determine if recurring UV active spots with the same Rf value, occurring in both western and subalpine larch, were the same compound. The procedure was also used to establish if a UV active spot found in both subalpine larch study trees was the same compound. The technique was not used for identifying chemical compounds. The procedure for obtaining the compounds for spectral analysis was relatively simple. The chromatographic spots were encircled under UV light, cut out, and eluted with ethyl alcohol from the chromatographic sheet, and analyzed with a Hitachi Recording Spectrophotometer in ethanol.

Vegetative Bud Analysis

Vegetative buds from western and subalpine larch, as well as one putative hybrid were sent to the laboratory of Dr. B. L. Turner in Austin, Texas, for gas chromatographic analysis.[1] Buds from six plants were analyzed; they were from three subalpine larch, two western larch

[1]Voucher specimens may be obtained from Dr. G. M. Blake of the School of Forestry, University of Montana, Missoula.

and one putative hybrid.[1]

The "single leaf" procedure entailed dissecting three or four buds and placing the green aromatic leaf primordia into a capsule of an indium alloy. The capsules were placed into the inductor unit of the chromatograph and heated to the melting point. The boiling point of the terpenoid fraction is approximately at the melting point of the indium alloy. When the capsule melts, the terpenoid material being in a vapor state, is injected into the chromatograph.

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[1]The samples were analyzed using a Varian Aerograph "Inductor" and gas chromatograph model 600D with a flame ionization detector, nitrogen carrier gas (20-25 ml/min), helium flow 25 ml/min, 5% polyethlene glycol (Carbowax 20M) on 80/100 gas chrom Q, DMCS treated, 15 feet x 1/8 inch stainless steel column.

CHAPTER IV

RESULTS AND DISCUSSION

I. RESULTS

Heartwood Analysis

One-dimensional chromatograms were established of the petroleum ether insoluble material. The results are presented with regard to the particular developing reagent applied to the chromatogram.

Sulfuric acid-ethyl alcohol reagent. A tracing of a representative chromatogram is illustrated in Figure 7. The ability to distinguish between the two species, based upon this technique appears obvious; however, the patterns were not always uniform.

When run on the same sheet of chromatographic paper, the patterns for the two subalpine larch were invariably the same. Comparisons made between runs indicated that although the patterns were similar, the number of spots varied. Chromatographic patterns for western larch were even more variable. There were always differences between the patterns of the two western larch on the same experiment and this difference was more evident between trials.

Some of the variation between experiments may be attributed to the presence of compounds in quantities at the threshold of detection. Although thin-layer development time is rapid, environmental fluctuations between experiments to alter the chromatographic patterns. All the compounds were probably present on the sheet; however, differences

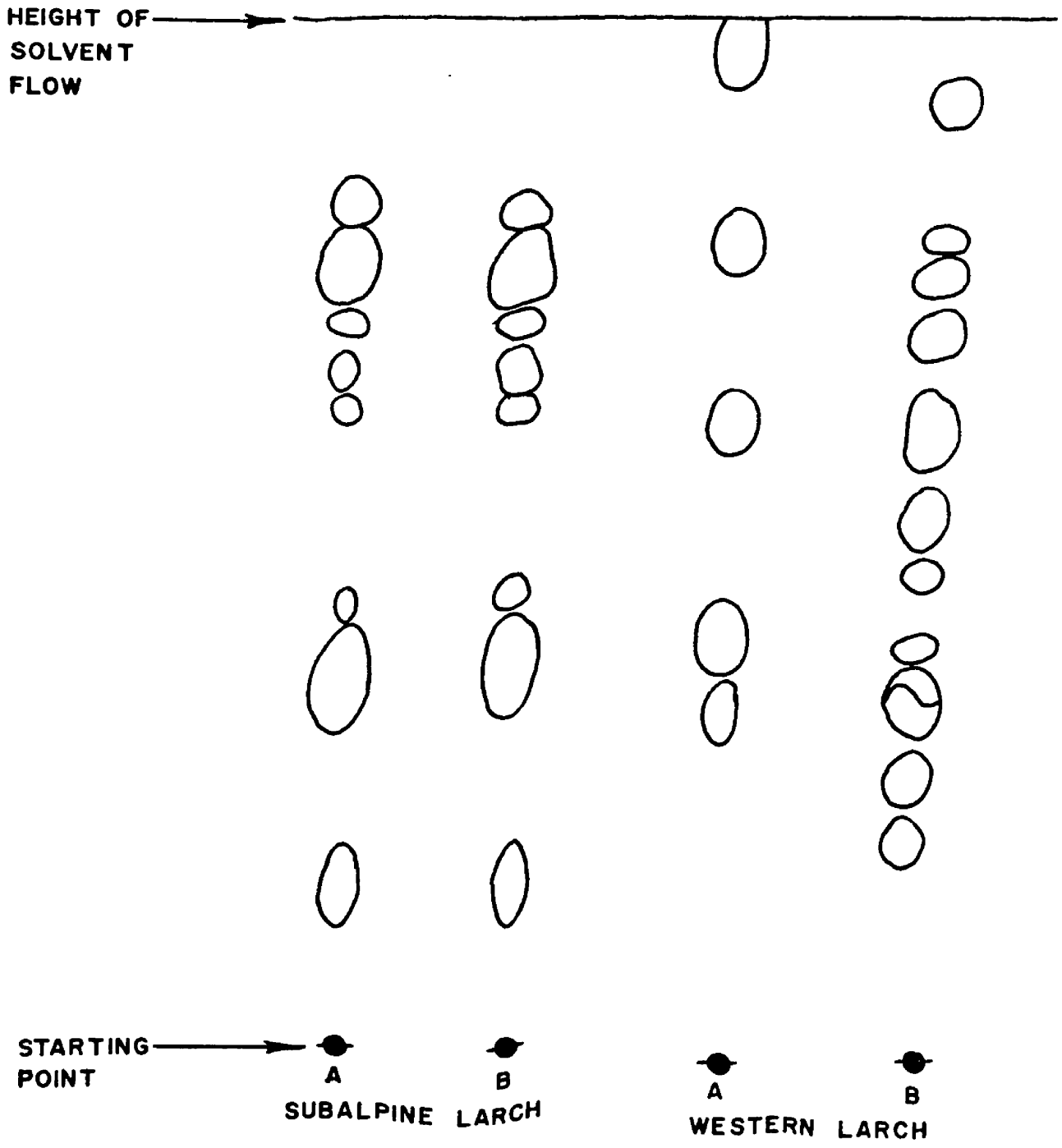

Figure 7. Tracing of a typical chromatogram developed with sulfuric acid-ethyl alcohol reagent.

in the chromatographic environment between trials, may have not always allowed certain substances to separate from one another.

The variation between the western larch study trees might be attributed to the presence of greater genetic and resulting chemical diversity within the species itself. The selection pressures acting upon subalpine larch are of such a specific nature that parallel development of similar genotypes may have occurred. Western larch, on the other hand, is found within a variety of sites and the selection pressures are somewhat more moderate and diverse. The pressure, being less selective, has allowed the evolution of a species with a greater genetic diversity.

Bis-diazotized benzidine and ultra-violet light. The use of bis-diazotized benzidine, a developing reagent that is specific for a group of phenolic compounds, was limited to an exploratory study of the two subalpine larch trees. The results are given since they illustrate a striking similarity between the two subalpine larch and they have a bearing on the ultra-violet analysis of the chromatograms.

Perhaps the most consistent differences found between western and subalpine larch were discovered when the chromatograms were viewed under ultra-violet light. Both species contained a UV active compound with a Rf value of .98 (Compound I); however, subalpine larch alone contained a compound with a Rf value of .70 (Compound II). Compound I was pale yellow in color while Compound II had a light blue appearance. The presence of Compound II was also evident when the chromatograms were developed with bis-diazotized benzidine, indicating that it is a phenolic compound (Figure 8).

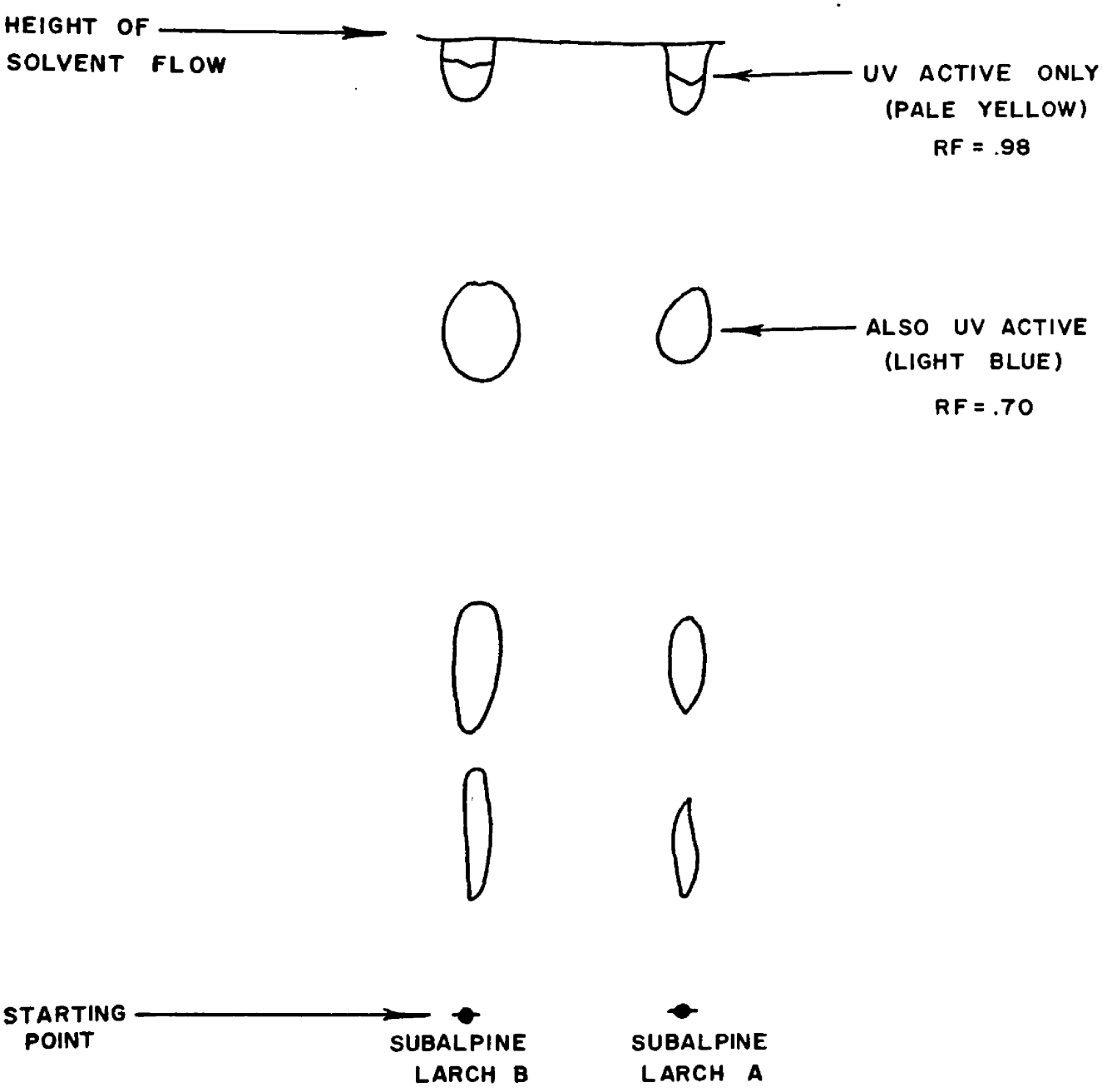

Figure 8. Tracing of a typical chromatogram developed with ultra-violet light. The subalpine larch material was also developed with bis-diazotized benzidine. Although not shown, the only UV active spot in western larch was at Rf = .98.

The results of the spectral analysis indicated that Compound I is the same in both species and Compound II is present in both of the subalpine larch trees studied (Figure 9).

Vegetative Bud Analysis

The findings of a gas chromatographic analysis of the vegetative bud terpenoid composition are shown in Figure 10. The chromatographic patterns shown are representative tracings for each of the species involved. Variation between the chromatograms within species was encountered although the patterns seemed relatively constant.

It appears that there are some qualitative and quantitative chemical differences between the parental species. The putative hybrid, due to probable genetic recombination, appears to contain more compounds than either parent. Although the preliminary chromatograms are promising, more study on analysis and identification of the individual compounds involved will be necessary before a significant taxonomic conclusion can be reached.

II. DISCUSSION

Heartwood Analysis

Although it was initially felt that sufficient hybrids contained heartwood for the study, it is possible that selecting this material was somewhat of an oversight. A technique involving heartwood is limited to the study of only those trees containing heartwood. Imposing such a limitation on a study would eliminate the investigation of instances where hybridization is very recent and the trees involved are only of seedling or sapling size.

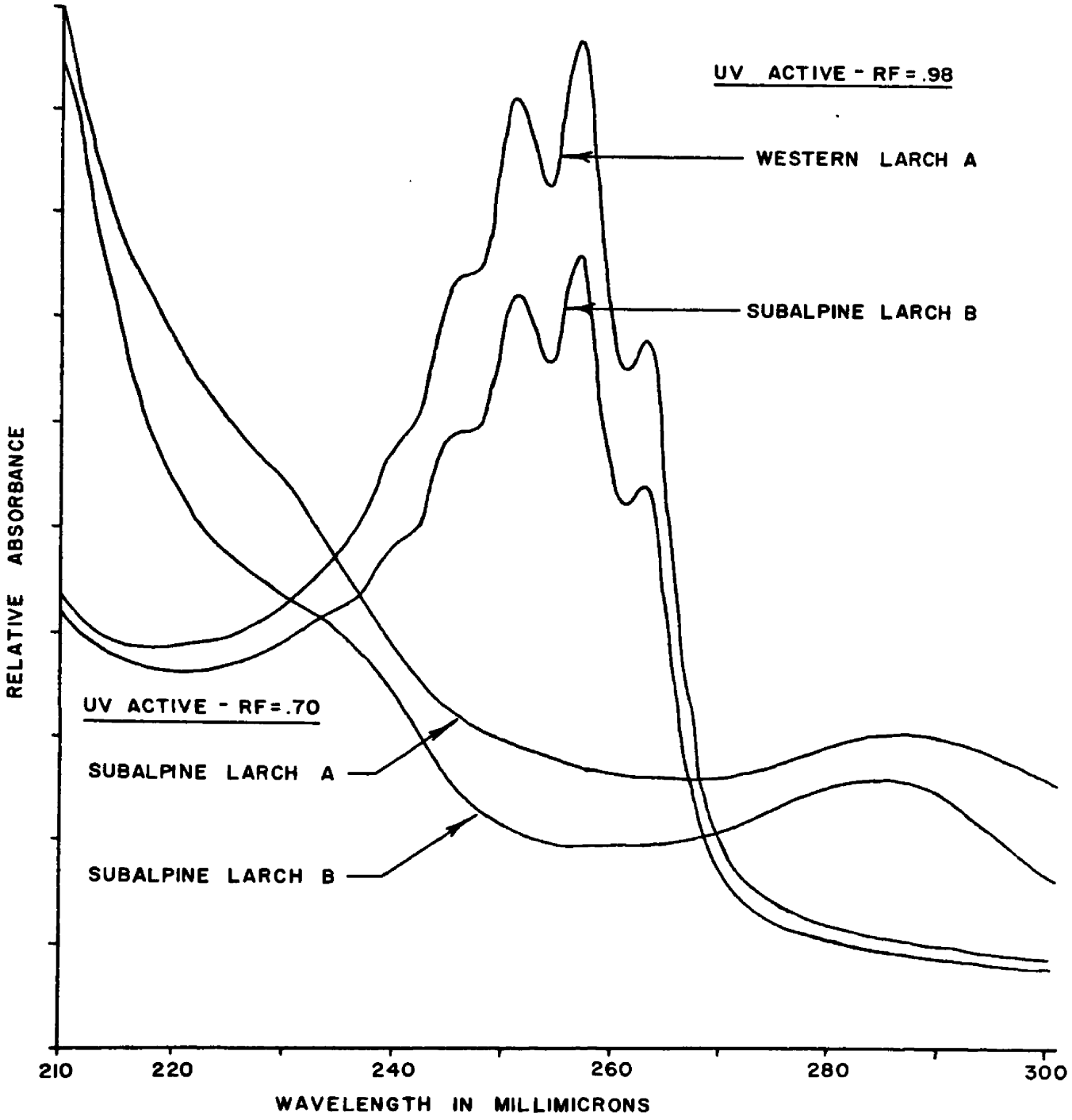

Figure 9. Ultra-violet absorbtion spectra of compound I (Rf = .98) and compound II (Rf = .70).

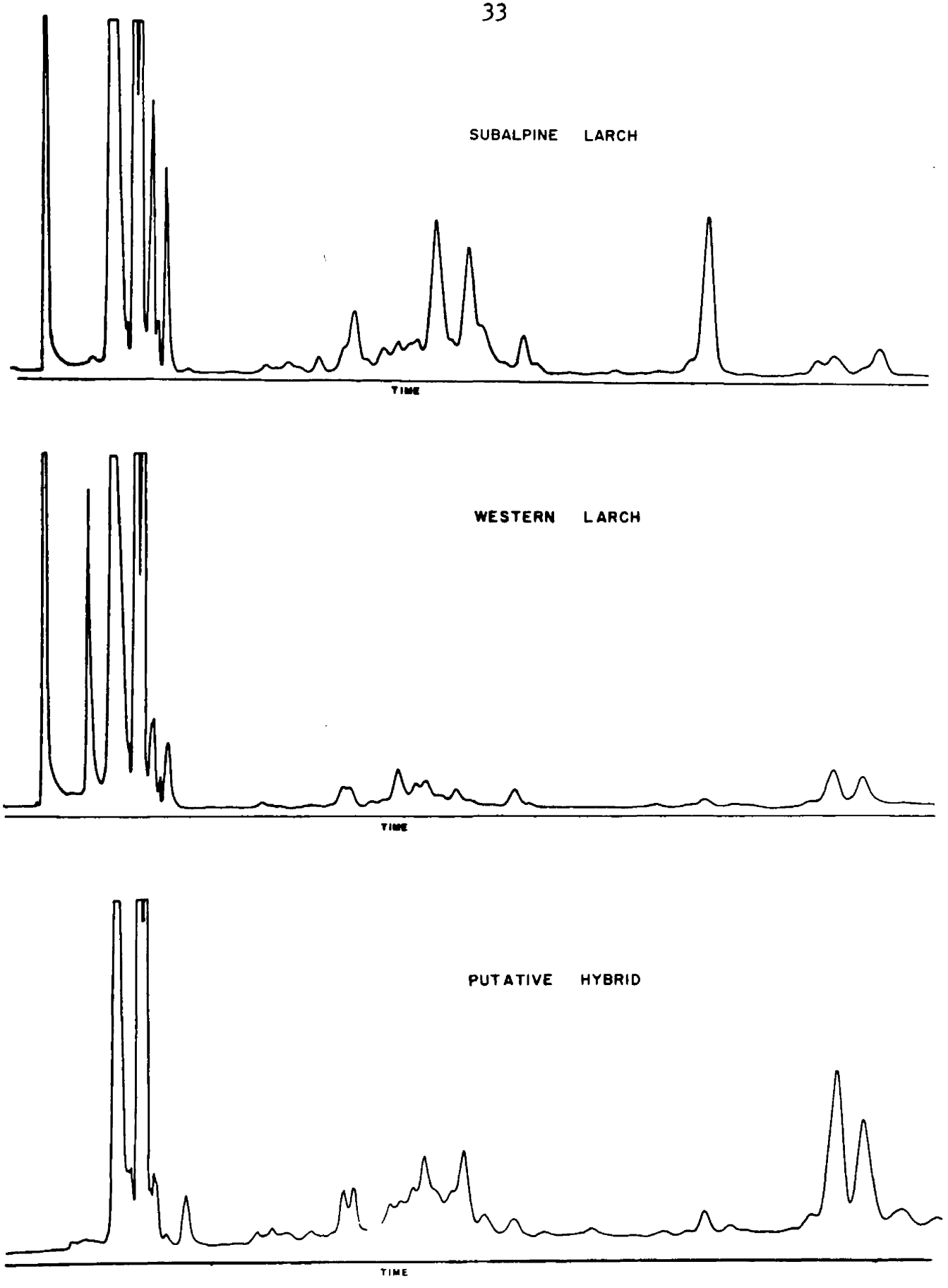

Figure 10. Gas chromatographic terpenoid profile of the leaf primordia of western and subalpine larch and a putative hybrid.

Difficulty was encountered in obtaining consistent chromatographic patterns. Perhaps the extraction procedure was carried too far. The presence of the phenolic compounds studied may have been significantly influenced by the environment and, therefore, were not reliable chemical characters. This may have been the case with western larch although the converse appears to hold true for subalpine larch.

Although a difficulty was encountered in the analysis of heartwood phenolics, it does not mean that subsequent endeavors with this problem should not include a study of phenolic compounds. Phenolic substances, in addition to being quite stable, have been investigated more thoroughly than many other substances currently utilized as taxonomic criteria. Alston and Turner (1963) noted that, "Certain of the phenolics have been the objects of a large number of productive biochemical genetic studies and also recently there have been important new advances in the knowledge of the biosynthesis of these compounds."

A possible alternative to the heartwood analysis might be study of the phenolic substances within the needles. Melnikoff and Shafizadeh (1968) adapted a procedure described by Seshadri (1962) for extracting phenolic compounds from the leaves of several Artemisia species with hot ether. A thin-layer chromatographic analysis of these phenolics was found to be repeatable and taxonomically valuable. In addition to being simple and fast, the procedure is non-destructive.

Vegetable Bud Analysis

Discovering an inadequate amount of heartwood in the hybrids directed efforts toward devising a non-destructive technique. The apparent consistency of the preliminary chromatograms and the presence of several

terpenoid compounds appears quite promising. Although the trees were from diverse and isolated sites, the terpene composition of the needle primoria appeared to be relatively constant. Similar chromatographic patterns obtained from a krummholz subalpine larch and a tree over 80 feet tall, both of which occurred over 90 miles from one another lends an air of optimism to the findings.

The results indicate that future efforts toward making chemotaxonomic distinctions between the three individuals might also involve a study of the needle terpenes. Since the preliminary findings were variable, Adams (1968) recommended that a single leaf analysis be abandoned in favor of an investigation of the steam distillate (terpenes) from 20 to 50 grams of fresh foliage. Variation in the terpenoid content of single leaves might be a result of their position on the tree. Studying terpenes of needles from throughout the crown allows one to base conclusions on the composition of much of the tree. Decisions based on the terpene composition of single needles may result in considerable intra-tree variation. Studies of foliage terpenes in Juniperus species have produced repeatable and very consistent results (von Rudloff et al., 1967). The necessity of collecting foliage in the same stage of development such that it is comparable chemically would be insured by collecting mature, healthy needles in mid-July. Deterioration of the foliage could be prevented by freezing it in plastic bags until needed for analysis.

CHAPTER V

SUMMARY AND CONCLUSIONS

The study is part of a long-term project studying the genetic relationships and silviculture of western larch. The purpose of the study was to determine if a chemotaxonomic procedure is feasible for determining if western larch and subalpine larch hybridize naturally.

The first phase of the study involved the use of thin-layer chromatography for analyzing heartwood extractives (phenolics) and determining if consistent chemical differences exist between the two species. There was considerable variability in the chromatographic patterns obtained and it was difficult finding consistent similarities within the species. The lack of sufficient heartwood in the putative hybrid precluded further investigation of the heartwood extractives. An alternative to an analysis of heartwood phenolics is a study of needle, twig or bark phenolic substances.

The second phase of the study involved the gas chromatographic analysis of vegetative bud terpenes. This procedure, like a study of needle phenolics, is applicable to studies of recent hybridization where the trees under consideration contain insufficient heartwood. Since the method is non-destructive, it does not eliminate trees from future studies. The chromatographic patterns, although somewhat variable, were promising.

The study has shown that a chemotaxonomic technique offers promise toward resolving this case of apparent interspecific hybridization.

The gas chromatograms were too variable and the sample too small to state conclusively whether hybridization has occurred. Further study of the needle terpenes from larger samples, in addition to a qualitative analysis of the compounds involved, may allow a more definite taxonomic conclusion to be reached.

Recently several areas where hybridization is possible were located (Arno, 1968). Convergence of western and subalpine larch has been observed in the Bitterroot and Cabinet Mountains of western Montana. The species converge on a rocky outcrop near 7000 feet and on an old burn at 7200 feet in the north Bitterroots. It is very likely that the two species exist sympatrically on the north side of little Saint Joseph Peak also in the north Bitterroots. Two areas of range overlap in the Cabinet Mountains west and southwest of Libby, Montana, were located recently by aerial reconnaissance. Convergence was observed on the northeast side of McDonald or Cable Mountain at 6000 feet. An extensive range overlap was also sighted on a broad, apparently undisturbed slope on the east-northeast side of Indian Head Peak at an elevation of about 6000 feet.

If either the needle terpene or a plant phenolic analysis proves successful for analyzing this instance of suspected natural hybridization, efforts toward establishing where further hybridization is occurring will be greatly facilitated.

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APPENDIX

LITERATURE CITED

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APPENDIX

TABLE I

PROCEDURE FOR PREPARING BIS-DIAZOTIZED BENZIDINE

1. Prepare the following solution:
   a. 5 g benzidine
   b. 14 ml conc. HCl
   c. Filter
   d. Dilute to 1000 ml with distilled water
2. Add 2 parts of the above solution to 3 parts 10% $NaNO_2$
3. Use the solution within 2 to 3 hours.