Is *Alnus viridis* 'a' Glacial Relict in the Black Forest?

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Dedicated to my husband and my son

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CONTENTS

3. RESULTS

List of abbreviations

R	red light
FR	far-red light
B	blue light
UV-A	ultraviolet A (indicate the wavelength range)
UV-B	ultraviolet B (indicate the wavelength range)
EDTA	Ethylendiamin-tetraacetic acid
PCR	polymerase chain reaction
TRIS	tris-(hydroxymethyl)-aminomethan
TE	Tris-ethylendiamintetraacetic acid
RPM	rotation per minute
kB	kilobase
M	molar
CTAB	Cetyltrimethylammoniumbromide
bp	base pair
Myr	Million years

1. Introduction

1.1 Ice ages and vegetation

Ice ages are geological periods when large portions of land were covered by ice (http://www.museum.state.il.us/exhibits/ice_ages). The earliest ice ages can be dated back to Precambrian times (more than 600 million years ago), followed by a second ice age during the early Cambrian (about 600 million years ago), and a third ice age during the Carboniferous and Permian periods (about 350 and 230 million years ago). The most recent ice age occurred during the Pleistocene beginning approximately 1.6 million years ago begin and ending only ten to tweenty thousand years before our time. The pleistocene ice age consisted of several glacial and interglacial stages. During the period of an ice age, ice forms on the mountains and flows downwards along frozen rivers called glaciers. At the time of the glacial stages, the ice layer was quite thick covering wide areas of land for some ten thousand years. At the time of the interglacial stages lasting for some twenty thousand years or more, most of the ice melted and became confined to the tops of mountains and the polar regions.

The transition between glacial and interglacial periods are not really understood, but are probably related to subtle fluctuations in the rotation of the Earth around the sun (Kutzbach et al., 1991; Harrison, 1995). For over a century, geologists have turned to astronomy for explanations for the cause of glaciation during the Pleistocene Epoch (the past 1.6 million years). Fluctuations in the amount of insolation (incoming solar radiation) are the most likely cause of large-scale changes in the terrestrial climate and variations in intensity and timing of solar heat are the most likely trigger for the glacial/interglacial cycles of the Pleistocene Epoch. Numerous theories have been suggested for the primary causes of the ice ages. Already, in the 1870ies, James Croll first suggested that the ice ages were caused by changes in the amount of solar irradiance received at the poles as a result of changes in the shape (with a frequency of about 100,000 years), tilt and wobble (with frequencies of about 40,000 and 20,000 years) of the Earth's orbit around the sun. However, most of the scientist suggest that not only increases of solar radiation caused the ice ages, but also changes of continental position, uplift of continental blocks, and reduction of CO_2 in the atmosphere complemented the changes of terrestrial orbit. In the 1930ies, Milankovitch suggested that orbitally induced variations of solar irradiance at 60^{o} N latitude drove growth or shrinking of ice sheets in North America and Europe.

1.2 Survival strategies of glacial relicts

The ice ages with their dramatic and large-scale changes of climate have had a major impact on the distribution of all organisms, may it be plants or animals. For plants as sessile organisms, this impact is even more pronounced as compared to animals that to a certain extent can respond by individual migration. It is certainly not exaggerated to state that in addition to continental shifts, the ice ages are the main factor shaping biogeographic areals of plant species. A given plant species will be able to tolerate the changes of climate occurring during glaciation to a certain degree that is defined by its physiology. For instance, chilling-sensitive species (such as most tropical crops, e.g. cucumber, cotton, most fruits) will already suffer irreversible damage, when temperatures fall below a certain threshold that is still above the freezing point. In contrast, freezing-resistant species are able to tolerate certain periods, where temperature falls below zero. Nevertheless, virtually all plant life gets extinct, when an area is covered by a solid cover of ice. This means that extending ice front will "push" away vegetation from their indigenous area. Along the edge of the ice shield a gradient of species will emerge with freezing-tolerant species being able to settle closer to the ice, whereas sensitive species will move away to more distant areas with warmer climate. During an interglacial period, the gradient will follow the retracting ice front and repopulate the previously ice-covered area. It is clear that these area shifts will lead to disintegration of distributional areas depending on the topography of the area passed during these movements.

Therefore, numerous biogeographic studies on plants have focussed on the postglacial spread from peripheral or periglacial refugia or on the possibility of long-term survival in so called *nunataks* (an Inuit word meaning ice-free mountains emerging from the surface of a glacier) within glaciated areas (Bennett et al., 1991; Abbott et al., 1995; Demesure et al., 1996; Gabrielsen et al., 1997; Soltis et al., 1997; Tollefsrud et al., 1998). Currently, two main models are discussed for the glacial survival of plant taxa (Stehlik, 2000). The *tabula rasa* hypothesis postulates that a given species survives mostly in peripheral refugia and subsequently reimmigrates into the vacant areas after the retreat of glaciers. The *nunatak* hypothesis assumes that a species persists over a long time *in situ* within the glaciated regions in ice-free locations above the ice-shield (*nunataks*) and from their spreads into neighbouring, vegetation-free areas after glaciations (**Figure 1**).

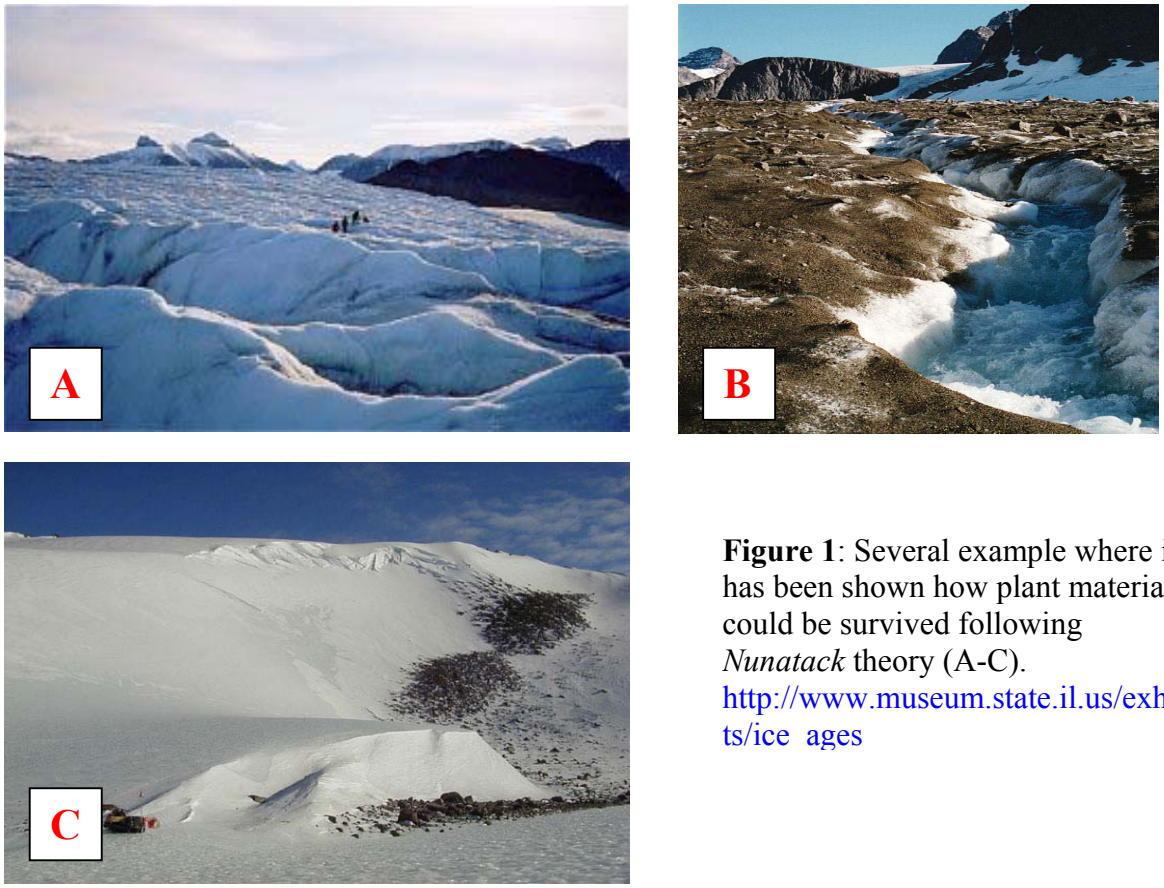


Figure 1: Several example where it has been shown how plant materials could be survived following *Nunatack* theory (A-C). http://www.museum.state.il.us/exhibits/ice_ages

1.3 *A. viridis* as a model for glacial relicts

Among other species, the Green Alder (*Alnus viridis*) represents a classical example, where the influence of glaciation upon recent distribution can be studied. Belonging to the Betulaceae, the genus *Alnus* together with the genus *Betula* forms a distinct tribe, the Betulae (Crane, 1989), that can be distinguished from the Corylae (*Corylus*, *Ostryopsis*, *Carpinus* and *Ostrya*). The Alder (*Alnus*) is an early-successional monoecious species that lives in symbiosis with the nitrogen-fixing actinomycete *Frankia* (Normand and Lalonde, 1986; Normand and Bousquet, 1989). Most species of the *Alnus* genus are distributed in temperate regions of the Northern Hemisphere (Asia, Europe and North America), although some species occur as far South as Northern South America (Bousquet et al., 1988; Woodland, 1991). However, Green Alder (*A. viridis*) is mainly confined to Europe and central Quebec in North America. It shows several features that are typical for a pioneer species: it typically forms a shrub, often with multiple stems that may form dense thickets. Plants may grow up to 2.5 m, occasionally up to 4 m at lower elevations (at lake shores), but usually occurs at middle to high elevations. The bark is brownish in colour, leaves ovate or elliptic, glabrous above but pubescent below, the margins

serrated with very fine teeth. As a consequence of the symbiosis, the chlorophyll in the leaves is not mobilized in fall, such that the leaves are still green, when they drop in autumn. The twigs possess sessile, pointed axillary buds with male catkins and female flowers on same plant (**Figure 2**). Flowering takes place from May to June with fruits maturating in July. Fruits are small and dry woody cones. Green Alder reproduces sexually by seeds, but also vegetatively by rhizomes. The seeds of *A. viridis* are light, ripen during late August and September depending on latitude and elevation and are dispersed by wind and water through April of the next year. Germination usually requires exposed mineral soil. This means, it will take place mainly in disturbed habitats such as they are naturally found along streams or lake shores or in consequence of anthropogenic influence along road sides. It can also occur along muskeg margins or as an understory component in conifer forests, usually associated with some source of moisture with sandy to gravelly or rocky soil. The nitrogen-fixing activity of the symbiot is mainly manifest under humid conditions. Plants are semi-shade tolerant but cannot cope with closed canopies. After fire, sprouts from the root crown along with seeds from plants in adjacent unburned sites rapidly resettle the destroyed area. These shrubs provide shade that reduces soil temperatures, allowing spruce and other trees to establish. Green Alder thus represents a typical pioneer species that occurs after disturbances of the vegetation, may it be by natural conditions or in consequence of human influence.

In Europe, this species is mainly found in the sub-alpine region of the European Alps (1500-2000 m altitude above sea level, also up to 2800 m), the Carpathian mountains, and the Dinarid mountains (Richard, 1967). Moreover, this plant is also distributed through the Alpine foreland, ranging from 500 m to 900 m above sea level (Dörr and Müller, 1982; Bresinsky, 1965), and in isolated areas in the German *Mittelgebirge*, respectively (see **map 1**). Different scientist suggest that the Alpine foreland populations are secondary (Bresinsky, 1965), and have immigrated from refugial areas into the Alpine foreland, after the long Mindle ice age that contiguously covered the Alpine foreland.

The rich fossile record of angiospermic pollen had been used to deduce the distribution dynamics of *Alnus* during the Pleistocene. During the temperate stages of the earlier Pleistocene, *Alnus* pollen is steadily found throughout the interglacial with highest frequencies in the later interglacials. During the Cromerian Interglacials (oxygen isotope stage 11), *Alnus* was abundant at several sites in the Cromer Forest Bed (Godwin, 1975). *Alnus* pollen is recorded throughout the Hoxnian Interglacial (oxygen isotope stages 7 to 9) and particularly

abundant in the mid-interglacial warm phases at several sites in East Anglia and the Midlands of England along with a strong macrofossil record (Godwin, 1975).

Figure 2: Morphology of *A. viridis* (Chaix DC.).

In the Ipswichian Interglacial (oxygen isotope stage 5), *Alnus* pollen frequencies are much lower except where directly associated with alder macrofossils and *Alnus* may not have been generally abundant during that period (Godwin, 1975; West, 1978). *Alnus* pollen does occur in glacial stages of the Pleistocene, including the mid-Devensian. Late Devensian records are quite numerous, and occasional macrofossils also occur, for instance at Willow Garth on the Yorkshire Worlds (Bush and Colinvaux, 1990). These records may be derived from earlier deposits or results from long distance transport (Tallantier, 1978). *Alnus* macrofossils have been reported in an early Holocene context in the south of England (Clarke and Jones 1980a) but *Alnus* pollen frequencies, although widely present remain very low in the early stages of the present interglacial (Bennett et al., 1991). As *Alnus* pollen is well transported by wind and water, these low frequencies suggest no major local populations of alder around these sites at this time, although the existence of small isolated populations remains possible. Such very early centers of *Alnus* growth, probably controlled by local edaphic conditions, would not be easily visible palynologically unless located very close to pollen sampling sites. The existence of local isolated centers of alder growth suggested by the early Holocen records may have provided locations from which *Alnus* could have expanded quickly without immigration from external sources.

Under certain circumstances *Alnus* may be regarded as a pioneer tree favoured by vegetation disturbance due to human or animal (beaver) influence (Bennett et al., 1991). *Alnus* is light-demanding and without disturbance may not be able to colonize the closed canopies of deciduous forests.

From these findings, the following scenario has been suggested for the last glacial that was very cold and dry throughout Europe: Large ice sheets were present throughout much of Northern Europe, and ice caps covered the Alps and Pyrennes mountains. Forest and woodland were almost non-existent, except for isolated pockets of woody vegetation or close to the mountain ranges of Southern Europe. Instead, a sparse grassland or semi-desert covered most of Southern Europe, whilst a mixture of dry, open *Steppe* tundra and polar desert covered those parts of Northern Europe that were not occupied by ice sheets. During that time a boreal conifer forest (birch, pine and other conifers) was present in central France, and a similar vegetation may have extended across much of central Europe, whereas Northern Europe was covered by tundra. Various geomorphological and isotopic temperature indicators suggest that all parts of Europe, especially South central Germany, were much colder than today in last glacial period. In that time the Northern-most region of Poland and Germany, close to the Scandinavian ice sheet, was a shrub tundra (Madeyska, 1990). These forest and shrub tundra lakes were dominated by *Alnus*. Plant seeds may have migrated from that refugia after the retreat of the glaciers. Alternatively, *Alnus* may have survived in *nunataks* in the glaciated area.

Interestingly, a *A. viridis* population is found in the central and Southern parts of the Black Forest ranging from 300 m to 1200 m above sea level (Oberdorfer, 1957; Wilmanns, 1977) (**map 1**). A wealth of ecological, floristic and sociological data (including interviews with old inhabitants from that region) collected over nearly two and a half decades led to the belief that the small *A. viridis* populations found in the Black Forest between 550 m to 1000 m altitude, represent glacial relicts (Hegi, 1957; Bresinsky, 1965; Braun, 1975; Wilmanns, 1977).

However, although this conclusion is plausible, it leaves open several alternative possibilities: For instance, it would be possible that several secondary populations of Green Alder exist in the Black Forest that have migrated from peripheral refugia after the retreat of the glacials. Or the divergence from the Alpine population occurred during the interglacial period when the warm climate in the lower areas allowed a dense forest to develop that would not allow Green Alder to survive. Or the species survived over a long time by vegetative propagation from

rhizomes that regenerated occasionally, when the conditions were favourable (e.g. local disturbance of the canopy).

To discriminate between these and possibly additional alternatives, it is important to assess the relationship between the populations in the Black Forest and Alpine populations of Green Alder. A morphological comparison between *A. viridis* from different sites in the Black Forest with specimens collected in the Lechtaler and Swiss Alps (Brüchert et al., 1994; Gallenmüller et al., 1999; Speck, 1996) suggest that these specimens differ with respect to their morphology and biomechanics. However, it remains unclear, whether these differences are due to genetics or caused by differences in the environment, where these specimens were collected. The Alpine specimens were collected at high altitudes (1900 m altitude above sea level) in avalanche tracks, where they were exposed to heavy snow loads or creeping wet snow (Brüchert et al., 1994; Gallenmüller et al., 1999). In contrast, Green Alder in the Black Forest typically grows along river banks, road margins or the edge forest canopies. It is not astonishing that these plants differ in terms of morphology and biomechanics from their Alpine counterparts. It will be very difficult, if not impossible to assess the evolutionary relationship between different specimens grown under different environmental regimes by means of biomechanics.

1.4 Scope of the study

To assess the evolutionary relationship between different species or populations of a species, it is necessary to analyze genetic diversity in the context of population history and present-day distribution (Soltis et al., 1997; Taberlet et al., 1998). For this purpose, molecular markers provide powerful tools to assess genetic diversity. Using a chloroplast DNA-marker, I therefore ventured to understand the evolutionary relationship between different populations of Green Alder (*A. viridis*) from the Black Forest and the European Alps.

The following goals should be achieved-

- To produce a sufficient dataset for this chloroplast DNA marker for different populations.
- To complement previous studies by a novel approach using this molecular marker to characterize the evolutionary relationship between the different populations.
- To estimate the evolutionary time scale for the divergence of these populations.

- To clarify, whether the Black Forest populations are true glacial relicts that had diverged from the Alpine populations during the ice ages or whether they have immigrated more recently, i.e. after the end of glaciation.
- To obtain information on the extent of gene flow between the different populations of the Black Forest.
- To test, whether the divergence has already been accompanied by differences in the physiological response using photomorphogenesis (hypocotyl growth, secondary metabolism) as trait.

This study aimed to a novel and experimentally based insight into the evolutionary dynamics of *A. viridis* and test, among others, the idea that this species is a progressive glacial relict (Wilmanns, 1977). The results have also strong impact for nature conservation. The thesis is subdivided into two main parts. The first part will deal with photomorphogenesis and ask, whether the Black Forest population of Green Alder have already diverged to such an extent from their Alpine counterpart that physiological parameters of adaptive value are altered. The second part will deal with the findings based on the use of molecular markers.

2. Material and Methods

2.1 Material

2.1.1 Chemicals

Acetic-acid	Roth, Karlsruhe
Agarose	Sigma, München
β-mercapthoethanol	Merck, Darmstadt
Cetyl trimethyl ammoniumbromide (CTAB)	Sigma, München
Chloroform, p.a.	Roth, Karlsruhe
Deoxyribonucleotides (ATP, CTP, DTT, GTP: dNTPs)	Invitrogen, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethylene diamintetra-acetic acid (EDTA)	Roth, Karlsruhe
Ethidiumbromide	Sigma, Münche
Formaldehyde	Roth, Karlsruhe
Hydrochloric acid	Roth, Karlsruhe
Isopropanol (2-propanol)	Roth, Karlsruhe
Lithium chloride	Roth, Karlsruhe
Loading buffer	MBI, Fermentas, Wilnius
Magnesium chloride	Invitrogen, Karlsruhe
PCR buffer	Invitrogen, Karlsruhe
Sodium chloride	Roth, Karlsruhe
Tris-(hydroxymethyl)-aminomethane	Roth, Karlsruhe
TE Buffer (pH 8.5)	Pharmacia-Biotech, Freiburg
Isoamylalcohol	Merck, Darmstadt

2.1.2 Kits

PCR purification Kit (QIAGEN Kit)	Qiagen, Hilden
PCR purification Kit (Amersham Kit)	Pharmacia-Biotech, Freiburg

2.1.3 Enzyme

Taq polymerase	MBI, Fermentas, Wilnius
RNase A	Roche Mol. Biochem, Mannheim

2.1.4 Primers/Oligonucleotide:

The following Primers were used in the present study. All primers were made by Dr. G. Igloi, sequence lab, Institute for Biology III, Freiburg University and Sequence laboratories GmbH., Gottingen, Germany.

Region	Primer name and sequence (5′ to 3′)	Designed by
trnT-trnF		
A (F)	CAT TAC AAA TGC GAT GCT CT	Taberlet et al.(1991)
B (R)	TCT ACC GAT TTC GCC ATA TC	Taberlet et al.(1991)
C (F)	CGA AAT CGG TAG ACG CTA CG	Taberlet et al.(1991)
D (R)	GGG GAT AGA GGG ACT TGA AC	Taberlet et al.(1991)
E (F)	GGT TCA AGT CCC TCT ATC CC	Taberlet et al.(1991)
F (R)	ATT TGA ACT GGT GAC ACG AG	Taberlet et al.(1991)

2.1.5 DNA Marker

Lambda DNA/*Eco*471 (AvaII) Marker, 13 (MBI, Fermentas, Wilnius)

Bands were 8126, 6555, 6442, 3676, 2606, 2555, 2134, 2005, 1951, 1611, 1420, 1284, 985, 974, 894, 597, 590, 513, 511, 433, 398, 345, 310, 308, 272, 242, 215, 151, 88, 73, 67, 45, 42, 32, 29, 23 bp, respectively.

2.1.6 Accessory

Centrifuge	ZK 15, Rotor Nr. 12145, 11411	Sigma, Deisendorf
Centrifuge	5415D	Eppendorf, Hamburg
Centrifuge	5804R	Eppendorf, Hamburg
pH- electrode	pH-meter CG 820	Schott Geräte, Mainz
Vortex	VF2	Eppendorf, Hamburg
Speed Vac Concentrator	SVC 300H	Bachhofer, Mannhaim
Spectrophotometer	Uvikon Nr. 940	Kontron, UK
Thermomix	T-5437, 5435	Eppendorf, Hamburg
Balances	853062	Braun, Melsungen
Universal UV-Lampe	TL-900, 254 u. 360 nm	Camag, Berlin
Chromatography paper	2043 BMGL	Schleicher & Schuell
Chromatography tank (Glass)		Desaga, Heidelberg
Eppendorf tube	1.5 ml, Nr.3810	Eppendorf
Eppendorf tube	2 ml, safe lock	Eppendorf
Falcon tube	15 /50 ml	Greiner, Germany
Plastic boxes	10 x 10 x 12 cm	Gerda, Schwelm
Spectophotometer	model 35	Beckman, Fullerton, CA, USA
Cuvette	1 cm width	Hellma
UV-absorption filters	WG 360 WG 305 10 x10 x 0.3 cm	Schott, Mainz
Blue, Red and Far-red light absorption filter	10 x10 x 0.3 cm	Schott, Mainz
PCR cycler		Cyclon 25 Peqlab, Biotech. GmbH.
Water bath		

2.1.7 Light sources

Far-red (FR)

Red bulb: TL 36/15 (Philipps), covered with 3 mm color glass PG 501 (Röhm & Haas, Darmstadt)

Maximum intensity λ_{max} = 658 nm

Halfband width = 24.5nm,

Fluence rate = 6.5 W m^{-2}.

Red (R)

Red bulb: 120 W/235 V (Linestra, Osram), combined with 3 mm heat-filtering glass KG 3/2 (Schott, Mainz) and Plexiglass PG 501/3 and 2x PG 627/3 (Röhm & Haas, Darmstadt).

Maximum intensity λ_{max} = 740 nm

Half band width =123 nm

Fluence rate = 3.5 W m^{-2}

Blue (BL)

Red bulb: 9x TL 40 W/18 (Philipps) covered with 3 mm colour glass PG 501 (Röhm & Haas, Darmstadt).

Maximum intensity λ_{max} = 450 nm

Half band width =123 nm

Fluence rate = 10 W m^{-2}

***Safety Green light*:**

The green light sources used in these experiments are described in Mohr & Appuhn (1963).

Red bulb: D-40 W/235 v (Osram) combined with Plexiglass PG 303/3 and PG 0248/3 (Röhm & Haas, Darmstadt).

UV-B/White light(UV/WL):

Red bulb: a) 2x L 40 W/ 73 (UV-A), Osram

b) 2x TL 40 W/ 18 (blau), Philipps

c) 1x TL 40 W/12 (UV-B), Philipps

This light source was filtered through 3 mm (Schott and Gen., Mainz, Germany) WG type transmission cutoff filters. The fluence rate of each filter from the measuring distance (28 cm) was:

WG 305: 11.9 Wm^{-2}

WG 360: 8.3 Wm^{-2}

The transmission characteristic of the WG-filters are given in the catalogue ``Farb- und Filterglas`` from Schott.

UV-A:

Red bulb: 6x L 36 W/73 (Osram) combined with WG 335.

Maximum intensity λ_{max} = 360 nm

Half band width = 40 nm

Fluence rate = 19.6 W m^{-2}

Radiation was measured by a Tektroniks Digital Photometer (Rode & Schwarz, GmbH., Germany).

2.1.8 Plant material /Sampling

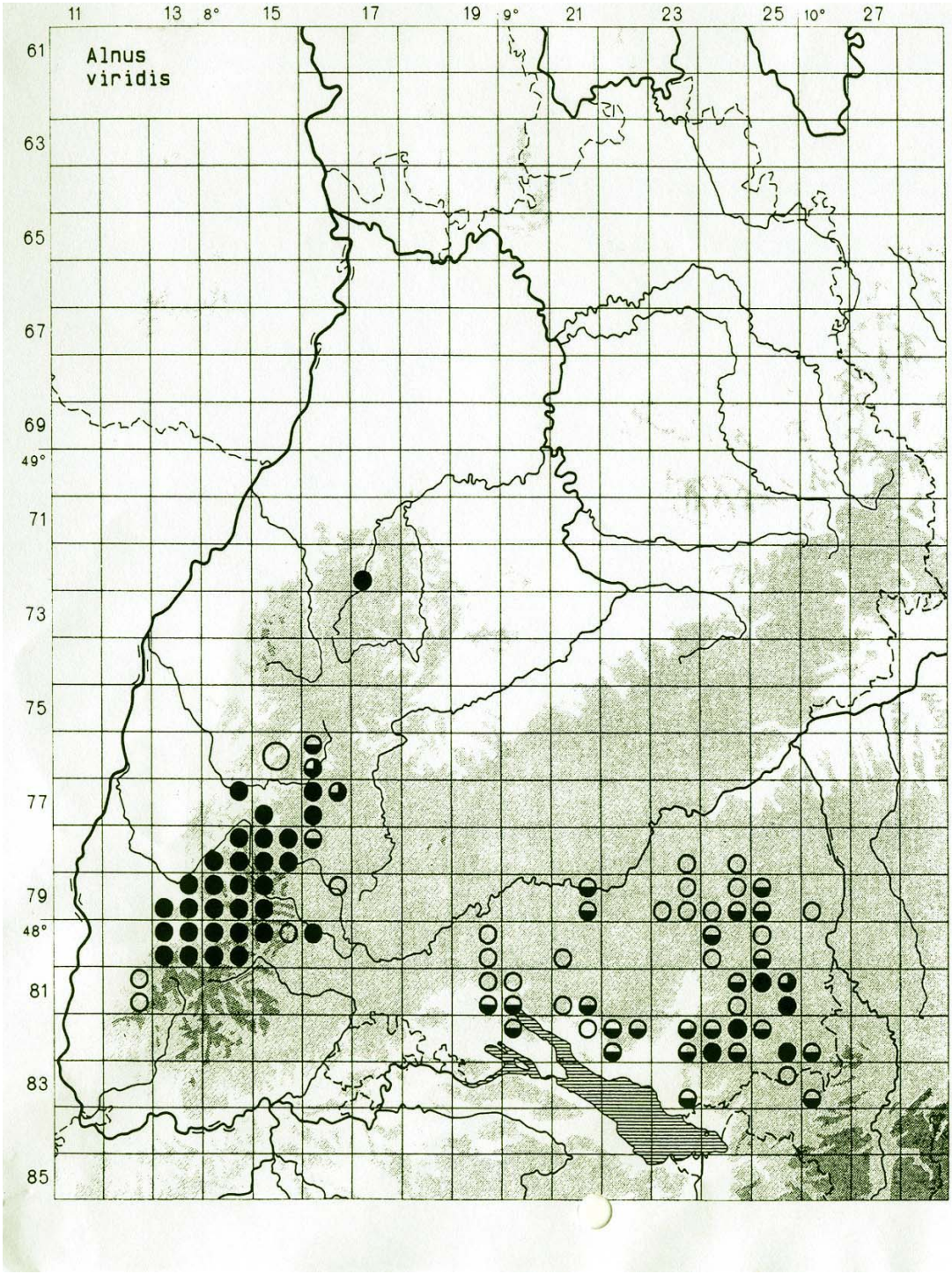
For the work on photomorphogenesis, plant materials (i.e. buds and seeds from *A. viridis*) were collected from the Pre Alpine/Alpine foreland (Allgäu, 200 m distance from the railway station of Lanzenhofen in the direction of Leutkirch, ca. 600 m to 700 m altitude above sea level), from the Black Forest (Sägendobel 550 m to 980 m altitude above sea level), or the Lechtaler Alpen (Freiburger Hütte, 1900 m altitude above sea level) (see **Table 1**). Seeds were stored at 4^{o} C. Immediately after collection, the buds were transported to the laboratory and used for the isolation of anthocyanin.

For the molecular work, fresh leaves and buds were used for the isolation of genomic DNA. Selected sites are listed in **Table 1.** Sites were selected particularly for studies on glacial history and immigration waves of Black Forest, Alpine, Alpine foreland (Allgäu), and Bavaria. Specimens were identified either by using regional keys (Hegi, 1951) or the Herbar of the Landessammlungen für Naturkunde in Karlsruhe. The Black Forest locations were identified according to Braun (1975) and Wilmans (1977). However, Allgäu location was identified according to Sebald et al., 1998. During recent field observations we could not locate the reported *Alnus viridis* sites at Rinken and Hinterwaldkopf (personal observation) in Black Forest. Five individuals were sampled per population with exception to the samples from the

Alpine (two individuals), Bavaria (two individuals) and St Blasien from South Black Forest (one individuals).

Table 1. Different *Alnus* population and species, location, geographical region, elevation, population size and collector were arranged in the following table.

Species	Location	Region	Elevation	Population size	Collector
A. viridis	Sägendobel	Black Forest	760 m	>10	Sultana Kamruzzahan, Abdul Ahad, Biology II, Freiburg University
A .viridis	Löffeltal	Black Forest	820 m	<10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer, NatureConservation,
A. viridis	Schulterdoble	Black Forest	745 m	>10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer
A .viridis	Alpersbach	Black Forest	1052 m	<10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer
A. viridis	Breitnau	Black Forest	1018 m	>10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer
A. viridis	On the way of Hinterwaldkopf	Black Forest	912 m	<10	SultanaKamruzzahan, Abdul Ahad & Markus Mayer
A. viridis	Ravenaschlucht	Black Forest	823 m	<10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer
A. viridis	Wildgutach	Black Forest	583 m	>10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer
A .viridis	Sank Peter	Black Forest	758 m	<10	Sultana Kamruzzahan, Abdul Ahad & Markus Mayer
A. viridis	St Blasien	South Black Forest	900 m	Herberium material	Dr. F. Schuhwerk Botanische Staatssammlung, München
A. viridis	Freiburger Hütte	Lechtaler Alps	1900 m	>10	Prof. A. Bogenrieder, Geobotany, Freiburg University, Freiburg.
A. viridis	Lanzenhöffen	Allgäu	667 m	<10	Sultana Kamruzzahan, Abdul Ahad
A. viridis	Brechtes garten	Bavaria	900 m	Gift material	Dr. Wolf Reutz, Bayerishes Amt für Saat – und Pflanzenzucht, Byarn.
A. incana	Siegsdorf	Bavaria	900 m	Gift material	Dr. W. Reutz,
A. glutinosa	Alpenvorland	Bavaria	900 m	Gift material	Dr. W. Reutz,



Map 1: Earlier report showed the above mention *Alnus viridis* distribution in Baden-Württemberg (according to Sebald et al., 1998). According to distribution full black ball indicate the current area of distribution (after 1970). Three quadrat black ball indicate the distribution from 1.1.1945 to 31.12. 1969. Half black ball indicate the distribution between 1900 to 1944. Small empty ball indicate extinct before 1900 and big enmpty ball showed the area where *Alnus* were already extinct after 1945.

2.2 Methods

2.2.1 Photobiological methods

2.2.1.1 Seed germination:

The seeds were germinated on filter paper with distilled water in transparent plastic boxes. During the imbibation period dormancy was broken by irradiation for 24 h with R (3.5 $W.m^{-2}$) light and subsequent incubation in darkness (using black boxes) at 25°C. As 4 days old etiolated seedlings were found to be most photoresponsive, all the experiments were conducted with seedlings of that age. The amount of flavonoids was determined for the period 6 days after imbibation. Seedlings were selected for homogenous size under dim green safe light (λ_{max} 550) prior to irradiation.

2.2.1.2 Irradiation conditions

Etiolated seedlings were irradiated for 48 h with continuous R (λ_{max} = 740 nm, fluence rate = 3.5 W m^{-2}), FR (λ_{max} = 658 nm, fluence rate = 6.5 W m^{-2}), UV-A (λ_{max} = 360 nm, fluence rate = 8.3 W m^{-2}), UV-B (λ_{max} = 310 nm, fluence rate = 11,9 W m^{-2}) or BL (λ_{max} = 450 nm, fluence rate = 10 W m^{-2}), while control seedlings were kept in darkness and not irradiated. For UV irradiation, seedlings were placed on wet filter paper and covered with 3 mm transmission cut-off filters of the WG series of $\lambda > 310$, $\lambda > 360$ nm, respectively. In a parallel set of experiments, radiation was reduced to 33 % and 11 % by covering with screening nets. Temperature was maintained 25° C during the light treatments.

2.2.1.3 Hypocotyl growth under various light qualities

Etiolated seedlings of four days age were irradiated for 48 h under various light qualities (R, FR, UV-A, UV-B and Blue). Hypocotyl length was measured by using a ruler scale before and after treatments.

2.2.1.4 Flavonoid determination:

For the determination of flavonoids, at least 20 irradiated hypocotyls or cotyledons were transferred into an Eppendorf tube, and 60 µl of extraction buffer were added (79% ethanol, 20% H_20 and 1% acetic acid). The tubes were incubated for 30 min at 85°C. After centrifugation for 10 min at 15300 rpm, the supernatant was used for descending paper chromatography. Air-dried chromatography papers were run with 15 % acetic acid (Markham, 1982). The flavonoid-containing region was excised and transferred into an Eppendorf tube with 1.5 ml of the same extraction buffer. The tubes were heated for 30 min at 85°C. The same

volume of extraction buffer was used as a negative control. After eluting the flavonoid spots, extracts were determined by their absorption spectra and quantified using a spectrophotometer (model 35, Beckman, Fullerton, CA, USA) at 360 nm wavelength.

2.2.1.5 Determination of anthocyanin content

For the determination of anthocyanin content, 20 irradiated hypocotyls were extracted in 500 µl extraction buffer (79% ethanol, 20% H_20 and 1% HCl) for 30 min at 85°C. After centrifugation, the supernatants were analyzed for their absorption spectra and anthocyanin quantified spectrophotometrically (model 35, Beckman, Fullerton, CA, USA) at 533 nm wavelength. In additional experiments, anthocyanin was measured in the same way after extraction from scale leaves (20 per sample) with 1 ml of extraction buffer.

2.2.2 Molecular biological methods

2.2.2.1 Genomic DNA isolation

For genomic DNA isolation, fresh leaves or buds were collected from their natural habitat, wrapped into moist paper and kept in plastic bags. Immediately after transfer to the lab, the materials were frozen in liquid nitrogen and stored at -80^{o}C**.** Genomic DNA was isolated from frozen (stored at –80^{o}C) leaf tissues or buds by using a modified CTAB method. Around 1-2 g of fresh leaf tissues, were ground in a mortar in liquid nitrogen. The tissue powder was transferred to pre-warmed 15-ml Falcon tubes with 5 ml of CTAB solution (2 % w/v Cetyltrimethylammoniumbromide, 1% w/v polyvinylpyrrolidone, 100 mM Tris (pH 8), 20 mM EDTA, 1.4 M NaCl) and 0.2 v/v % β-mercapthoethanol. The Falcon tube was incubated at 67°C for 40 min. Equal volumes of chloroform/isoamyl alcohol (24:1) were added and the tubes shaken very slowly for 10 min at room temperature. Then, the samples were centrifuged for 10 min at 5000 rpm. The supernatant was transferred into a new Falcon tube. In addition, 500 µl of 10% CTAB buffer was added and again incubated for 15 min at 67°C using a water bath. An equal volume of the chloroform/isoamyl alcohol (24:1) solution was added and the mixture gently shaken for 10 min. The supernatant was transferred into a fresh Falcon tube after 10 min centrifugation. A 0.7 volume of 100% isopropanol was added to the supernatant and the mixture gently shaken for 3-4 times, and incubated for 15 min at room temperature. Then it was centrifuged at maximum speed (15300 rpm) for 30 min. The sediment was washed with 70 % ethanol after discarding the supernatant. The washed sediment was air-dried and then dissolved in 500 µl *aqua bidest.* For the precipitation of RNA, at least 2 ml of 4 M LiCl were added to the DNA and incubated at 4°C for several hours or over night. After 5 min

centrifugation at 4°C at 15300 rpm, the supernatant was transferred to a new tube. For DNA precipitation 0.7 volume of 100% isopropanol were added to the tube. The DNA sediment was washed with 70% ethanol after 20 min centrifugation at 15300 rpm at 4°C. The sediment was air-dried and the DNA was dissolved with 100-200 µl of *aqua bidest.* The concentration of DNA (µg/µl) was determined spectophotometrically.

2.2.2.2 RNAse treatment:

For DNA purification 3 µl RNAse (20mg/ml) was added to the DNA solution and incubated for 30 min at 37^0 C. After incubation 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuged at 6000 rpm for 10 min. After centrifugation the supernatant was taken carefully by cut a tip and the same volume of chloroform/isoamyl alcohol (24:1) was added. After 10 min centrifugation the supernatant was taken into a fresh tube and the same volume of 100 % isopropanol was added. After 20 min of centrifugation at full speed in room temperature the pellet was washed with 70 % ethanol. The pellet was air dried, dissolved with 100-200 µl of *aqua bidest*. The concentration of DNA (µg/µl) was determined spectophotometrically.

2.2.2.3 *cpDNA* regions and primers:

For molecular evolution study of *A. incana*, *A. glutinosa* and for different *A. viridis* population, the noncoding chloroplast region, *trnT-F* was chosen. The *trnT-F* region consists of the *trnL* (UAA) intron, the intergenic spacer between *trnT* (UGU) and *trn*L 5` exon, and another intergenic spacer *trn*L 3` exon and *trn*F (GAA) (**Figure 14**).

The primer pair A (forward primer)/ B (reverse primer) were used for PCR to amplify the intergenic spacer between the *trnT* (UGU) gene and *trnL* (UAA) 5` exon. The primer pair C (F) and D (R) were used to amplify the intron of the *trnL* gene. Primers E (F) and F (R) were used to amplify the intergenic spacer between the *trnL* (UAA) 3´exon and the *trnF* (GAA) gene (Taberlet et al., 1991).

The primer for the *trnL* (UAA) intron and the primer for the intergenic spacer *trnL-trnF* worked for all three species *A. viridis, A. incana* and *A. glutinosa*. Each region was sequenced seperately as it was not possible to amplify the whole *trnT-F* region. In most cases amplification of the *trnT-trnL* region together with intron and intergenic spacer with primers A and D did not work, and often produced two or three fragments of very similar size. On the

other hand the amplification of the *trn*L intron together with the *trnL-trn*F intergenic spacer with the primers C and F work for all species. Consequently, primers A and B were used to amplify the *trnT-trnL* region for few populations of *A. viridis* because other cases often produced two fragments of very similar size.

2.2.2.4 PCR amplification and DNA sequencing

For PCR amplification of *trn*T-*trn*F the protocol of Taberlet et al., 1991 was followed. Double-stranded DNA was directly amplified by symetric PCR using universal primers (Taberlet et al., 1991). The *trn*T-*trn*F region was amplified by PCR in a 50 µl reaction volume and containing-

5 µl Mg-free 10 X Taq buffer,

3 µl 25 mM $MgCl_2$,

2.0 µl forward primer (20 pm/µl),

2.0 µl reverse primer (20 pm/µl),

10 µl dNTPs (each 1.25 mM),

0.3 µl tag (5 units/µl) and

2 µl solution of DNA template 1:10 (approx. 10 ng of genomic DNA) and

rest of H_20.

The PCR temperature profile for amplification was as follows-

TRNTF: Step 1 = 94^{o}C (1 min), step 2 = 52^{o}C (1 min), step 3 = 72^{o}C (2 min), step 4 = 34 times to 1, step 5 = 72^{o}C (15 min).

TRNTF reaction was carried out in a Thermocycler Cyclone, 25 Peqlab, Biotech. GmbH. The name to the left are the programme name.

PCR fragments size were seperated in 1% agarose gel, excised with a razor blade and purified with a Qia-Quick gel extraction kit following the manufacturer's instructions (QIAGEN, Inc., Pharmacia, Freiburg) or GFX PCR-DNA and Gel-Band Purification Kit (Amersham Biosciences, Freiburg, Germany). The sequencing reaction was done by Sequence laboratories GmbH, Göttingen. Purified PCR product was sequenced using an ABI Prism ™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA polymerase FS (Perkin Elmer, Norwalk, Connecticut). Primer A was used to sequence the *trn*T-L spacer, primer C for the *trn*L-intron and primer E for the *trn*L-F spacer for all samples. If there were

any ambiguous sites, primers B, D, and F were used for sequencing the opposite strand. In this case 80% of the sequence were overlapped for verification. Two to three repetitions showed the same results for *trn*T-*trn*F region. The intergenic spacer *trn*T-*trn*L (5´exon) consisted of 920 bp, the *trn*L intron consisted of 592 bp and the *trn*L (3´exon)- *trn*F intergenic spacer consisted with 456 bp respectively.

2.2.2.5 **Sequence alignment and indel coding**

Sequence similarities were analyzed using the BLAST search engine at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Nucleotide sequences were aligned using the sequence alignment program (William Pearson's *lalign* program, Huang and Miller, published in Adv. Appl. Math. (1991) 12:337-357), with possible slight manual modifications to minimize the number of gaps. They were further edited by eye. Aligned sequences of *trn*T-*trn*F for all *A. viridis* populations including *A. incana* and *A. glutinosa* (only *trn*L intron and *trn*L-*trn*F region) are shown in (**Data set 1 & 2).** Several indels were required to align sequences of *trn*T-*trn*F within *A. viridis* as well as *A. incana* and *A. glutinosa*. As noted by Taberlet et al., (1991) and confirmed by others for different plant groups (e.g., Mc Dade and Moody, 1999; Sytsma et al., 2002), the *trn*L-*trn*F region has a relatively high frequency of insertions and/or deletions (indels). Indels were treated as missing data in each analysis. Indels that were potentially parsimoniously informative were scored and added to the end of the data sets as presence/absence characters. Adding new sequences to the alignment was relatively easy because of conserved regions and shared indels. In all the cases there were smaller indels and at least 1-15 base pair insertion variations. Areas of ambiguous alignment or containing poly-n strings in *trnL-trnF* were excluded from all analyses.

2.2.2.6 Outgroup selection

For our molecular phylogenetic studies we took *C. cornuta* which is belongs to the family Betulaceae, subfamily-Betuloideae as outgroup. This clade is well supported (by ITS) with *Alnus* as its out group (Chen et al., 1999). In data base only one could obtain the complete sequence of ((*trn*L intron and *trn*L-*trn*F) region of *C. cornuta* as out group. This was used for comparison with produced sequence data. For our molecular phylogenetic studies we took sequences of *trn*L intron and the intergenic spacer between the *trn*L (UAA) 3`exon and *trn*F (GAA) of *Alnus* compare with *C. cornuta*. However examplerary showes the complete sequence of the tobacco chloroplast genome (Wakasugi et al., 1998). In dataset 1 and 2 I did not put sequence data from *C. cornuta*.

2.2.2.7 Molecular clock or Divergence time determination

For molecular clock determinations the calibration was done with a known time of divergence and then used to estimate divergence times of other species. *B. occidentalis* has a known divergence time (fossile record) and is used for calibrating estimates of divergence time of *Alnus* species (Kumer and Hedges, 1998). After calibreating divergence time of *A. incana* were used to calibreat unknowen divergence time from *Alnus* populations. *Betula* being a sister group of the monophyletic Betulaceae sensu strictu (Hutchinson, 1967; Dahlgren, 1983) and its substitution variation was compared with *A. incana, A. glutinosa* and different *A. viridis* populations.

The following formula was used for determination of divergence time:

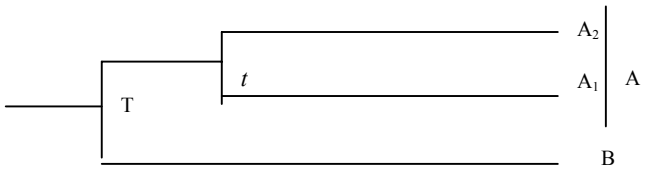


Figure 3. Divergence time estimation. t is unknown divergence time between A_1 and A_2 when A and B diverged at calibration time T (Milion years ago). In the average distance method, $t = d\ 12/(2r)$, where $r = (d_{1B}+d_{2B})/4T)$ is the rate of change for lineages A and B, and dij is the number of substitutions per site between sequences i and j.

2.2.2.8 Phylogenetic tree analysis

Phylogenetic trees were performed by using sequence variation with the Phylip 3.5 version (http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/win/setup.zip) programme. Sequences from twelve populations of *A. viridis* were used for producing a phylogenetic tree. One population did not (Schulterdobel) follow the general phylogenetic tree. Analyses were carried out based on substitutions variations only, with gaps coded as missing data. As most of the substitution variation was found in *trn*L-*trn*F region, only these data were used to make tree. Whereas, *trn*L intron sequences are shown in **dataset 1.**

Statistics

The experiment on hypocotyl elongation consisted of three replicates (with 20 seedlings per replicate). Flavonoid and anthocyanin determinations represent three independent experiments.

Kinetics for flavonoid induction were elaborated in two independent experiments with similar results. For all experiments mean values and standard errors are presented.

Following formula was used to calculate the standard error-

$$s = \frac{s}{\sqrt{n}} = \sqrt{\frac{\sum (x-\bar{x})^2}{n(n-1)}}$$

Where,

x = total number of individual

$\bar{x}$ = mean value

n = number of sample

A. Photobiological Study

3.1 Introduction

Light is the most important environmental parameter for plants, as it provides the ultimate source of biological energy. Photosynthesis is the photobiological process responsible for the transfer of energy from light to the biosphere, however it is not only photobiological process occurring in plants. Plants have evolved a remarkable capacity to track and respond to fluctuations in multiple parameters of the light environment. They can monitor the presence, absence, spectral quality (wavelength), fluence rate (intensity), directionality and diurnal duration of the incident light signals, and can modulate their growth and development appropriately to reach optimal radiant energy capture, and to ensure survival and reproduction. This non-photosynthetic action of light on plant development is known as photomorphogenic action (Mohr and Shropshire, 1983). Light also inhibits the rate of hypocotyl growth, stimulates the opening of the hook, the expansion of the cotyledons, induces the differentiation of leaves and chloroplasts, and the formation of pigments and trichomes. All of these processes are accomplished by and dependent on the differential expression of a large number of genes. Several other aspects of plant growth and development are affected by light, such as seed germination, sensing and response to neighbouring plants, phototropism (the bending response in relation to the direction of the light) and induction of flowering (Chory et al.,1996; Chory, 1997; Kendrick and Kronenberg, 1994; McNellis and Deng, 1995; Millar et al., 1994; Quail, 1994a; Smith, 1995).

3.1.1 Photoreceptors

The photomorphogenic responses of plants are mediated by the actions of several distinct classes of photoreceptors, the UV-B photoreceptors (280-320 nm of spectrum) which regulate the formation of UV-shielding pigments (Beggs and Wellmann, 1994; Christie and Jenkins, 1996; Quail, 1994a), cryptochromes and phototropins which monitor the B /UV-A region (320-390 nm of spectrum) of the spectrum, (Ahmad and Cashmore, 1993, 1996a; Ahmad et al., 1998; Batschauer, 1993; Christie et al., 1998; Hoffman et al., 1996; Lin et al., 1996a, 1996b; Lin, 2000), and the phytochromes which monitor the R and FR region of the spectrum (400-700 nm of spectrum), (Furuya, 1993; Millar et al., 1994; Quail et al., 1995; Quail, 2002). In recent years, the function of individual phytochrome and cryptochromes/phototropins have been defined in plants along with the molecular and cellular basis of light perception and

transduction (Chory and Wu, 2001; Deng and Quail, 1999; Fankhauser, 2001; Neff et al., 2000; Smith, 2000). In contrast, UV-B photoreceptors are still not identified..

3.1.1.1 Phytochromes

The phytochrome molecule is a soluble, dimeric chromoprotein, which consists of two ~ 125-kDa polypeptides. Each polypeptide folds into two main structural domains: -an amino-terminal photosensory domain that harbours a single, covalently attached tetrapyrrole chromophore, phytochromobilin; and a carboxy-terminal domain that mediates dimerisation. The photosensory activity of the molecules results from its capacity to undergo light-induced, reversible switching between two conformations: the biologically inactive Pr (red-light-absorbing, λ_{max}= 660 nm) form and the biologically active Pfr (far-red-light absorbing, λ_{max}= 730 nm) form (Furuya, 1993; Quail, 2002a). Although the two forms of phytochrome have different spectral absorption ranges, these also overlaps. Due to this overlap, the photoequilibrium of Pfr /P_{tot} depends on the wavelength and is about 80% in R, 3% in FR and about 40% in blue (450 nm) light. Thus blue (and UV-A) light is quite effective in the phototransformation of Pr. Sometimes its very difficult to distinguish between blue-light responses mediated by phytochrome and those triggered by a specific blue-light receptor, or a co-action between both. Pfr formation (signal perception) initiates an intracellular transduction process culminating in altered expression of specific genes that are responsible for directing the morphogenesis that is appropriate for the prevailing conditions, whereas reconversion to Pr can abrogate this process.

In *Arabidopsis*, the most throughly investigated plant system, the *phy* family (phytochrome apoproteins are encoded by five genes), consists of five members, designated *phyA* to *phy E* (Batschauer, 1998; Clack et al., 1994; Gärtner et al., 1996; Hill et al., 1994; Parks and Quail 1993; Quail 1994a, 2002a). Different phytochromes have discrete biochemical and physiological properties, are differentially expressed, and are involved in the perception of different light signals. During germination and seedling development, phytochrome A (PhyA) and phytochrome B (PhyB) play a predominant role in *Arabidopsis* seedlings (Reed et al., 1993; Shinomura et al., 1996). The function of PhyE seems to be primarily confined to regulating stem elongation (Devlin et al., 1998; Smith, 2000). PhyB is necessary for the perception of continuous red light (Rc) whereas phyA efficiently senses continuous far-red light (FRc) perception. Thus, although both phyA and phyB absorb R and FR light, and although the morphogenic response of wild-type seedlings to both Rc and FRc is similar in regard to the deetiolation process, the two phytochromes monitor distinct facets of the light

environment (Quail et al., 1995; Smith, 2000; Whitelam and Devlin, 1997). This control of plant growth and development occurs throughout the life cycle from seed germination and seedling de-etiolation, through sculpturing the vegetational architecture to floral induction. Based on the quantity of red light, which is necessary to produce photoresponses (intensities in the range from 10^{-10} mol m^{-2} to > 10^{-4} mol m^{-1}), they are divided into three categories: 1) very low fluence response (VLFR - which are irreversible due to the fact that even under FR illumination a Pfr concentration sufficient for the physiological effect is achieved), 2) low fluence responses (LFR - typical photoreversible p reactions), and 3) high irradiance responses (HIR), dependent on the rate of cycling between P_r and P_{fr}. In low red/far-red conditions, both the PhyB-mediated low fluence response (LFR) and the PhyA-mediated high irradiance response (HIR) are acting antagonistically to regulate the growth of the plant (Nemhauser and Chory, 2002).

3.1.1.2 Cryptochromes and phototropins

On the basis of molecular genetic studies in *Arabidopsis*, it is clear now that there are two types of blue light receptor in plants: cryptochromes and phototropins. Cryptochromes, which were historically defined by their action spectra, are photolyase-like blue light receptors (Briggs and Huala, 1999; Cashmore et al., 1999; Gressel, 1979; Lin, 2000b). Most organisms examined to date have more than one cryptochrome, and different cryptochromes of the same organism often mediate related light responses. Cryptochromes are found not only in plants but also in animals, including humans, making them ubiquitous photoreceptors throughout higher eukaryotes. In *Arabidopsis*, cryptochromes are encoded by two genes, *CRY1* and *CRY2*. The expression of cryptochrome genes can be regulated by light on different levels from transcription to mRNA-degradation. Cryptochromes work together with the phytochromes to regulate photomorphogenetic responses, including the regulation of cell elongation and photoperiodic flowering. Similar to the cryptochromes, phototropins are also found in a range of organisms and regulate responses to environmental stimuli, such as light and oxygen. Cryptochromes are characterised by the sequence similarity to DNA photolyases and are now believed to be involved in the synchronisation of the circadian clock (Ahmad and Cashmore, 1993; Hoffman et al., 1996; Lin et al., 1996a). In contrast, the phototropins are involved in phototropism (Huala et al., 1997) and share no sequence similarity with the cryptochrome group. Blue light affects many aspects of plant growth and development such as inhibition of hypocotyl elongation, stimulation of cotyledon expansion, regulation of flowering time, phototropic curvature, stomatal opening, entrainment of the circadian clock and regulation of

gene expression (Briggs and Liscum, 1997b; Jenkins et al., 1995; Liscum and Hangarter, 1993; Liscum and Briggs, 1996). Blue-light receptors have been cloned from *Arabidopsis,* and for each of them mutants have been identified. In screens for long hypocotyls, Koorneef et al. (1980) had isolated several *hy* mutants, one of which is *hy4*. In this mutant the blue-light-dependent inhibition of hypocotyl elongation, the formation of anthocyanin and the expression of chalcone synthase (CHS) as key enzyme of flavonoid biosynthesis are affected (Ahmad and Cashmore, 1993; Ahmad et al., 1995; Koornneef et al., 1980).

3.1.1.3 UV-B receptor

Ultraviolet-B radiation (280-320 nm) is increasing in the biosphere as a result of stratospheric ozon depletion by chlorofluocarbons and has been shown to cause several effects in plants such as damage to DNA and other molecules which can lead to cellular injury, mutagenesis and death (Jordan, 1995). UV-B causes both visible changes in leaf and plant morphology (Ballaré et al., 1991) and intracellular alterations including changes in gene expression (Stride, 1993) or synthesis of protective pigments mainly in the vacuoles and epidermal cells such as flavonoids and anthocyanins (Jansen et al., 1998; Krizek et al., 1998; Reuber et al., 1996; Strid et al., 1992; Wellmann, 1971). Low UV-B irradiance causes inhibition of hypocotyl elongation in tomato seedlings (Ballaré et al., 1995). UV-B induced inhibition may also result from direct cellular damage at the level of meristems and elongating organs. In fact, the action spectrum for the inhibition of hypocotyl elongation in etiolated seedlings of *Lepidium sativum* resembles spectra for DNA damage in other systems (Caldwell, 1971). Flavonoid and other phenolic compounds have been discussed as protective shields against UV-B radiation in *Arabidopsis* (Li et al., 1993).

3.1.2 Synthesis of flavonoids/anthocyanin and hypocotyl inhibition

Light-induced flavonoid and anthocyanin biosynthesis have been studied extensively in many plants. Flavonoids and anthocyanins are a diverse group of plant secondary metabolites derived from phenylalanine branching off the shikimate pathway by the action of the key enzyme chalcone synthase (CHS). The flavonoid pathway plays an important role in plant growth, reproduction and survival (defence against developmental stresses such as cold, heat, UV radiation, high intensity of light and wounding). These diverse compounds are present in different forms in different plant species, but typically include flavonols (UV-absorbing compounds), anthocyanin (red, blue or purple pigments), while 3-deoxyanthocyanidins are produced in a very limited number of plant species, notably maize and *Sorghum* and

isoflavonoids are found in the seeds of legumes such as soybean (Stafford, 1990). The synthesis of anthocyanins is affected by environmental factors other than light, such as temperature, nutrition, pathogen infection and drought. Light regulation of flavonoid such as flavones, flavonols and also anthocyanin biosynthesis have been a classical response to study photomorphogenesis. Flavonoids are synthesised at defined times in response to both internal metabolic cues and external signals in specific tissues i.e. hypocotyls, mesocotyl, coleoptiles and cotyledons of developing seedlings, or in the seed coat, flower petals and senescing leaves (Caldwell et al., 1989; Lie et al., 1993; Robberecht and Caldwell, 1983, 1989; van Tunen and Mol, 1991). Phytochromes, cryptochromes and UV-B receptor are all involved in the control of flavonoid synthesis (Buchholz et al., 1995; Dixon and Christopher, 1999; Kubasek et al., 1992, 1998; Lois, 1994; Rozema et al., 1997; Schnitzler et al., 1997; Shirley, 1996; Shirley et al., 1195; Yoshitama, 2000). Genes encoding flavonoid structural and regulatory proteins have been cloned and characterised in a variety of plants and found to be expressed in response to a wide range of external and internal cues.

Normally, flavonoids accumulate preferentially in the epidermis. Anthocyanin is widely distributed in floral and fruit tissues and also located in root, shoot and leaves. Anthocyanin have been even reported in the root cap of *Impatitiens* seedlings and in roots under osmotic or toxin stress (Kaliamoorthy et al., 1994; Mumford, 1990; Wetzel et al., 1995). Light-induced accumulation of anthocyanin was found in root cap and roots of *A. viridis* seedlings. However, anthocyanin accumulation in *A. viridis* seedlings is driven independently through B/UV-A (cryptochromes) and UV-B (UV-B receptor). In most cases, phytochromes, B/UV-A receptors and UV-B receptors are all involved in the control of anthocyanin synthesis (Beggs and Wellmann, 1985). Nevertheless, anthocyanin synthesis can be induced by monochromatic irradiation with R, FR, B, or with UV light in various plants. The involvement of UV-B could, for instance, be demonstrated by a tomato mutant that is deficient in phytochrome-induced anthocyanin accumulation (Brandt et al., 1995). However, in some cases, anthocyanin synthesis was shown to be efficiently induced by the co-operative action of phytochrome and B/UV receptors (Mohr, 1994). Anthocyanin synthesis remains usually silent in the dark (Camm et al., 1993; Dong et al., 1998; Kakegawa et al., 1987). The light regulation of anthocyanin and flavonoid synthesis varies from species to species and sometimes even from variety to variety within a single specis (Beggs and Wellmann, 1985). Therefore, the regulatory network has to be established for each experimental system independently. In seedlings of *A. viridis*, at least three photoreceptors (phytochromes, cryptorchromes, and UV-B receptor)

regulate the accumulation of flavonoids and anthocyanin. This complexity is complemented by a tissue pattern of competence: Whereas phytochrome does not inhibit flavonoid accumulation in the hypocotyl it does so in the cotyledons of *A. viridis*.

Light induced biosynthesis of anthocyanin and flavonoids in the epidermis and outer tissues is considered to be a ubiquitous protection mechanism against intense solar radiation (UV-B) via DNA damage (Buchholz et al., 1995; Caldwell, 1981; Hada et al., 1996; Moorthy and Kathiresan, 1997). However, flavonoids have been shown to be relevant for the interaction between Leguminous plants and nitrogen-fixing bacteria (Yoshitama, 2000, Vierheiling et al., 1998), as antioxidants (Rice- Evans and Miller, 1998), as regulators of pollen germination and pollen-tube elongation. (Ylstra et al., 1992), and as antifungal agents like phytoalexins. Some plants require the activation of a BL/UV-A photoreceptor to release the competence of phytochrome in the regulation of flavonoid and anthocyanin (e.g. in maize or millet). Other plants such as egg plant, parsley, soybean, as well as some varieties of roses or certain flavonoid-producing parsley cell lines, require UV-B irradiation in addition to red or visible light in order to initiate mass production of anthocyanins or other flavonoids (Drumm-Herrel and Mohr, 1981; Wellmann, 1971).

Similar to flavonoid biosynthesis, light can inhibit hypocotyl and stem elongation in higher plants with all three photoreceptor classes being involved (Beggs et al., 1980; Lin et al., 1995; Mohr, 1984; Schäfer and Haupt, 1983). This inhibitory effect of light is driven by the independent photoreceptor along with complex cooperation between the photoreceptors.

3.1.3 Scope of the study - photomorphogenesis as sensitive indicator of adaptive evolution

If one wanted to see fast evolutionary changes related to the adaption to a changed habitat, one would search for a trait that is fundamental for survival and in addition buffered by interactive, partially complex regulatory pathways. Photomorphogenesis meets both requirements. Moreover, the ecology of *Alnus viridis* as a pioneering species that has difficulties to cope with a closed canopy, suggests that photomorphogenesis would be expected among the traits that most evolve in the adaptation to possibly altered habitat conditions.

In high elevation (about 3000 m altitude above sea level) UV-B intensity is much higher than at sea level (Caldwell et al., 1980). At this high altitude plants have to cope with increased

levels of UV-B, such that the impact of a UV-B receptor is expected to be more pronounced as compared to plants from lower altitude.

I therefore analyzed the pattern of light regulation for flavonoid and anthocyanin synthesis along with hypocotyl elongation in three populations of *Alnus viridis* of Alpine, pre-Alpine and Black Forest origin. My results presented in this part of the thesis show that these populations differ with respect to competence and sensitivity for certain light qualities. They also indicate that during the separation between the populations, the role of the individual photoreceptors has been shifted from the UV-B receptor system (more pronounced in the Alpine population) in favour of the BL/UV-A receptors.

3.2 Results

3.2.1 Spectral dependence of hypocotyl inhibition

To determine hypocotyl growth, etiolated seedlings (6 d after sowing) from an Alpine ('Alps'), a pre-Alpine ('Allgäu') and a Black-Forest population were continuously irradiated with various light qualities (R, FR, white light λ > 310 nm, λ > 360 nm and Blue) and the length increment of the hypocotyl over the following 48 h was determined. Under short wavelengths (UV-B, UV-A and Blue) as well as under far-red, hypocotyl growth was significantly inhibited while red light was ineffective for all populations. In the Black Forest population, the inhibition of hypocotyl growth was slightly more pronounced under short wavelength (UV-B, UV-A and Blue) as compared to the other two populations (**Figure 4**). However, for far-red irradiation the inhibition was comparable for all three populations.

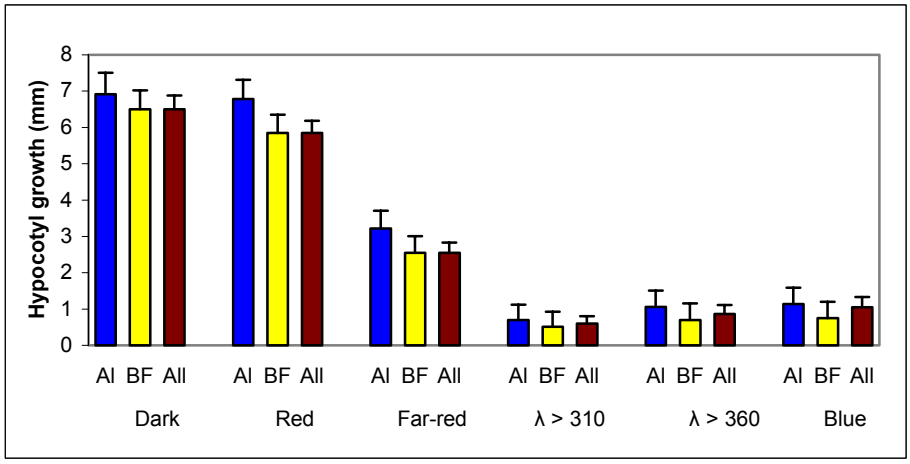


Figure 4. Hypocotyl growth in response to different light qualities in seedlings from the Alpine (Alps, Al), Black-Forest (BF) and pre-Alpine (Allgäu, All) populations. After 48 h of irradiation (6 d after sowing) 20 seedlings were measured per experiment. Mean values of three replicates ± SE. The mean length at the onset of irradiation was comparable (5 mm) for all populations.

3.2.2 Spectral dependence of flavonoid formation

To determine the light qualities that were effective in flavonoid formation in hypocotyls and cotyledons, etiolated seedlings of the same age (6 days after sowing) were irradiated continuously for 48 h with R, FR, λ > 310 nm, λ > 360 nm and Blue light. In case of hypocotyls, for all populations, the response was most pronounced for UV-B (λ > 310) with the highest flavonoid content reached in the Alpine population as compared to the pre-Alpine or the Black-Forest population (**Figure 5).** A stimulation of flavonoid synthesis was also observed under UV-A (λ > 360) and Blue light. In general, the short-wavelength response in seedlings

from the Black-Forest was less pronounced as compared to the other two populations. Light of longer wavelength (R or FR) was observed to be ineffective in all three populations.

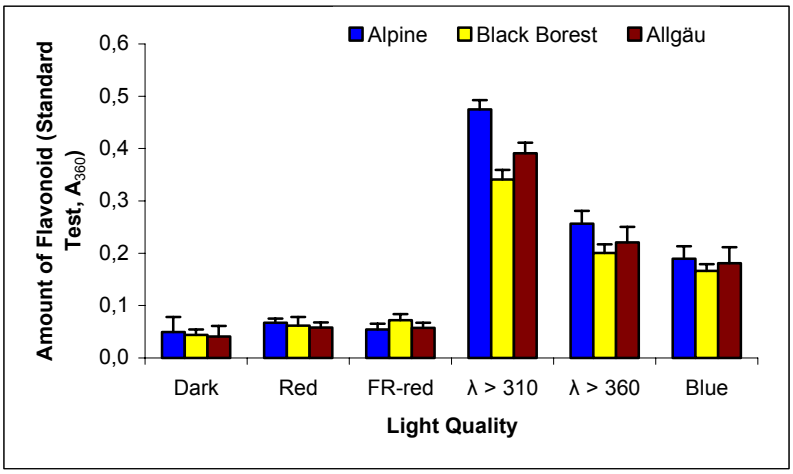


Figure 5. Spectral dependence of light-induced flavonoid formation in the hypocotyl for the Alpine, the Black-Forest and the pre-Alpine (Allgäu) population. Seedlings (6 d after sowing) were irradiated under standard light sources as described in Methods. Mean values of three replicates ± SE.

In contrast, both longer and shorter wavelengths (R, FR, B, UV-A and UV-B) were effective for flavonoid formation when cotyledons were analyzed. Here, the strongest response was observed under FR or under Blue light for the Alpine population (**Figure 6)**. Continuous R light was the least effective light quality for all the populations. Dark grown seedling used as a control showed very small flavonoid content for all three populations.

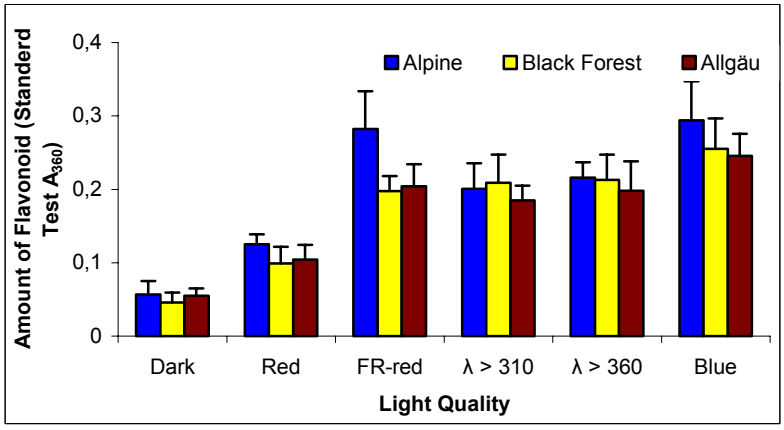
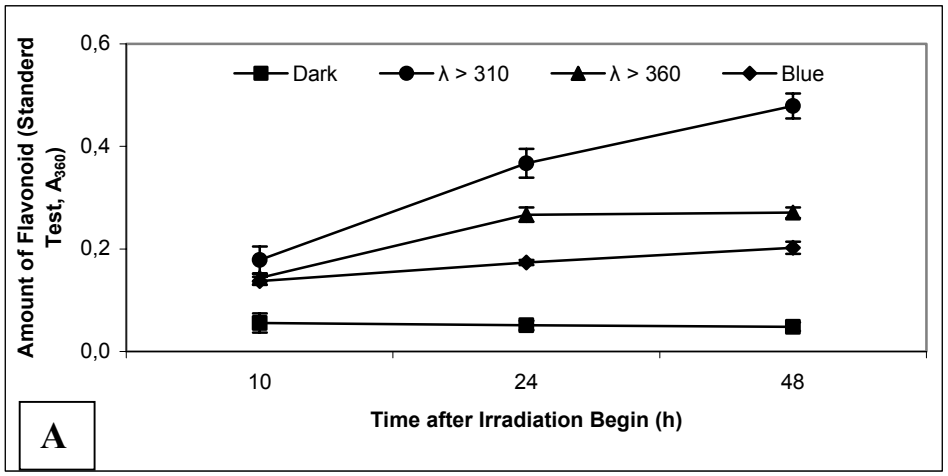


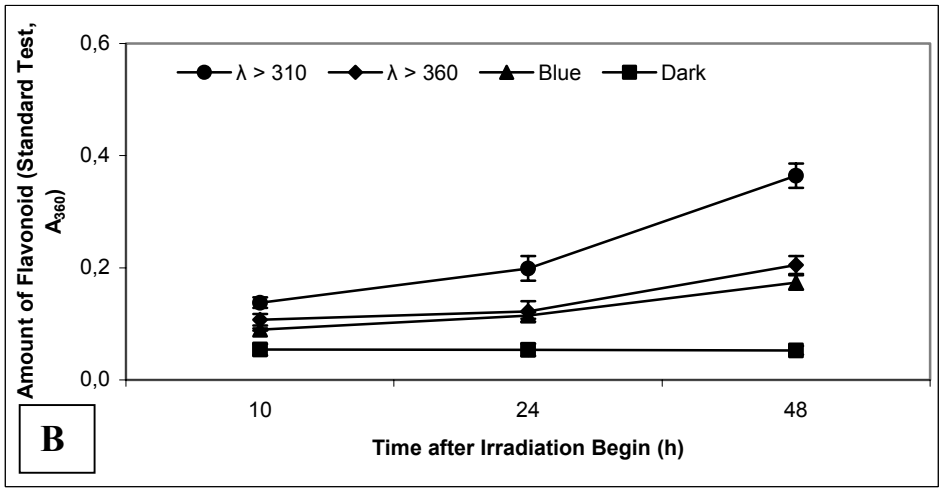
Figure 6. Spectral dependence of light induced flavonoid formation in the cotyledons for the Alpine, the Black-Forest, and the pre-Alpine (Allgäue) population. For details refer to the legend of **Figure 5**.

The time course of flavonoid accumulation under UV-A, UV-B and B light was deterimined in hypocotyls for all three populations. For all three light qualities, flavonoid became induced within 10 h irrespective of the population. Consistent with the end-point experiment (**Figure 5**), UV-B induced higher flavonoid contents for all time points (10 h, 24 h, 48 h) with maximal values obtained in the Alpine population after 48 h of irradiation (**Figure 7A, 7B and 7C)**. This difference was also observed, although less prominent for UV-A and Blue light. No flavonoids were detected when seedlings were irradiated with R and FR light or kept in darkness.

In the next step, the sensitivity of flavonoid formation was determined in seedlings irradiated with either UV-B, UV-A or blue light. The fluence rate was adjusted by nets to either 100 %, 33 % or 11 % of the intensity used in the previous experiments. For UV-B the Alpine (**Figure 8A**) and the pre-Alpine (**Figure 8C**) populations show a similar sensitivity. In contrast, the Black-Forest population (**Figure 8B**) is definitely less sensitive to UV-B, because the full fluence rate was necessary to obtain a significant increase of flavonoid content, whereas for 33 % or 11 % more or less similar flavonoid levels were measured. For UV-A and blue light already the lowest fluence rate (11 % of the maximum) was obviously saturating, because it was not possible to obtain higher values by raising the fluence rate.

When this experiment was analysed for flavonoid synthesis in the cotyledons, again the Black-forest population (**Figure 9B**) was observed to be less sensitive as compared to the Alpine (**Figure 9A**) or the pre-Alpine population (**Figure 9C)**. As compared to the hypocotyl, the sensitivity to UV-A or blue light was somewhat higher, because the fluence rates used were still not saturating the flavonoid synthesis. However, the sensitivity between the three populations seems to be comparable.





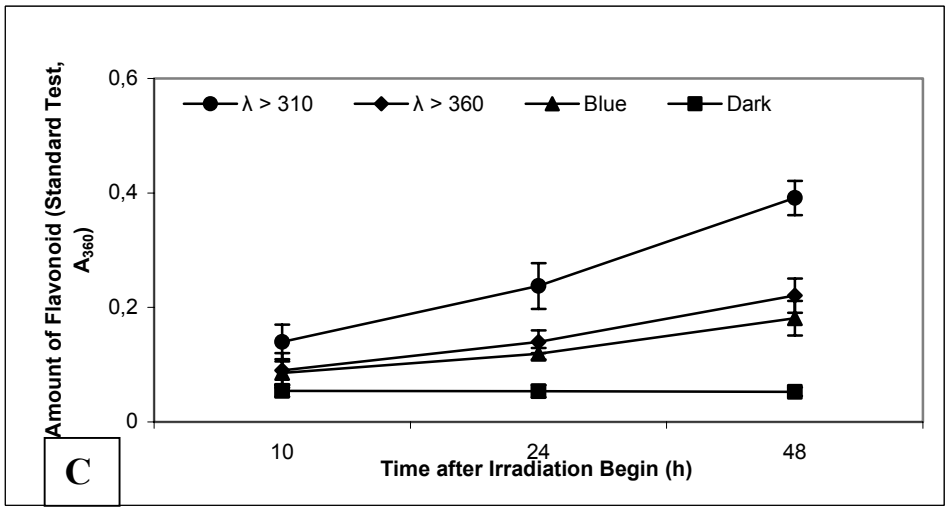
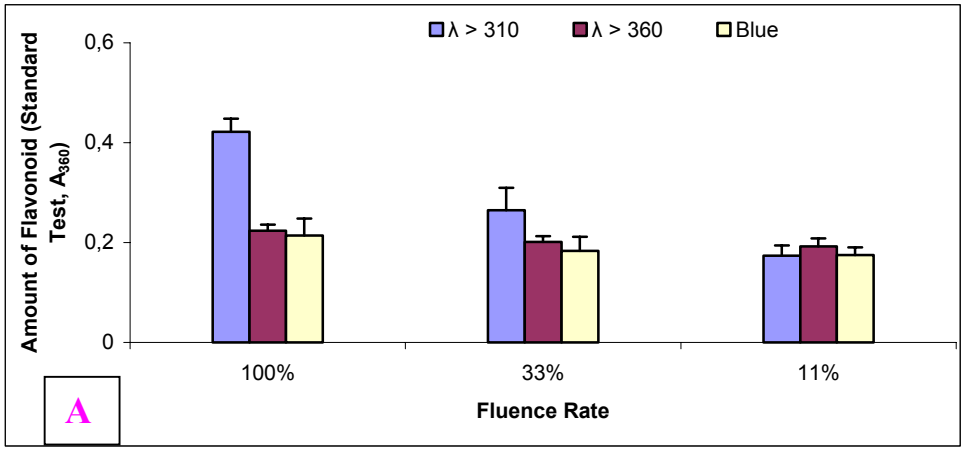
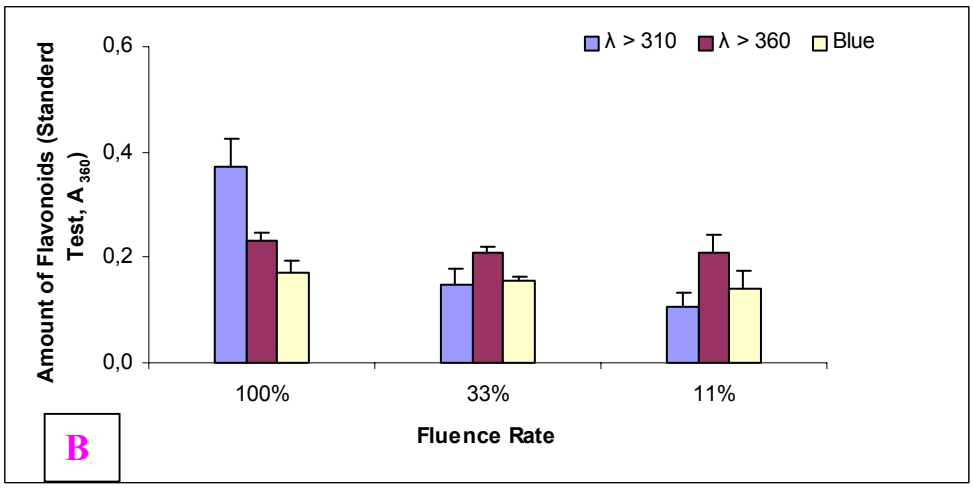


Figure 7. Time course of flavonoid formation in response to irradiation with UV-B (λ > 310) UV-A (λ > 360) or blue light for the Alpine (**Figure 7A**), the Black-Forest (**Figure 7B**) and the pre-Alpine (Allgäu) population (**Figure 7C**). Seedlings were irradiated 6 d after sowing with standard light sources as described in Methods. Seedlings were harvested after the indicated time intervals (10, 24, 48 h) for flavonoid determination. Mean values of three replicates ± SE.





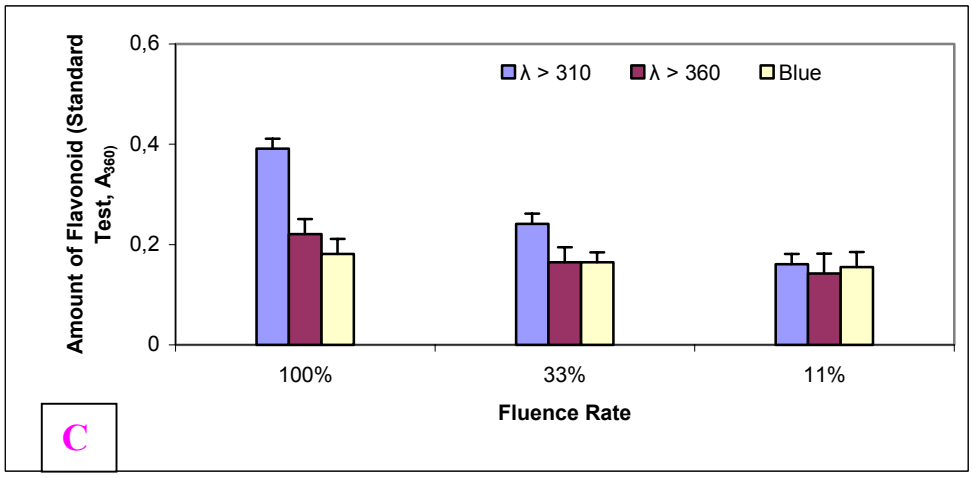


Figure 8. Fluence-rate dependency of flavonoid formation in the hypocotyl for the Alpine (A), the Black-Forest (B) and the pre-Alpine (Allgäu) population (C). After 48 h of irradiation 20 seedlings (8 d after sowing) were measured for each data point. Mean values of three replicates ± SE.

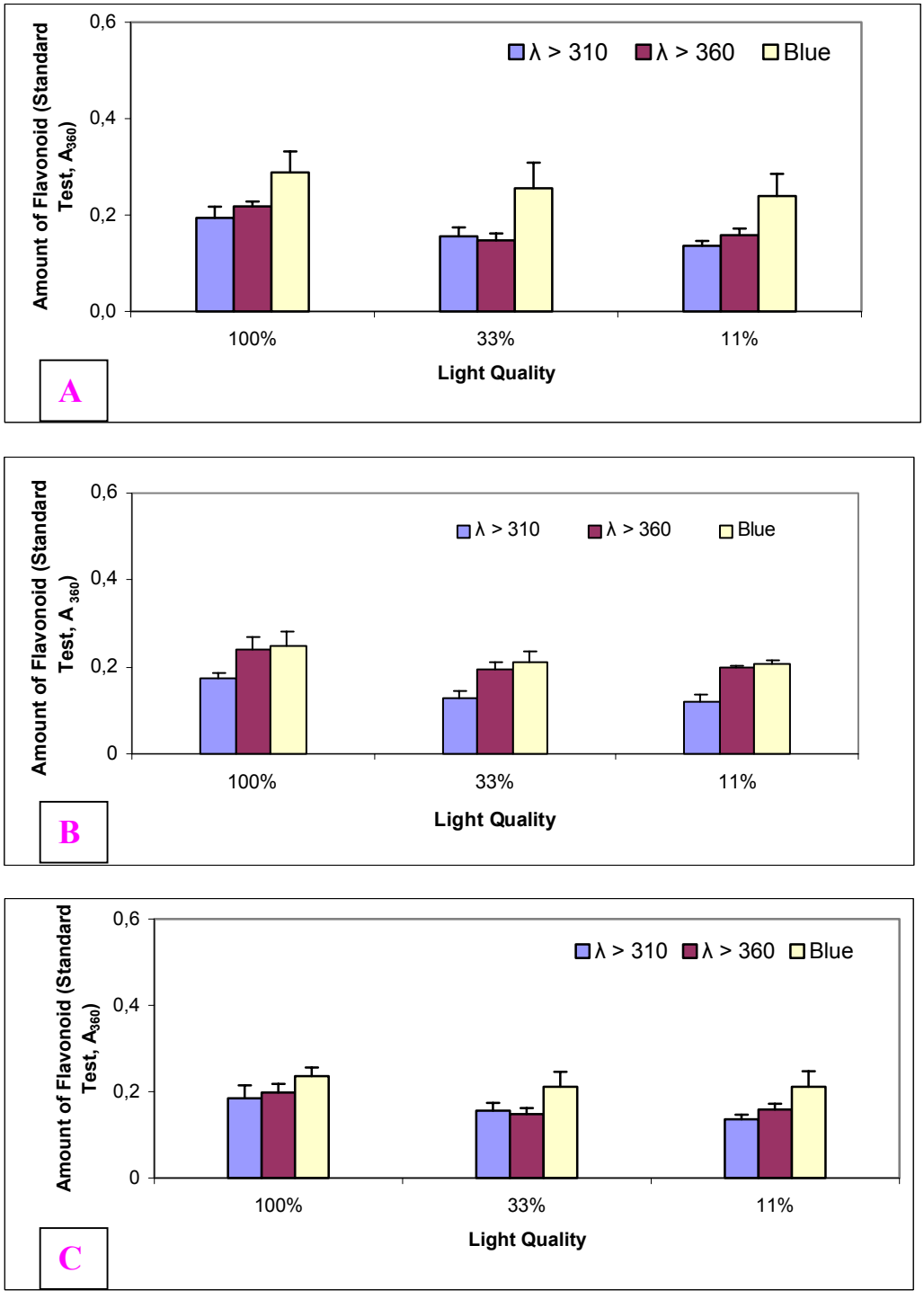


Figure 9. Fluence-rate dependency of flavonoid formation in the cotyledons for the Alpine (A), the Black-Forest (B) and the pre-Alpine (Allgäu) population (C). For details refer to the legend of **Figure 8**.

3.2.3 Spectral dependence of anthocyanin formation

To determine the spectral dependence anthocyanin formation hypocotyls from seedlings irradiated with longer (R and FR) or shorter wavelengths (UV-B, UV-A and B) were extracted for all three populations. Similar to flavonoid synthesis (**Figure 5**), only the short wavelengths were effective (**Figure 10**). However, there was a subtle difference between the populations: Whereas in the Alpine population UV-B was the most effective light quality, the Black-Forest and the pre-Alpine populations showed a stronger response for UV-A and, even more pronounced, for blue light. This was especially true for the Black-Forest population, whereas the pre-Alpine population represented some kind of intermediate pattern.

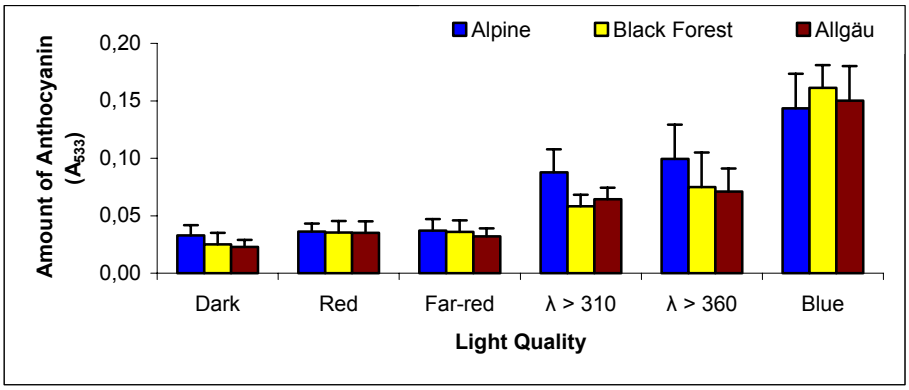


Figure 10. Spectral dependence of light-induced anthocyanin formation in the hypocotyl for the Alpine, the Black-Forest, and the pre-Alpine (Allgäue) population. For details refer to the legend of **Figure 5**.

3.2.4 Dark germination and scale-leaf anthocyanin differs in the Black-Forest population

The germination rate in the dark was determined for all three populations (**Figure 11**) and found to be much higher in the Black-Forest population as compared to the other two populations. In addition, the anthocyanin content in scale leaves collected from the indigenous site (**Figure 12**), was observed to be drastically increased in the Black-Forest population as compared to the other two populations.

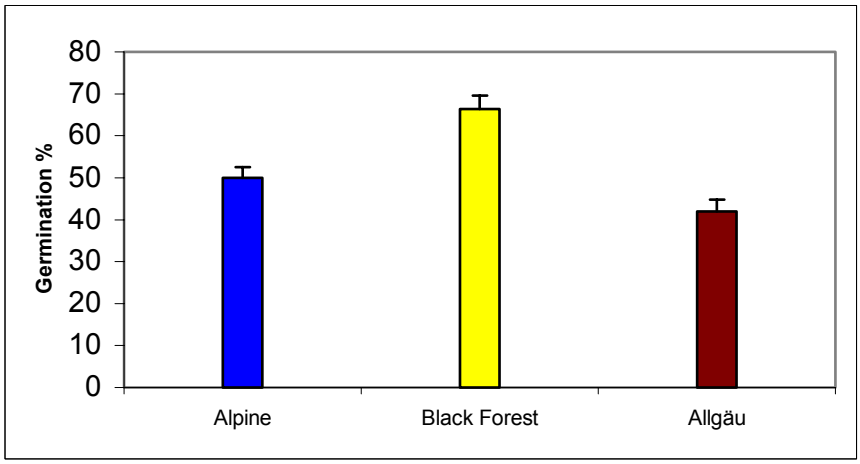


Figure 11. Frequency of germination in the dark for seeds from the Alpine, the Black-Forest, and the pre-Alpine (Allgäu) population.

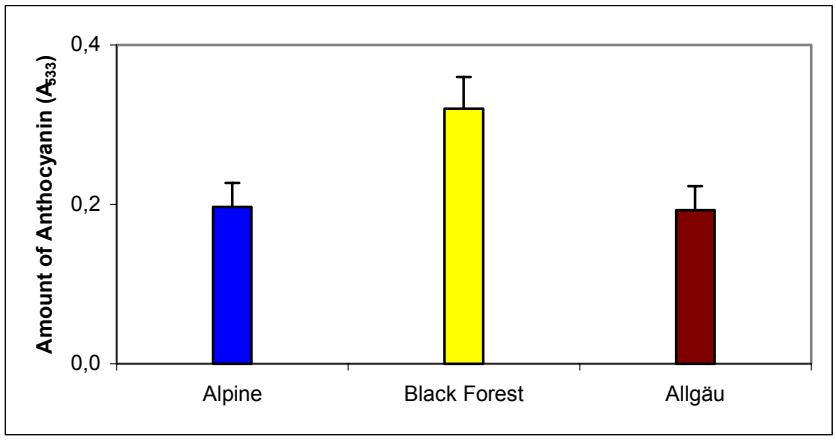


Figure 12. Anthocyanin content in scale leaves collected in situ for the Alpine, the Black-Forest and the pre-Alpine (Allgäu) population.

3.3 Discussion

To use photomorphogenesis as indicative trait to test, whether the divergence between the Alpine (Lecturer Alps) and the Black-Forest (Sägendobel) population of Green Alder has already resulted in adaptive changes related to the changed habitat conditions. Results can be summarized in the following way-

1. Flavonoid induction in the hypocotyl is under control of short-wavelength light. The most important photoreceptor seems to be the UV-B receptor, whose action is complemented by UV-A/BL receptor(s). Phytochromes are not involved. A similar result is observed for one of the end products, the anthocyanin. 2. In contrast, phytochromes are clearly involved in the regulation of flavonoid induction in the cotyledons. 3. There are subtle, but significant differences in responsiveness and sensitivity between the Alpine, the Black-Forest, and the pre-Alpine (Allgäu) population: Whereas flavonoid and anthocyanin synthesis in the hypocotyl are dominated by the UV-B receptor in the Alpine population, the Black-Forest population is characterized by a stronger influence of the UV-A/BL receptor system. The pre-Alpine population is somewhat intermediate, in terms of flavonoid induction it resembles the Alpine population, in terms of anthocyanin synthesis, it is closer to the Black-Forest population. Interestingly, the rate of dark germination is much higher in the Black-Forest population as compared to the other two populations.

3.3.1 What are the responsible photoreceptors in different population?

Under short wavelengths (UV-B, UV-A and Blue) as well as under far-red, hypocotyl growth was significantly inhibited in *A. viridis* from all the three population. However, careful observation showed the UV-B induced slightly pronounced pattern of hypocotyl inhibition activity in all population (**Figure 4**). This result interpreted that *Alnus* hypocotyls are highly sensitive to short wave length light therefore hypocotyls growth significantly inhibited. This pattern is very specific for different altitude base population of *Alnus* and the involvements important photoreceptor is UV-B. Involvements of UV-A and blue light also showed the significant inhibition of growth pressumely also active with the complementation of UV-B. However, no significant phytochrome effects have been detected except slight inhibition with FR. Similar specific photoreceptor induced hypocotyl inhibition result also reported earlier by Ballare´, et al., 1991;1995 in cucumber and in tomato seedlings and Boccalandro et al., 2001 in *Arabidopsis*. The same researcher also argued high fluence UV-B can not mediated by

phytochrome. In *Arabidopsis* hypocotyl growth inhibition and cotyledon expansion are controlled by blue, R and FR light also reported by Neff and Chory, 1998. Blue light, perceived by cry1 was reported to have similar hypocotyl growth inhibition (Hennig et al., 1999).

Pattern of UV-A photoreceptor activity in *Alnus* for hypocotyl inhibition showed remarkable inhibition in all three population. Earlier report by Lercari et al., (1989) also showed the same UV-A induced inhibitory effects in cabbage. This effect of UV-A seems to be complemented with UV-B photoreceptor. Several earlier report also showed B light induced hypocotyl inhibition in cucumis (Gaba et al., 1984), in *Prunus* (Rapparini et al., 1999) and in *Arabidopsis* (Lin et al. (1995). In the same report they also suggested the small additive effect of phytochrome which could not be ruled out. This result indicated that the 'classical' high irradiance reaction (HIR) of phytochrome, as found in etiolated seedlings with a strong pick of action in the far-red spectral range around 720 nm (Mohr, 1984). Under R light there is no inhibitory effect in *Alnus* hypocotyl showed no photochrome involvements. Report in *Sinapis alba* showed both R and FR light induced significant inhibition of hypocotyl (Beggs et al., 1980) and in *Avena* seedlings (Schäfer et al., 1983).

The pronounced pattern of UV-B ($\lambda > 310$) photoreceptor action was detected for flavonoid induction in hypocotyl of *Alnus*. Taking consideration the UV-B photoreceptor is most importan in *Alnus* whose action was found to be highly specialized on population level. Under this light the highest flavonoid content were detected in the Alpine population as compared to the pre-Alpine (Allgäu) or the Black-Forest population (**Figure 5**). A stimulation of flavonoid synthesis was also observed under UV-A ($\lambda > 360$) and Blue light. This short-wavelength response in the Black-Forest population was less pronounced as compared to the other two populations. The Allgäu population holds intermediate position. This result explain in one hand the especial action of UV-B photreceptor in Alps population and other hand also explain the action accompained with UVA and B light photoreceptor. In an earlier report by Larson and his collegue (1990) showed the same UV-B mediated major flavonoid production in an alpine than non alpine species of *Aquilegia*. Age, light intensity and irradiation duration dependent accumulation of flavonoid also described for *Alnus* in this thesis (**Figure 8 and 9**). High intensity induce low flavonoid production has been reported in *Arabidopsis* (Lois, 1994). Buchholz et al., (1995), also shown the evidence that UV-B mediated phytochromes involvement and higher flavonoid production in *Sinapis alba.* B light induced enhancement of

flavonoid production was shown in different plant species (Khurana and Poff, 1999). Seedling experiments under different light conditions indicated most probably flavonoid synthesis regulated by independent pattern of photoreceptors in *Alnus* seedlings.

Cotyledons experiment under different light conditions strongly suggest phytochrome involvement during flavonoid production in different *Alnus* population. Continuous R light was the least effective light quality for all the populations (**Figure 6**). Highest response was observed under FR light for the Alpine population means the involvement of phytochrome. In parallel with short wavelengths the same Alpine population showed also highest sensitivity indicated the complementation with short wavelengths action in cotyledons for flavonoid production. UV-B induced kinetics also clearly showed Alpine population produced maximam flavonoid after 48 h of irradiation (**Figure 7A, 7B and 7C**). Less difference was observed for UV-A and Blue light. For UV-B, pre-Alpine populations showed very close sensitivity to Alpine. In contrast, the Black-Forest population is definitely less sensitive to UV-B.

Under short wavelengths light, subtle difference of anthocyanin between the populations were determined: whereas in the Alpine population UV-B was the most effective light quality, the Black-Forest and the pre-Alpine populations showed a stronger response for UV-A and, even more pronounced for blue light. This was especially true for the Black-Forest population, whereas the pre-Alpine population represented some kind of intermediate pattern. To evaluate above result it may clearly suggest *Alnus* does not have any kind of phytochrome effect for anthocyanin production or in other way it may have some kind complemented effect only with UVA/B light photoreceptors. This result might followd earlier demonstrated CRY1 that regulates *chs* exppression in *Arabidopsis* (Jackson and Jenkins, 1995) and also suggest that *chs* enzyme accumulate for anthocyanin synthesis. Anthocyanin synthesis and *chs* expression are strongly control by UV-A/B light and UV-B also showed in cultured cells of *Parsley* (Ohl et al., 1989) and in mature leaves of *Sinapis*, *Parsley*, *Arabidopsis* and *Petunia* (Batschauer et al., 1996; Jenkins et al., 1995; Koes et al., 1989; Krizek et al., 1998; Wellmann et al., 1976) again suggest the possibility to involvement with anthocyanin production in *Alnus*. In contrast accumulation of anthocyanin by the induction of R, FR and B light have been reported in other plants (Drumm and Mohr, 1981; Mol et al., 1996; Lin et al., 1995). Anthocyanin synthesis may regulated by independent photoreceptor or by the action of other photoreceptor also reported by various authors (Beggs & Wellmann, 1985; Jain & Guruprasad, 1990; Kubasek et al., 1992;

Mancinelli, 1994; Buchholz et al., 1995). Scale leaves anthocyanin accumulation showed the Black Forest population is most sensitive than other two population (**Figure 12**).

The four days old seedlings of *A viridis* were found to be most photoresponsive for anthocyanin production in all populations. Age-dependent potentiality and induction of anthocyanin synthesis was first described Malaviya and Laloraya (1966) in studies with *Celosia plumosa* seedlings. Reddy et al. (1994) also reported a similar age-dependent induction of anthocyanin levels in rice seedlings. This pattern may be dependent on the distribution of different photoreceptors and the competence for the photoreceptors regarding to anthocyanin accumulation in each part of the hypocotyls and other tissues (Yamaguchi, et al., 2000).

It has often been suggested that anthocyanin formation in response to UV-B radiation represents a protective response whereby plants shield sensitive molecules from damaging effects of UV-B radiation. Alps population feet very well with this suggesion as Alps *Alnus* should cope with altitude dependent physiology and high UV-B. In contrast Allgäu and Black Forest may not required such protection, therefore less sensitivity could be occurred in general. Anthocyanin accumulation during dehydration also suggest in *Craterostigma wimsii* and *Xerophyta viscosa* plants that of resurrection is thought to shield these dehydrated plants from photoinhibition (Sherwin et al., 1998).

3.3.2 How do population differ due to the habitats differ?

From earlier report (Oberdorfer, 1957; Wilmanns, 1977) it has been known to be well distribution of *A. viridis* especially in the Black Forest area ranging from 300 m to 1200 m above sea level. In this low elevation UV-B light intensity is lower (reduced) than high elevation (Caldwell, 1981) and there is typically shading. On the other hand Alpine (as sample were collected 1900 m altitude from base level) and pre Alpine population faced higher UV-B intensity than Black Forest population. Not only that UV-B radiation is increases with altitude. In this high mountain areas there is no shading. This seems to be true to Alpine population for higher anthocyanin production while exposed to UV-B light (**Figure 10**). Alpine plants are dwarfed, develop small and thick leaves and colourful flowers because of possible enhanced UV radiation. UV-B intensity depends on season, weather, habitat (sun, shade) and altitude and latitudinal differences. In this high elevation plant leaves absorb the UV-B and finally mesophyll tissue can damage. In this circumstances plant produce more flavonoids and related phenolic compounds to protect the cell from UV-B (Robberecht and Caldwell, 1983; Larson et al., 1990). The pre Alpine population situated in the 667 m high from base level. This

population shows intermediate sensitivity under UV-B, although there is no shading. From this result one could estimate UV-B response also depends on altitude. In the Black Forest there is a typical shading including less intensity of UV-B may be related to the dark germination. Because the Black Forest population shows higher germination rate than Alps and Allgäu. Again Allgäu population shows the intermediate germination rate.

3.3.3 What is the possible adaptive function of the observed differences?

One point could be important to discuss about UV-B as a responsible photoreceptor which is reduced in the Black Forest population in favour of UV-A and B light (see the anthocyain spectral dependency). This could be a preliminary indicator for specific adaptation of Black Forest which also nicely linked to the altitude base UV-B differences (seems also be an adaptive change). Natural condition the Black-Forest *Alnus* situated typically in the steep canopy rather than in exposed sites (what could seen in the Alpine population), therefore, it is clear that seed must able to germinate even shaded by a canopy (**Figure 11**). Thus, the dark germination would be an favourable adaptation to this new ecological niche of Black Forest population. In this contents the Allgäu population somehow intermediate, because the flavonoids in the hypocotyl are dominated by UV-B (i.e. it is still like the Alpine population). The anthocyanin, however, is better induced by UV-A and B light (i.e. it already has acquired the Black-Forest trait). This is one hand meaningful (lower altitude of the habitat), on the other hand it is also evolutionary meaningful - the Allgäu population represents an evolutionary bridge and shows how the Black-Forest population looked like just after divergence: first, a new trait (increased BL/UV-A competence) was introduced into the end of the metabolic pathway (anthocyanin), whereas the earlier steps of the pathway remain still under UV-B control.

4. Molecular Systematics of Green Alder

4.1 Introduction

4.1.1 Chloroplast DNA (cpDNA) is a marker for genetic variation

The climatic changes during the Pleistocene ice ages had a dramatic influence on many species (Dynesius and Jansson, 2000) causing separation, migration and extinction of populations (Bennett, 1997; Taberlet et al., 1998) as well as accelerating evolution (Comes and Kadereit, 1998; Hewitt, 2000). The dramatic changes of habitat during the ice ages have also caused diverging evolutionary developments within species (Hewitt, 2000). Ice ages occur at regular intervals of 100,000 years interrupted by warm interglacial periods lasting for 10-20,000 years and are thought to result from instabilities of terrestrial climate caused by the Milankovitch cycles (Bennett, 1990). Various molecular methods offer the possibility to track diverging intraspecific evolution (Schaal et al., 1998; Newton et al., 1999). The genetic structure of a plant species, as revealed in recent phylogeographic studies (Comes and Kadereit, 1998; Schaal et al., 1998) can be very different, ranging from strong variance within small geographic areas (Travis et al., 1996; Bauert et al., 1998; Stehlik et al., 2001a) to weak structural patterns in large areas due to a high extent of (long-distance) migration and gene flow (Gabrielsen et al., 1997; Hagen et al., 2001). Many species that are today common in Northern Europe survived the glacial periods in small, low-density populations in refugia in the mountains of Southern Europe (Bennett et al., 1991). Recent studies on different plant species in Europe and North America have shown that refugial areas and postglacial migration routes can be identified using DNA markers (Dumolin Lapegue et al., 1997; Ferris et al., 1998; Demesure et al., 1996; Sewell et al., 1996). Moreover, the evolutionary relationship between different species or populations of a species as well can be identified using DNA markers (Solties et al. 1992; Soltis et al., 1997; Taberlet et al., 1998; Palmer, 1987). For instance, fossile pollen maps for European deciduous oaks indicate refugia in Southern Spain, Southern Italy and the Balkan Peninsula (Huntly and Birks, 1983 ; Bennett et al., 1991). Two separate studies based on cpDNA variation have confirmed the existence of these three refugia (Dumolin et al., 1997; Ferris et al., 1998) and also identified areas in Northern Europe that were colonized by oaks from these refugia. Similar studies have also been carried out on *Fagus sylvatica* in Europe (Demesure et al., 1996) and *Liriodendron tulipifera* in North America (Sewell et al., 1996).

In case of Alder, Huntley and Birks (1983) provide evidence for at least three glacial refugia for *Alnus* based on fossile pollen data, including Corsica, the Carpathian Mountains and Southwestern Russia and the Bay of Biscay region. A previous study of isozyme variation in *A.*

glutinosa (Prat et al., 1992) demonstrated strong differentiation between populations that was attributed to both ecological and historical events affecting population evolution. This contrasted with the results of Bousquet et al., 1990 who found very little population differentiation in the North American *A. sinuata* and *A. crispa*. A recent study of cpDNA variation in *A. glutinosa* from different European populations indicated that most of the populations in Northern and central Europe were colonized from a refugium in the Carpathian Mountains (King and Ferris, 1998).

The classical work by Chase et al. (1993) who inferred the first overall angiosperm phylogeny based on sequences of the chloroplast gene rbcL created a new interest in angiosperm systematics, since there was a clearly defined hypothesis to be tested. At the same time the field of molecular systematics developed rapidly. It became obvious that analysing the sequences of only one gene basically reflects the phylogeny of this particular gene, and further attempts are necessary to test if this gene phylogeny is congruent with the organismic phylogeny (Doyle, 1992; Kellogg et al., 1996; Rieseberg and Soltis, 1991). It was shown that it may be crucial to consider chloroplast capture resulting from hybridization in particular at lower taxonomic levels by comparing nuclear and plastid based phylogenics (Soltis and Kuzolf, 1995). From these studies, the *trn*T-*trn*F of chloroplast DNA has emerged as versatile and useful marker that is now widely used for phylogenetic relationship as family, species and population level.

4.1.2 Chloroplast DNA (cpDNA) structure

Chloroplast DNA (cpDNA) has been used extensively to infer plant phylogenics at different taxonomic levels. However, as compared to nuclear DNA (Wolfe et al., 1987) cpDNA has experienced limited use in population studies at the intraspecific level (Palmer, 1987). Several studies have shown differing levels of intraspecific cpDNA variation in a wide range of plant species (Solties et al., 1992). Chloroplasts are cytoplasmic organelles present in green plants and contain their own genetic system. The chloroplast genome varies little in size, structure, and gene content among angiosperms (**Figure 13:** exemplary shown a tobacco chloroplast genome). It consists of homogenous circular double-stranded DNA molecules of 120-190 kb in size that contain 3-5 different rRNA genes, about 30 different tRNA genes and up to 100 different polypeptide genes (Palmer, 1985; Sugiura 1989; Shimada and Sugiura, 1991). Chloroplast DNA is maternally inherited in the majority of flowering plants and the predicted

organelle genes (chloroplast and mitochondrial) are highly structured when compared to nuclear genes (Petit et al., 1993).

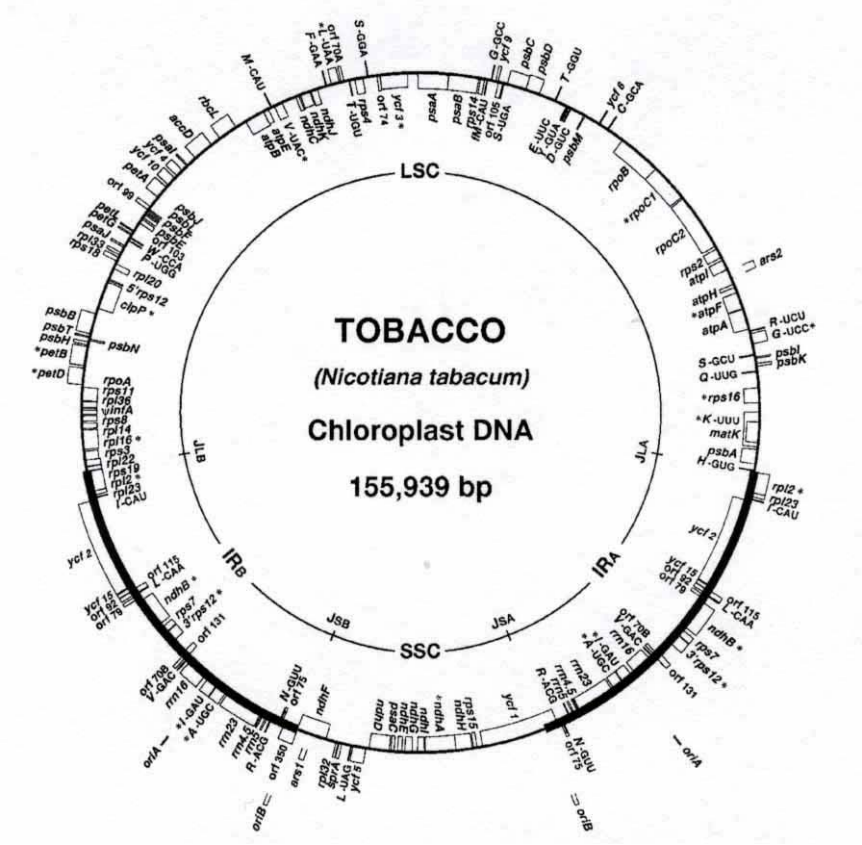


Figure 13: Tobacco chloroplast genome and their gene map (according to Wakasugi et al., 1998).

Direct sequencing of chloroplast DNA after amplification by the polymerase chain reaction (PCR) has now become a rapidly expanding area of plant systematics and evolution (Clegg and Zurawski, 1991; Palmer, 1991). Due to their rare occurrence major structural rearrangements in the cpDNA have proven to be extremely powerful markers for evolutionary relationships at higher taxonomic levels (Jansen and Palmer, 1987). In addition, sequence variations of chloroplast DNA spacer and intron sequences have been used in several studies on plant molecular evolution (Morton & Clegg. 1993; Van Ham et al., 1994).

The size of the non-coding regions of the chloroplast genome is small enough to allow complete cloning to identify the regions with the highest frequency of mutations (Palmer et al., 1988; Clegg et al., 1991). Non-coding regions tend to evolve more rapidly than do coding

sequences. Therefore, by amplification and direct sequencing of these non-coding regions, the resolution of cpDNA can be increased both for evolutionary studies, and for identifying intraspecific genetic markers (Saiki et al., 1988). The major rearrangements include inversions, translocations, loss of one copy of the large inverted repeat, along with gene and intron gain/losses (Downie and Palmer, 1991) which is of interest for evolutionary studies (Zurawski and Clegg, 1987; Clegg and Zurawski, 1991). At the level of closely related taxa, the evolutionary divergence of cpDNA is mainly due to nucleotide substitutions and insertion/deletions (indels) in noncoding regions (Clegg and Zurawsky, 1991; Palmer, 1991). Indels have been frequently observed in numerous studies of overall cpDNA variation. Several studies that have focused on the molecular evolution of variable noncoding regions of the chloroplast genome between closely-related taxa (Zurawski et al., 1984; Doebley et al., 1987; Aldrich et al., 1988; Ogihara et al., 1988, 1991; vom Stein and Hachtel, 1988; Wolfson et al., 1991; Nimzyk et al., 1993), have uncovered that small length mutations of 1-10 bp occur most frequently and are often found in close proximity to short repeated sequences. They may be caused by slipped-strand mispairing during replication and repair (Takaiwa and Sugiura, 1982; Zurawski et al., 1884). Larger length mutations (10-1000 bp) are less frequent and are most probably caused by recombination (Palmer, 1991). In this process short repeats may also be involved (Ogihara et al. 1988; vom Stein and Hachtel, 1988).

As a powerful tool for inferring global groupings of closely-related taxa (populations, species or genera), major rearrangements in the cpDNA might complement the small and medium-sized length mutations in noncoding regions (Ogihara et al., 1988; Wolfson et al., 1991). However, little is known about the frequency of parallel evolution between these classes of length mutations. The knowledge about this frequency would be needed in order to determine the reliability of small and medium-sized indels as taxonomic markers.

Nearly one decade before, Taberlet and his collegues (1991) designed primer sets for the PCR-amplification of three noncoding cpDNA sequences, the *trn*T-*trn*L spacer, the *trn*L 3´exon to 5´exon intron and the *trn*L-*trn*F spacer sequences. The primers are based on consensus sequences of highly-conserved, adjacent tRNA genes that flank the short, noncoding regions. The *trn*T-*trn*F-region was studied most extensively leading to the largest data set for this chloroplast region available for flowering plants so far (Taberlet et al., 1991). Therefore, I used these primer sets to analyze overall size variation in PCR products of cpDNA from different populations of *A. viridis* or species of *Alnus*. The presumably variable noncoding regions were

thought to be a particular interest to study genetic variation of cpDNA within and among closely-related species or different *A. viridis* populations.

4.1.3 Molecular clocks and the estimation of divergence times are necessary for evolutionary studies

The hypothesis of the molecular clock (MC) is based on the neutral theory of evolution. It holds that, in any given sequence, mutations accumulate at an approximately constant rate as long as the DNA sequence retains its original functions. The difference between the sequences of a given DNA segment between two species would then be proportional to the time that had elapsed after the species had diverged from a common ancestor. This time may be measured in arbitrary units and can then be calibrated in millions of years for any given gene when the fossil record of that species happens is sufficiently rich. A time scale is necessary to estimate rates of molecular and morphological change in organisms and to interpret patterns of macroevolution and biogeography (Novacek, 1992; Avise, 1994; Hallam, 1994; Easteal, et al., 1995). Therefore, I used the molecular-clock approach to calculate the time when different populations diverged from a common ancestor.

4.1.4 Scope of the study

To assess the evolutionary relationship between different species and populations of *Alnus*, a cpDNA sequence data set was produced to analyse genetic diversity in the context of population history and present-day distribution. Since intraspecific variability had been reported for various plant species especially in the *trn*L-*trn*F spacer and the *trn*L intron region, several populations of *A. viridis* and two *Alnus* species from different sites in the European Alps and the Black Forest were investigated on their evolutionary relationship.

The following goals should be achieved-

- To produce a sufficient data set for this chloroplast DNA in different populations.
- To complement previous studies by a novel approach using this molecular marker to characterize the evolutionary relationship between the different populations.
- To estimate the evolutionary time scale for the divergence of these populations.
- To clarify, whether the Black Forest populations are true glacial relicts that had diverged from the Alpine populations during the ice ages or whether they have immigrated more recently, i.e. after the end of glaciation.
- To obtain information on the extent of gene flow between the different populations of the Black Forest.

4.2 Results

4.2.1 Characterization of the *trn*T-*trn*F-region of the chloroplast genome in *Alnus*

The structure of the *trn*T-*trn*F-region with the length relations of spacers (bold blue lines) and intron (bold red line), the coding regions for the tRNA genes *trnT* and *trnF*, and the exons *trnL*-5`and *trnL*-3' (boxes) are shown for the genus *Alnus* in **Figure 14.** For all the mentioned species as well as different populations of *A. viridis* amplificates of double-stranded DNA could be obtained and sequenced for all three regions using the different primer pairs shown in **Figure 14.** After sequencing all three regions, the full-length sequence for this region of the cpDNA could be obtained, which represents the first complete *trn*T-*trn*F sequence from the genus *Alnus*. The whole *trn*T-*trn*F region could be aligned with that of *Nicotiana tabacum* as outgroup sample (data not shown). The overall length of the *trn*T-*trn*F region (including partial sequence of the tRNA genes; **Figure 14**) was 1968 bp in *Alnus* (partial sequence of *trn*T-*trn*L spacer and tRNA gene). In angiosperms, mutations causing differences in length are frequent in this region of the cpDNA. In fact, the *trn*L-*trn*F spacer region of *Alnus* showed major variations due to substitutions and insertions. Therefore, the focus of the work described below was in this region. In addition, for two additional regions sequence data were obtained (see data set 1 and 2). For each of the two spacers and the intron the variability within *Alnus* and particular between different populations of *Alnus viridis* was characterized as follows.

4.2.2 Analysis of the *trn*T-*trn*L intergenic spacer

The *trn*T-*trn*L is known to be most variable in terms of length among the three parts of *trn*T-*trn*F in other plants. Repeated sequencing (using the primer pairs A and B) result showed that Alnus has preserved this region with a length of 920 bp. This is nearly half of the total length of *trn*T-*trn*F region. The sequences for the populations from the Alps, the Allgäu, Sägendobel, and Löffeltal were aligned together with that from *A. incana* (data not shown). Between different angiosperms the length of this region can vary from 379 to 1411 bp. Up to two base pair insertions or deletions could be detected between positions 750 and 920 of this region. In total, ten variable sites were detected in this region for the investigated Alnus populations. This region offenly occurred transitional (Pyrimidine to pyrimidine or Purine to purine) and transvertional neucleotide substitution (Pyrimmidin to purine or purine to pyrimidin) variation.

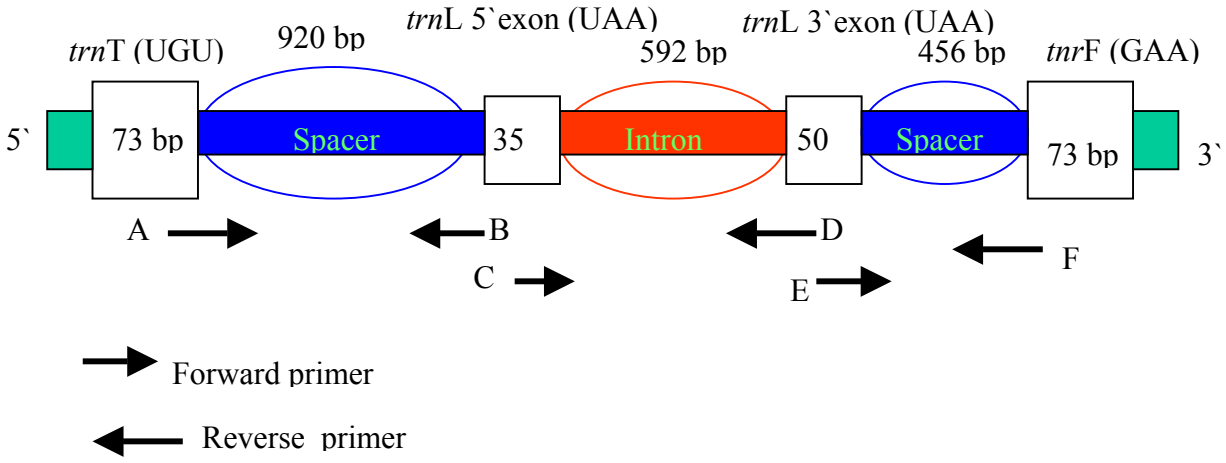


Figure 14: Schematic representation of the *trnT-trnF* region of the cpDNA in the genus *Alnus*. tRNA genes (*trnT* and *trnF* are each 73 bp long) and exons (*trn*L-5` is 35 bp and 3`is 50 bp) are represented by empty boxes. Spacers are represented by bold blue lines, the intron by a bold red line.

4.2.3 Analysis of the *trn*L intron

The primer pairs **C** and **D** were used to amplify this region of the cpDNA of *Alnus*. The length of this region consists of 592 bp (see data set 1). In addition to the species *A. viridis*, *A. incana* and *A. glutinosa*, different populations of *A. viridis* from the Alps, Allgäu, Bavaria, Sägendobel, Löffeltal, Alpersbach, Ravennaschlucht, Breitnau and Hinterwaldkopf were sequenced to produce a large data set. The alignment was performed using *Corylus cornuta* as outgroup sample. The sequences from the Alpersbach and Ravennaschlucht populations exhibited an insertion of five base pairs (GTAGG) at alignment position 491, whereas the other populations contained AGAGT in the same alignment position. In addition, eight variable sites were detected in this region. Two base pair insertion (TT, alignment position 348) occurred in *A. glutinosa*. However, *A. incana* contained only one T at alignment position 348. The other *A. viridis* populations showed two base pair deletions in the same position. To compare the outgroup *C. cornuta* with *A. viridis* populations or *Alnus* species, at least seventeen variable sites were monitored. In addition to several point mutations could be detected. In this sequence transitional (Pyrimidine to pyrimidine or Purine to purine) and transvertional neucleotide substitution (Pyrimmidin to purine or purine to pyrimidin) variation could be occurred.

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Alps GACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATACCGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

Allg GACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTT-CAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATA-CGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

Bava GACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAA-GGATGAAGGATAACCGTATATACATATGTATA-CGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

Brei GACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGGCA
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATA-CGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGCAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACGATGA
AATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

Alpe GACTTAATTGGATtGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATA-CGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGC
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATTGTAGGCCCATTCTACATGTCAATACCGACAACGATGA
AATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

Rave GACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGGCA
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATACGTACTGAAATACTATC
TCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTGT
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CTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATTGTAGGCCCATTCTACATGTCAATACCGACAACGATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

Hint GACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATA-CGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT—-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592
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SägeGACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTT-CAAATTCAGAGAAACCCTGG-A
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GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
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TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

LöffGACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTT-CAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATATGTATACGTACTGAAATACTATC
TCAAAAAATTAATGACGACCTGAATCTTTATTTCTTTTTTTT--ATATTTATATAAAAACTGAATGAAAGAGTTGT
TGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCATAGTCTGATAGAT
CTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAAA
TTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCC 592

AlglGACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCAAGTGATAACTTTCAAATTCAGAGAAACCCTGG-A
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GACTAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATA-CGTATACGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTATTTTTTTTT-ATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCAGAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC 592

AlinGACTTAATTGGATTGAGCCTTGGTATGGAAACCTACCA-GTGATAACTTTCAAATTCAGAGAAACCCTGG-A
ATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCCGAAAACAAATAAAACAAATTGAAGGGTTCATAAAGCGA
GACTAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAATTGGGGTTGACTTCGTTCCGTTATTAAA
GTAATCCTTCTATCAAAACTACAGAAAGGATGAAGGATAACCGTATATACATACCGTATACGTACTGAAATACTAT
CTCAAAAAATTAATGACGACCTGAATCTTTATTTATTTTTTTTTTATATTTATATAAAAACTGAATGAAAGAGTTG
TTGTGAATCGATCCAAATTGAAGAAAGAATCGAATATTTATTAATCAAATTATTTACTCCATCAGAGTCTGATAGA
TCTTTTGAAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATACCGACAACAATGAA
ATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCC 592
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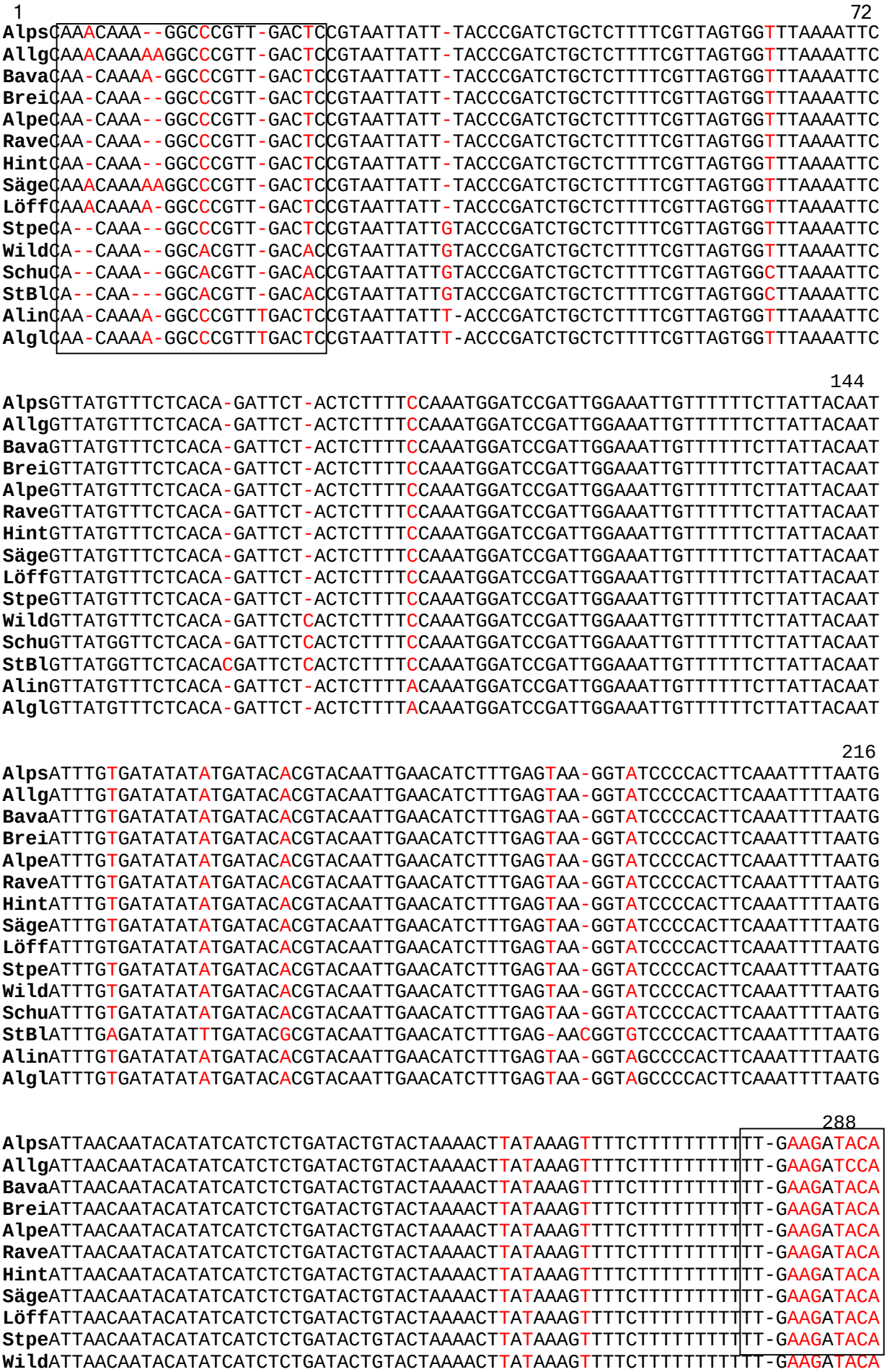
Data set 1: *trn*L region of the cpDNA from two species of *Alnus* and different *A. viridis* populations. Variable sites are marked in red. Alps- Alpine, Allg- Allgäu, Bava- Bavaria, Brei- Breitnau, Alpe- Alpersbach, Rave- Ravenaschlucht, Hint- Hinterwaldkopf, Säge- Sägendobel, Löff- Löffeltal population of *A. viridis*, and Algl- *A. glutinosa*, Alin- *A. incana* respectively. For details of sample origin see **tabel 1**.

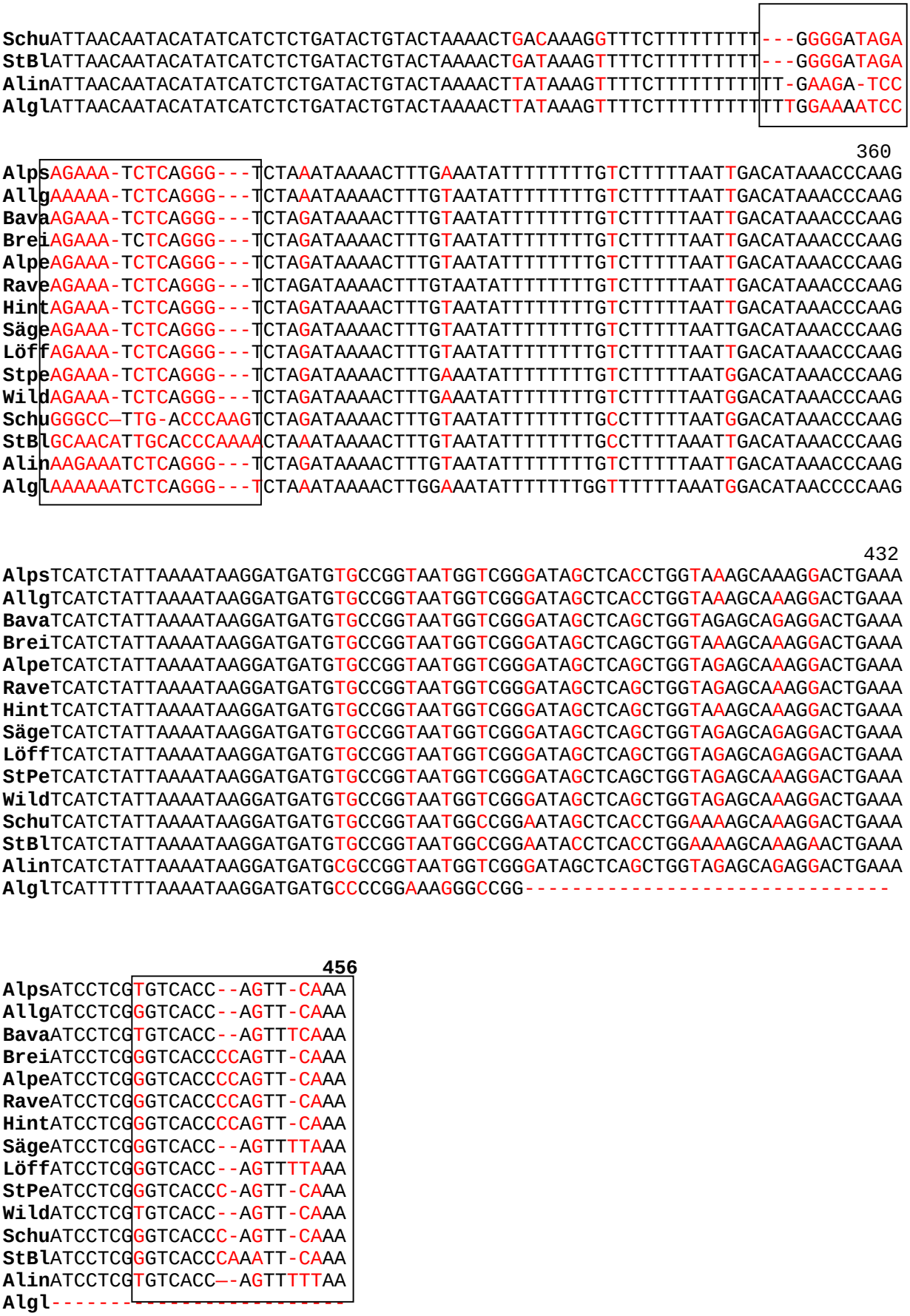
4.2.4 Analysis of the *trn*L-*trn*F intergenic spacer

The primer pairs **E** and **F** were used to amplify this region. All *Alnus* populations (see map) including two species of *A. glutinosa* and *A. incana* cpDNA were sequenced to produce an extensive data set to study the genetic relationship. From these data it is clear that this region of *Alnus* cpDNA consists of 456 bp except for *A. glutinosa* where it consists of only 390 bp (due to a different number of repetitive sequences). This part is the most variable part in *Alnus*. The variation are not only found on the level of species but even also occurred between the populations. Therefore, this data set was extensively used to study the genetic variation in order to identify glacial relict populations and to estimate the time of divergence. Although this region did not contain any inversion, it frequently exhibited several point mutation and

insertions (up to six bp). From the sequence analysis major mutational variation was located between bp 1-22, 270-304 and 440-456. For example, the St. Blasien population of *A. viridis* possessed two deletions of three base pairs (AAA and TTT, alignment positions 8 and 276, respectively). The same sequence also possessed seven base pairs long insertion (CCCAAA, alignment position 298). In addition, this sequence also contained five insertions of two base pairs between alignment positions 279 and 296. In total five insertions were found in the Schulterdobel population of *A. viridis*. The first insertion spanned six base pairs (CCCAAG, alignment position 298), and the second insertion three base pairs (GCC, alignment position 287), respectively. In addition to these insertions, the sequence also contained more than three additional nucleotides (GG pairs at alignment position 279, AG at alignment position 284, and TG at alignment position 294). Strikingly, *A. incana* contained a three base pair (TTT) single insertion close to the end of this region (alignment position 451). The Allgäu population of *A. viridis* contained two inserted nucleotide pairs (AA at positions 8, and CC at position 286). Interestingly, Allgäu, *A. incana* and *A. glutinosa* contain A whereas, in the same possition all other population contain G (alignment possition 290). With exception of the St. Blasien and Schulterdobel populations of *A. viridis*, all other *A. viridis*-populations and *Alnus* species showed a three base pair deletion at alignment position 300. In the Wildgutach, Schulterdobel and St.Blasien populations of *A. viridis* C at position 14 and T at position 22 were replaced by A. In the St.Peter, Wildgutach and Schulterdobel populations T at position 344 was replaced by G. *A. incana* possed an insertion TTT (alignment position 551). Whereas in the same position Sägendobel and Löffeltal contained only TT. Within different populations of *A. viridis* a maximum of 35 nucleotide variations were detected. On the other hand *A. incana* differd a maximum 42 nucleotide from *A. viridis* population.

Interestingly, the difference between the *A. viridis* population from St. Blasien to the other populations of the same species was more pronounced than that of *A. incana* with *A. viridis* in general.


1
72
Alps CAAACAAA--GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Allg CAAACAAAAAGGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Bava CAA-CAAAA-GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Brei CAA-CAAA--GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Alpe CAA-CAAA--GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Rave CAA-CAAA--GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Hint CAA-CAAA--GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Säge CAAACAAAAAGGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Löff CAAACAAAA-GGCCCGTT-GACTCCGTAATTATT-TACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Stpe CA--CAAA--GGCCCGTT-GACTCCGTAATTATTGTACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Wild CA--CAAA--GGCACGTT-GACACCGTAATTATTGTACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Schu CA--CAAA--GGCACGTT-GACACCGTAATTATTGTACCCGATCTGCTCTTTTCGTTAGTGGCTTAAAATTC
StBl CA--CAA---GGCACGTT-GACACCGTAATTATTGTACCCGATCTGCTCTTTTCGTTAGTGGCTTAAAATTC
Alin CAA-CAAAA-GGCCCGTTTGACTCCGTAATTATTT-ACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
Algl CAA-CAAAA-GGCCCGTTTGACTCCGTAATTATTT-ACCCGATCTGCTCTTTTCGTTAGTGGTTTAAAATTC
144
Alps GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Allg GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Bava GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Brei GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Alpe GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Rave GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Hint GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Säge GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Löff GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Stpe GTTATGTTTCTCACA-GATTCT-ACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Wild GTTATGTTTCTCACA-GATTCTCACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Schu GTTATGGTTCTCACA-GATTCTCACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
StBl GTTATGGTTCTCACACGATTCTCACTCTTTTCCAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Alin GTTATGTTTCTCACA-GATTCT-ACTCTTTTACAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
Algl GTTATGTTTCTCACA-GATTCT-ACTCTTTTACAAATGGATCCGATTGGAAATTGTTTTTTCTTATTACAAT
216
Alps ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Allg ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Bava ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Brei ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Alpe ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Rave ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Hint ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Säge ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Löff ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Stpe ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Wild ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
Schu ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTATCCCCACTTCAAATTTTAATG
StBl ATTTGAGATATATTTGATACGCGTACAATTGAACATCTTTGAG-AACGGTGTCCCCACTTCAAATTTTAATG
Alin ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTAGCCCCACTTCAAATTTTAATG
Algl ATTTGTGATATATATGATACACGTACAATTGAACATCTTTGAGTAA-GGTAGCCCCACTTCAAATTTTAATG
288
Alps ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Allg ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATCCA
Bava ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Brei ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Alpe ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Rave ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Hint ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Säge ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Löff ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Stpe ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA
Wild ATTAACAATACATATCATCTCTGATACTGTACTAAAACTTATAAAGTTTTCTTTTTTTTTTT-GAAGATACA



Data set 2 Alignment of the *trn*L-*trn*F region of cpDNA between different populations of *Alnus*. Highly variable sites are indicated by boxes and single nucleotide variation between different populations by red colour. For population always used only four digit i.e. Alps- Alpine, Allg- Allgäu, Bava- Bavaria, Brei- Breitnau, Alpe-

Alpersbach, Rave- Ravenaschlucht, Hint- Hinterwaldkopf, Säge- Sägendobel, Löff- Löffeltal, Stpe-Sankt Peter, Wild- Wildgutach, Schu-Schulterdobel, StBl- St. Blasien population of *A. viridis*, and Algl- *A. glutinosa*, Alin- *A. incana,* respectively. For details of sample origin see **table 1**.

4.2.5 Grouping/clustering of the populations

Based on the base-pair variations, in the *trn*L-*trn*F region all individual population of *Alnus viridis* could be clustered into several groups (**Figure 15)**. All the populations from the Black Forest and European Alps were classified under 9 different groups. However, within Black Forest at least six different groups of *A. viridis* were identified. For example, the St. Blasien population (Southern Black Forest) was clearly distinct from all other populations and constituted a separate group (group 4). Also the Schulterdobel population (Central Black Forest) was distant to the other populations but with 21 substitutions also far away from the St. Blasien population such that it had to be grouped separately (group 5). The populations from the Alps, Allgäu and Bavaria population formed individual groups (group 1, 2 and 3, respectively). The populations from Alps and Allgäu differed by four substitutions, whereas the distance between the Bavarian population to that from the Alps and that from the Allgäu was seven substitutions in both cases. The populations from Breitnau and Hinterwaldkopf fall into one group (group 7), and differed only in one nucleotide variation. However, group 7 differed by more than five nucleotides from group 9 comprising the Sägendobel and the Löffeltal population (group 9) that were identical in sequence. Individuals from Ravennaschluct and Alpersbach formed one group (group 8) and differed by only one nucleotide substitution variation, but by four nucleotides from group 9 only four nucleotides substitution variation. In contrast, groups seven and eight were mutually closely related . *A. incana* classified as separate species by the rRNA-ITS region Chen et al, 1999) clustered distantly from most *A. viridis* populations, but was definitely closer than the St. Blasien population and the Schulterdobel population of *A. viridis*.

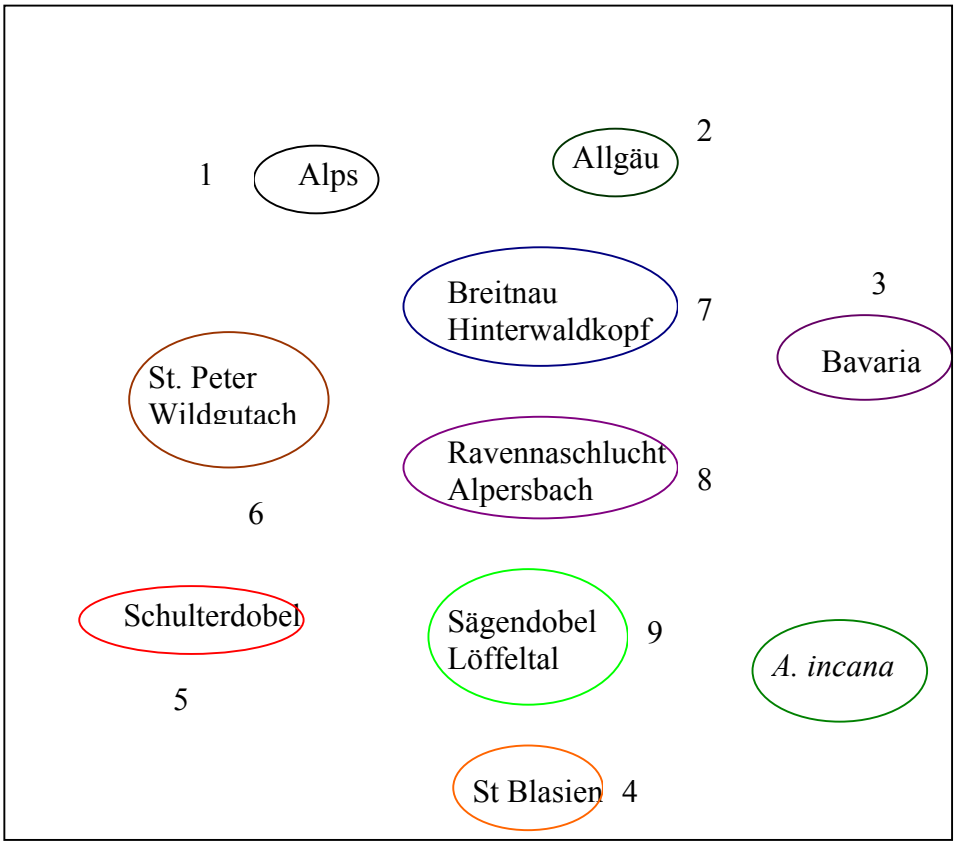


Figure 15: Relative proximity of the 13 investigated *A. viridis* populations from Black Forest and European Alps based on the *trn*L-*trn*F region. The position of the populations in the plot correspond to genetic distance. For details on the populations refer to the legend of **data set 2**.

4.2.6 Analysis of divergence times

The substitution variations were used to estimate the divergence times when two given populations presumably separated (Table 2). These data showed that the St. Blasien population separated a long time ago from all the investigated *A. viridis* populations in the Black Forest area and even earlier than the two species *A. incana* from *A. viridis*. The same is true for the Schulterdobel population. The estimated divergence between these two deviating populations occurred 4.9 Myr ago. However, the separation of the St. Blasien population from the other *A. viridis* populations happened much earlier (around 7 Myr ago). Therefore, the difference between the St. Blasien population and the remaining populations of *A. viridis* is considerably larger than that between the defined species *A. viridis* and *A. incana*. The Schulterdobel population became isolated between 4 and 5 Myr ago and thus seems to be an intermediate between the St. Blasien population and the other populations of *A. viridis*.

	Alps	Allg	Bava	Brei	Alpe	Rave	Hint	Säge	Löff	StPe	Wild	Schu	StBl	Alin
Alps		0.8	1.3	0.8	1.1	1.1	0.8	1.3	1.6	1.0	1.3	4.5	6.4	2.0
Allg	4		1.4	1.1	1.3	1.3	1.1	1.3	1.3	1.6	2.3	4.5	6.8	1.6
Bava	7	7		0.6	0.4	0.4	0.6	0.3	0.4	1.3	0.9	4.9	7.0	1.0
Brei	4	5	3		0.4	0.4	0.2	0.9	0.8	0.6	1.3	4.2	6.8	2.3
Alpe	5	6	2	1		0.2	0.2	0.7	0.5	0.4	0.9	4.1	7.6	2.0
Rave	5	6	2	1	1		0.2	0.7	0.5	0.4	1.1	4.3	6.9	2.0
Hint	4	5	3	1	1	1		0.9	0.8	0.6	1.3	4.2	6.8	2.3
Säge	7	7	2	5	4	4	5		0	1.4	1.7	5.4	6.4	1.0
Löff	8	7	2	4	3	3	4	4		1.3	1.6	4.7	7.1	1.0
StPe	5	8	7	3	2	2	3	8	7		0.6	4.4	7.2	1.5
Wild	6	11	5	6	4	5	6	9	8	3		4.1	7.3	1.7
Schu	23	24	28	22	23	23	22	30	27	24	22		4.9	4.7
StBl	29	31	35	30	34	31	30	33	35	33	32	21		8.4
Alin	11	9	6	12	11	11	12	6	6	8	9	30	42	

Table 2: Pair-wise substitution variation and divergence times (Million years) in the 13 investigated populations of *A. viridis* from the Black Forest and the European Alps. Values in the left lower half show the numbers of substitution between the two populations, the values in the right upper half the estimated divergence times are given in million years (Myr). For details on the populations refer to the legend of **data set 2**.

A third wave of isolations seemed to have occurred around 1.5 Myr ago, when the Wildgutach and St. Peter populations diverged from most of the other populations in the Black Forest, and those, in turn, became isolated from the Alps population. Interestingly, among the pre-Alpine populations, the sample from Bavaria seemed to have separated later (around 0.4-0.6 Myr ago) from most of the Black-forest populations as compared to the sample from the Allgäu (more than 1 Myr ago).

These results show that ancient (St. Blasien, Schulterdobel, Wildgutach/St. Peter) populations of *A. viridis* coexist with the relatively recently separated populations such as those from Löffeltal, Alpersbach, Ravennaschlucht, Hinterwaldkopf, and Sägendobel. We can thus distinguish at least four glacial relict populations of *A. viridis* in the Black Forest that coexist in sometimes close vicinity (**Figure 16**).

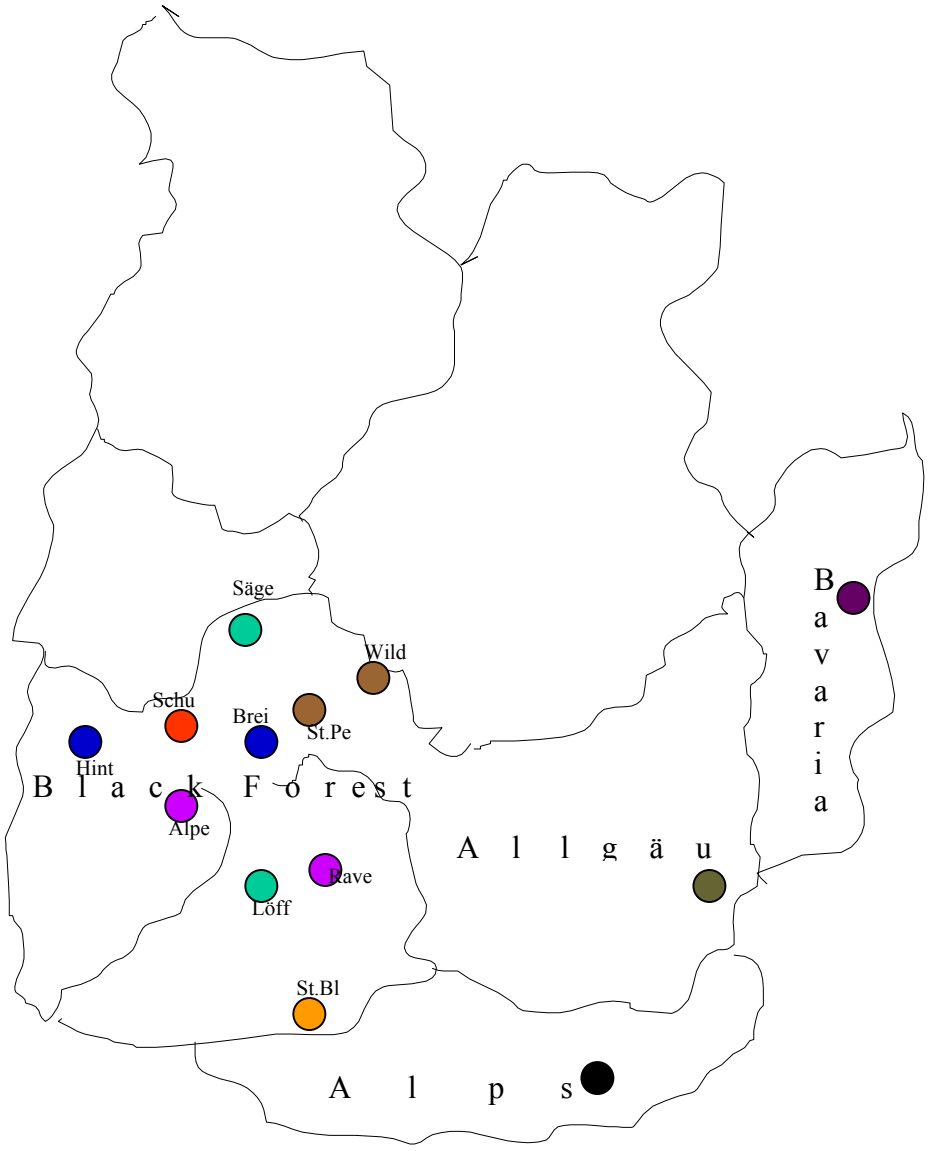


Figure 16: Schematic distribution of 13 *A. viridis* population studied in the Black Forest and the European Alps. All these population were belongs to 9 different groups. Black dot represents group 1, deep green dot 2, deep violet dot 3, orange dot 4, red dot 5, brown dots 6, blue dots 7, violet dots 8 and green dots 9. For details on the populations refer to the legend of data set 2.

4.3 Discussion

Molecular evolution of *trn*T-*trn*F non-coding chloroplast region in *Alnus*

Thirteen individual *Alnus* populations mainly distributed in the Black Forest and European Alps were taken under the *trn*T-*trn*F sequence study to determine their genetic relationship. This is the first cpDNA sequence used in *Alnus* to produce a large data set for the establishment of genetical relationships at population level. The Sequences of *trn*T-*trn*F non-coding cpDNA sequence that cover the two spacers and the intron over a broad evolutionary scale of *Alnus* population are presented (Figure 14). The *trn*T-*trn*F region is located in the large single copy region of the chloroplast genome, approximately 8 kb downstream of *rbc*L (based on the tobacco chloroplast genome, Wakasugi et al., 1998). Separated by spacers consisting of several hundred base pairs three highly conserved transfer RNA genes follow subsequently after each other: the tRNA gene for threonine (UGU), the tRNA gene for leucine (UAA), and the tRNA gene for phenylalanine (GAA). The *trn*L gene contains an intron that splits it into a 35 bp 5' exon and a 50 bp 3' exon. Bonnard et al. (1984) found that the *trn*L intron is a group I intron, and thereby differs from the other known introns in chloroplast tRNA genes. Kuhsel, Strickland, and Palmer (1990) found this intron to be conserved in secondary structure and primary sequence across cyanobacteria and chloroplasts, and thus considers it an ancient intron. The pattern of conservation may relate to functional needs in the self-splicing process during mRNA processing (Kuhsel, Strickland, and Palmer, 1990). *trn*L and *trn*F are both encoded on the A strand and are transcribed counter-clockwise, whereas *trn*T is encoded on the B strand and transcribed clockwise (Hiratsuka et al., 1989, Maier et al., 1995). Van Ham et al., (1994) found –35 and –10 promoter elements in a highly conserved region of the *trn*L-F spacer flanking the *trn*F gene.

Taberlet et al., (1991) designed primers for amplifying and sequencing the *trn*T-*trn*F region. These primers anneal to the highly conserved tRNA genes and can thus be used for a broad range of plants. The first phylogenetic studies utilising parts of the region were carried out on *Echium* (Boraginaceae) by Böhle et al., (1994; 1996), *Gentiana* and related genera by Gielly and Taberlet (1994), and on Crassulaceae (Mes and Hart, 1994; van Ham et al., 1994; Small et al., 1999). The *trn*T-L spacer has been sequenced rather rarely (Small et al., 1998; Renner 1999; Böhle et al., 1994). However, a recent publication from Borsch et al., (2003) shows that combined sequences from the *trn*T-*trn*F (*trn*T-L, *trn*L and *trn*L-F) region or were useful for inferring well resolved phylogenic trees for basal angiosperms.

The *trn*T-*trn*L region in *A. viridis* consists of a 920 bp first spacer, a 592 bp intron and the last spacer consists of 456 bp. However, in other angiosperm *trn*T-*trn*L region of cpDNA varied from 379-1411 bp. The intron region consists of 324-615 bp and the last spacer were consists 164-466 bp.

On the whole the *trn*L-*trn*F region offers more phylogenetically carring information for variation per nucleotide sequenced than the other two regions in *Alnus*. Sequences from the *trn*L-*trn*F spacer were useful to infer well resolved phylogenies of families like Crassulaceae (van Ham et al., 1994) and combined sequences from the *trn*L intron and *trn*L-*trn*F spacer were also useful for the phylogenetic relationship of families like Acanthaceae (McDade and Moody, 1999), Asteraceae (Bayer and Starr, 1998), and Gentianaceae (Gielly and Taberlet, 1996). Within Arecaceae the lack of resolution using the *trn*F-L region corresponds to the generally highly conserved chloroplast genome in this family (Baker et al., 1999). The region prooved also to be useful at lower levels: for some genera quite well resolved phylogenies are based on the *trn*L-*trn*F spacer (*Sedum*, *Monanthes*, Crassulaceae; Mes and Hart, 1994) and the combined *trn*L-*trn*F spacer (*Gentiana*, Gentianaceae, Gielly and Taberlet, 1996; Perlagonium, Geraniaceae, Bakker et al., 1999; Echium, Boraginaceae, Bohle et al., 1994). Moreover, intraspecific variability in the *trn*L intron (Polymorphic indels) has been observed in *Silene alba* (Caryophyllaceae; McCauley, 1994), and in the *trn*L-*trn*F spacer (substitutions) among geographically separated populations of *Bruguiera gymnorrhiza* (Rhizophoraceae). Terry et al., (2000) observed substitutions in both fragments that characterize geographically differentiated groups of populations in *Juniperus osteosperma* (Cupressaceae). Considerable variability in the *trn*L-*trn*F spacer sequences showed parsimony informative of different *Alnus* populations and species. Sequences of *trn*L-*trn*F region are likely to be useful for differenciating geographically separated populations within *Alnus* species.

The intron is relatively well-conserved in length whereas, the spacers are more variable. Possibly, the length conservation of the *trn*L intron corresponds to functional needs which was anticipated by Kuhsel et al., (1990) based on a relative conservation in secondary structure and primary sequence. From the two spacers the *trn*T-*trn*L spacer is considerably more variable in length (Borsch et al., 2003). In *Alnus* the intron is also relatively conserved in length (around 592 bp). The *trn*L-*trn*F length in turn, is slightly variable in different populations. This is caused by the insertion of a sequence in a certain area that seems to occur independently in different lineages and may promote insertions of larger fragments of so far unknown origin. In addition, 4-6 bp simple direct repeat motifs were noticed about 30 % less frequent in the intron

(Borsch, 2000). The degree of sequence divergence are proportionally less pronounced in the intron than in the spacers. In *Alnus*, the *trn*L intron region contained an insertion in the alignment position 491 (GTAGG) in the Alpersbach and the Ravennaschlucht population. However, other populations contain TAGAG in the same position. Only two base pair insertions (TT, alignment position 348) occurred in *A. glutinosa*. However, *A. incana* contains one T, alignment position 348. Other *Alnus* populations contain two base pair deletions in the same position.

The ***trn*L-*trn*F region is the most variable part of cpDNA in** *Alnus*

Sequences of *trn*L-*trn*F region (**data set 2**) showed more phylogenetically informative variation per nucleotide sequenced than the other two regions in *Alnus*. From the sequence results at least 43 variable sites were detected in this region. Out of 43 at least 26 sites were informative. Several studies also showed that the *trn*L-*trn*F region is a valuable source for phylogenetic studies on family, genus and species level (Baker et al., 1999; Gielly et al., 1996; Sang et al., 1997; Small et al., 1998). The St. Blasien population possessed two times three base pair deletions (AAA and TTT). In the same sequence it also possessed four base pair insertions (AAAA). It is genetically characterised by the deletion and insertion of a few base pairs in the *trn*L-*trn*F region. In addition the sequence also contained five times two base pairs insertion. It is completely different from other Alnus populations. Stehlik et al., (2002) found a genetically characterised haplotype with an additional copy of a TTATT repeat in the *trn*S-*trn*fM spacer region. Three base pair deletions occurred in position 8 of the St.Blasien alignment. However, the *trn*L-*trn*F region of the Sägendobel and Allgäu population contain additional three AAA. The Bavaria and Löffeltal population contain two additional AA in the same position. The other populations contain one additional A in the same position. On the other hand, in this region (*trn*L-*trn*F) three base pair insertion were found in the alignment position 289 (GCC) and 301 (AAG) in Schulterdobel. The Schulterdobel population also contained three base pair deletions (TTT) in the alignment position 277. Except from St.Blasien all populations contain AAA in the same alignment position 287 and a three base pair deletion in alignment position 300.

Are St. Blasien and Schulterdobel completely differ than others ?

Scientists had a long speculationthat glacial relict a population of *Alnus* exists in the Black Forest area (Hegi, 1957; Bresinsky, 1965; Braun, 1975; Wilmanns, 1977). In this study sequences from ten populations of *A. viridis* collected from this area were investigated.

Comparative result clearly classified the ten populations in six groups. The grouping depends on substitution variation (**Figure 15**). St. Blasien contained the highest nucleotide substitution variation as compared to the other populations. The evolutionary divergence value of this population showed maximum distance to the other populations. From this result it may be concluded that the St. Blasien population was isolated much earlier than the others. Therefore it formed a separat group. The St. Blasien population could have resisted in the ice ages in the *Nunatack* area or could have survived separately due to a few base pair deletions or insertions in the cpDNA . The second highest substitution variation of the Schulterdobel population showed the second highest evolutionary divergence distance to the other populations. From this it could be assumed that the Schulterdobel population is another ice age relict in this area. They could have separated from each other either in the same or in different ice ages. However,the Schulterdobel population also survived due to changes its cpDNA sequences by either the same insertion or deletion. One might question how several ice age relict populations could survive in the same Black Forest area. An earlier report by Stehlik et al., (2002) supports this idea. They showed that seven out of eleven haplotypes of *E. nanum* occurred in a small geographical area of the eastern and western central Alps. They also showed that both Alps haplotype are closely related groups. Although the groups in the Black Forest have been found to be closely related, they appearently did not have gene flow. Pollen fossil records suggest that *Alnus* pollen was first present in cretecious and persisted in the whole pleistocene. Other populations from Black Forest especially that from Sankt Peter and Wildgutach formed a group since they both show less variation in substitution. This group also showed a separate ice age relict. One could imagine that this group either immigrated after glaciation from the South Black Forest (St. Blasien) or it already survived there with *nunatack*. Holdereger et al., (2002) found two haplotypes in the study from *S. oppositifolia* in Alps. They also suggest that these haplotypes could have resisted the ice ages on high Alpine *nunatack* in the central chain and expanded their distribution after the retreat of the glaciers. The populations from the Alps, Allgäu and Bavaria formed a separate group due to the substitution variation. Divergence time results show that the Allgäu and Bavaria populations (although they are two different groups) are close to the Alps population. They might thus be immigrated from the Alps. However, populations like Löffeltal and Sägendobel, which have a low evolutionary divergence might have a gene flow.

Why did the *Alnus* sequence not follow a general phylogenetic tree-

The *trn*L-*trn*F region of cpDNA sequences were directly used for the production of a phylogenetic tree by the "Phylip program". Comparative tree results of genetical distances between populations revealed one major outgroup consisting of St.Blasien and Schulterdobel. All other populations (eleven) were placed in a cluster. The above mentioned tree studies on *Alnus* partially failed to find distinct phylogenetic relationships due to the outlier position of St.Blasien and Schulterdobel. The populations differed at least in 21 nucleotides although, they are placed in the same group with "Phylip program". RAPD based tree studies on *S. oppositifollia* in the Alps also failed to find distinct geographical patterns (Holderegger et al., 2002). Because of the above mentioned difficulties a tree was made without the Schulterdobel population. This result seems to be perfectly followed the above mentioned facts (nucleotide base differences) of their relationship.

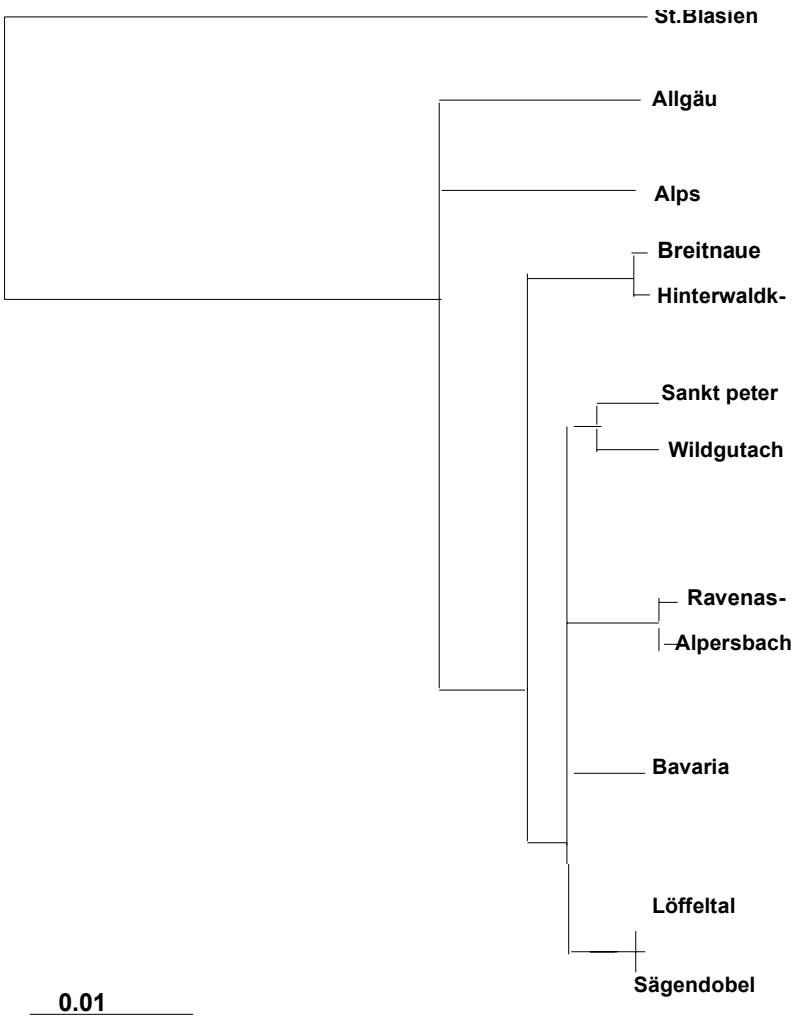


Figure 17: Schematic phylogenetic tree of twelve *A. viridis* populations.

Nevertheless, a relative distinct outgroup of the St. Blasien and Schulterdobel population in the Black Forest area was detected in this survey. Whether these findings indicate nunatack glacial survival of *A. viridis* on this altitude is unclear. That this population survived the ice age might be due to the accumulation of mutations in the chloroplast genome or the lack of local seed dispersal (special adaptations like selection pressure). I will take up more detail the nunatak versus lacking dispersal discussion in the general discussion.

5.General Discussion

Marker based study of phylogenetic relationships in the plant kingdom

One of the most exciting and revolutionizing developments in the past decade has been the application of nucleic acid sequence data for the investigation of phylogenetic relationships Early proponents of molecular systematics claimed that molecular data were more likely to reflect the true phylogeny than morphological data, because they reflected gene-level changes, which were thought to be less subject to convergence and parallelism than were morphological traits. This early assurance now appears to be wrong, and molecular data are in fact subject to most of the same problems that morphological data are. The big difference is that there are simply many more molecular characters available, and their interpretation is generally easier- "an adenine is an adenine", whereas compound leaves for example, can form in quite different ways in different plants. As a result molecular data are now widely used for generating phylogenetic hypotheses. The plant cell contains three different genomes: those of the chloroplast, the mitochondrion, and the nucleus. Systematists have used data from all three. The two organelles are generally inherited uniparentally (usually maternally in angiosperms), but the nucleus is biparental. The three genomes differ dramatically in size, with the nucleus being by far the largest-measure in megabases. The mitochondrial genome includes several hundred kilobase pairs of DNA (200-2500 kbp). The chloroplast genome is the smallest of the three plant genomes, which ranges in most plants from 120-190 kbp. Like eubacteria from which they are derived, mitochondria and chloroplast have circular genomes. The order of genes in the mitochondria is variable and they are separated by large regions of non-coding DNA. The mitochondrial genome also rearranges frequently, so that many rearranged forms can occur in the same cell. In contrast, the chloroplast genome is stable both within cells and within species. The most obvious feature of the chloroplast is the presence of two regions that encode the same genes, but in opposite direction which are known as inverted repeats. Between them are a small single copy region and a large single copy region (**see figure 14**). Rearrangements of the chloroplast genome are rare enough in evolution that they can be used to demarcate major groups. Also gain and losses of genes or their introns are common enough to be worth looking for, but rare enough to be a stable marker of evolutionary change. Chloroplast genes tend to accumulate mutations more rapidly than do mitochondrial genes in plants. DNA sequencing of genes or non coding regions is becoming more and more common and is now widely used in systematics. Sequencing determines the precise order of nucleotides

in a stretch of DNA. By using PCR -a method for enzymatical DNA-replication - simplified the cloning step itself. Within angiosperms the *rbc*L gene (which encodes the large subunit of ribulose 1, 5-biphosphate carboxylase, RUBISCO) has been widely sequenced and used for inferring plant phylogenies at higher taxonomic levels (Palmer et al., 1988; Clegg and Zurawski, 1991; Chase et al., 1993). Unfortunately, *rbc*L usually did not contain enough information to resolve relationships between closely related genera such as *Hordeum*, *Triticum* and *Aegilops* (Doebley et al., 1990; Gaut et al., 1992; Smith et al., 1993). Therefore, the analysis of non coding regions (i. e. *trn*T, *trn*L, *trn*F, *trn*K and *mat*K gene etc.) of cpDNA could extend the rapid utility of the molecule at lower taxonomic levels (Curtis and Clegg, 1984; Palmer, 1987; Wolfe et al., 1987; Stehlik, 2000; 2002; Stehlik et al., 2001a; 2001b; 2002).

The most striking results of the present analysis of basal angiosperms are based on noncoding sequences from *trn*T-*trn*F (region consist of two spacer and one intron). In bryophytes, in which the *trn*T-*trn*F region is less that half the size than in angiosperms. By all three regions have been sequenced in *Monoclea* spp. (Meißner et al., 1998) and in *Lembophyllum divulsum* (Quandt, 1999). Combined sequences from the *trn*L intron and the *trn*L-F spacer were useful for inferring well resolved phylogenies of families like Acanthaceae (McDade and Moody, 1999), Asteraceae (Bayer and Starr, 1998), Crassulaceae (van Ham et al., 1994), Gentianaceae (Gielly and Taberlet, 1996) and Nympheaceae (Borsch, et al., 2003). Betulaceae have been studied on the basis of ITS (internal transcribe sequence) and *rbc*L genes (Chen et al., 1999).

cpDNA sequence *(trnT-trn*F) and its relationship with angiosperms

Recent molecular approaches based on single and combined gene data sets have provided immense insight into the evolution of flowering plants. Hypotheses from the precladistic era recognized the Magnoliales (Takhtajan, 1980; Cronquist, 1981) with their large showy flowers and a high number of spirally arranged carpels to be the most ancestral flowering plants (so called Magnolialean Hypothesis for overview of angiosperm relationships, Qiu et al., 2000). Analyses of an 18S rDNA data set by Hamby and Zimmer (1992) resolved Nymphaeaceae as the sister group to all other angiosperms. However, results of the first large scale molecular phylogenetic analysis based on *rbc*L depicted the aquatic Ceratophyllum as the first branching angiosperm (Chase et al., 1993; Qiu et al., 1993). Subsequent intense efforts of sequencing multiple genes from different genomes culminated into a first general hypothesis of what could be the root of the angiosperms (Mathews and Donoghue, 1999; Qiu et al., 1999; Soltis et al.,

1999). The picture has changed not only by revealing *Amborella* as sister to all other angiosperms but also by providing strong corroborative evidence from various genomic regions, including *trn*T-*trn*F, in support of an *Amborella*, Nymphaeaceae and Illicicum-Schisandra-Austrobaileya (Borsch et al., 2003). Moreover, *Trimenia* has shown to be a member of the Illicum- Schisandra-Austrobaileya clade (Qiu et al., 1999; Renner, 1999; Zanis, et al., 2002). This finding provides a phylogenetic framework for one of the most species-rich groups of angiosperms. In order to reveal possible parallellisms in structural characters and to improve robustness of the molecularly derived phylogenies, the addition of genomic regions that evolve under different functional constraints as well as the integration of information from morphology, palaeobotany and developmental genetics are needed. Better understanding of sampling effects and patterns of molecular evolution in conjunction with the development of algorithms that more effectively reflect the evolutionary modes of the different genomic regions used in molecular systematics will perhaps allow further progress in this area.

Phylogenetic tree and genetic distance

The phylogenetic approach can be used to find answers to questions of evolutionary biology. In addition to resolving the taxonomic relationships of species, phylogenetics can be used to study the evolution of gene families and evaluate evolutionary rates in different lineages. The sequences can be used to compare the extend of divergence from a common ancestor within a given time period. Sequences accumulate changes over time through mutation and thus are of evolutionary relevance. Substitution is the most common kind of mutation. Mutations occur in an individual and are then either fixed (after a time be present in every individual in the population) or lost (after a time be eliminated from the population). Fixation probability depends on whether the mutation enhances the fitness (with positive selection) or diminishes the fitness (with negetive selection). To find out about losses and fixations of mutations one needs to allign all sequences prior to analysis. The correct alignment of the sequences is fundamental for the identification of homologous characters. When alignment has been made variation can be treated according to substitution type. Three basic methods were used to construct the phylogeny: 1) neighbor-joining method (a distance based method), 2) maximum parsimony, and 3) maximum likelihood. The first method identifies the closet pairs of neighbors. It is defined by two units connected through a single node in an un-rooted bifurcating tree, where two branches join at each interior node. This method continues by successive clustering of the lineage, setting branch lengths as the lineage join. The tree does not assume a constant substitution rate i.e. evolutionary clock. The phylogeny constructed from

several *Alnus* populations of the *trn*T-*trn*F region sequences by using the above mention method was somehow unable to give a clear idea on phylogenetic relationships. This issue is already discussed in the part of the discussion on molecular phylogeny: The St. Blasien population forms a completely separated group from the others. The same tree is unable to show clearly the differences between the St. Blasien and Schulterdobel populations although they differ at least with 21 nucleotide substitutions. The largest distance found by comparing 13 populations of *Alnus trn*L-*trn*F region sequence was between the St. Blasien population and the others.

Pollen fossil history and the relationship with glacial relicts of *Alnus*

The earliest occurrences for extant and extinct genera or species are currently understood from fossil records. Molecular and morphological phylogenies are compatible with fossil records. Molecular divergence data have provided an important tool for evolutionary studies, especially when the lack of fossil records hampers interpretation. The oldest known *Alnus* infructescences occur in the middle Eocene of Oregon, USA, where they co-occur with staminate catkins and leaves (Crane, 1989). But the pollen records suggest an earlier occurrence of the genus or perhaps related extinct genera. Stephanoporate pollen assigned to Alnipollenites R. Potonie with arci and vestibula diagnostic of *Alnus* is found in the Late Cretaceous (Santonian) of Japan (Miki, 1977) and becomes very common in Eurasia and North America by the latest Cretaceous (Bratzeva, 1967; Kedves and Uri-Kiss, 1968; Sun et el., 1979; Muller, 1981). However, evidence from different studies on fossil pollen suggests more diversity in the *Alnus* complex during the Late Cretaceous than at present. Pollen fossil records also showed that *Alnus* and *Betula* were differentiated as early as in the Santonian (80 Myr). On the other hand pollen fossil record suggest that all six extant genera of the Betulaceae had differentiated in the Oligocene. During the Cretaceous and early Tertiary, migration between Eurasia and North America was possible via the North Atlantic and Bering land bridges (Tiffney, 1985). The effect of these intercontinental migrations can still be observed among several genera of the family today, although the land connections have been severed. The migration of *Alnus* southward and into the southern Hemisphere might have begun from Oligocene with the climatic deterioration around the globe, and this process might have accelerated during the glacial epoch of the Quaternary. Based on fossil pollen evidence it is likely that *Alnus* reached Kalimantan during the Oligocene (Sun *et al*., 1981). *Alnus* arrived in Central America during the Miocene (Martin and Harrell, 1957; Graham, 1973). By the end of the Pleistocene, *Alnus* might have been dispersed to Africa and South America (van der Hammen, 1989) and to

Taiwan (Chung and Huang, 1972) when the sea level lowered sufficiently to permit overland migration. Records for the whole Pleistocene suggest that *Alnus* pollen frequencies are steady throughout the interglacial periods. Not only that *Alnus* pollen also occur in the glacial stages of the Pleistocene, including the mid-Devensian.

In this thesis the molecular divergence time of *Alnus* populations from the Black Forest and the European Alps based on cpDNA of *trn*L-*trn*F analyses is presented. Result showed that populations from St. Blasien and Schulterdobel might have differentiated from each other 4.9 Myr ago. That means they differentiated from each other in the Pliocene epoch. However, both populations are situated in the Black Forest area within 20 km distance. The St. Blasien and Alps populations were separated from each other 6.4 Myr ago and the Schulterdobel and Alps populations 4.5 Myr ago. The time period suggests that the St. Blasien and Schulterdobel populations differentiated from the Alps population during the Eocene epoch or both population survived separately as a nunatack. Comparative results from Allgäu with St. Blasien and Schulterdobel showed 6.8 and 4.5 Myr respectively. Possibly both populations differentiated from Allgäu during Eocene epoch. An example of an inconsistency between paleontological and molecular data, a fossile record dated to the late cretaceous included *Alnus* which followed in our result. However, other populations in the Black Forest like Ravennaschluct, Alpersbach, Breitnau, Hinterwaldkopf, Sägendobel and Löffeltal showed 900,000-200,000 Myr before they diverged from each other. This results support the fossil records of *Alnus* from Pleistocene (interglacial and glacial) which state that this species was present in different periods (Graham, 1973). The Breitnau and Hinterwaldkopf populations differentiated from each other at the same time therefore they cluster both in the same group. The same applies to the Sägendobel and Löffeltal populations.

A. viridis **in the Black Forest is not ``a``, it is several glacial relict populations !**

Many species that are common today in Northern Europe survived the glacial periods in small, low-density populations in refugia in the mountains of Southern Europe (Bennett et al., 1991). Alder, Huntley and Birks (1983) provided evidence for at least three glacial refugia for *Alnus* based on fossil pollen data, including Corsica, the Carpathian Mountains of Southwestern Russia and the Bay of Biscay region. A recent study of cpDNA variation in *A. glutinosa* from different European populations indicated that most of the populations in Northern and Central Europe were colonised from a refugia in the Carpathian Mountains (King and Ferris, 1998). However, in a long lasting speculated hypothesis – a glacial relict population of *Alnus* exists in

the Black Forest area (Hegi, 1957; Bresinsky, 1965; Braun, 1975; Wilmanns, 1977). Presented here are molecular data of the *trn*L-*trn*F region from cpDNA sequences which show the genetical relationship of *Alnus* populations within the area of the Black Forest. Divergence time from different populations showed that the St. Blasien population separated from all the others long time ago. Displaying a reasonable substitution variation the Schulterdobel population also formed a separate group. The genetical variation could have occurred in the glacial period when plants were unable to survive without any mutation that supplied adaptive traits to the changed environment. The cpDNA study from the group of Stehlik (2002) found very high genetic variation between 21 haplotypes of *Rumex nivalis* in the Alps mountain. In another study, her group successfully distinguished 11 haplotypes of *Eritrichium nanum* out of 37 populations covering the entire Alps. Holderegger et al., (2002) found only four haplotypes out of 15 populations in Alpine *Saxifraga oppositifolia*, while Abbott et al., (2000) identified 14 haplotypes in the same species at the circumpolar level. Study of 20 haplotypes of *Dryas integrifolia* showed very similar results to *R. nivalis* in North America. Huh and Huh (1999) showed that the genetic diversity is higher at the population level than at species level in *A. hirsuta* using allozyme variation. From the above mention published data one could also expect several separate *Alnus* populations in the Black Forest area since for long time different sites have been known.

The eight populations from Black Forest investigated formed four different groups. Although the results of this study does not allow to draw a very clear picture about gene flow it seems that there was no or only very little. Most probable, the St. Blasien population either survived as a *Nunatack* (an *inuit* word meaning an ice-free mountain prominently emerging above the surface of a glacier) in formarly glaciated areas or it separated/ immigrated from the main Carpathian Mountain population long time ago like it was shown for *E. nanum* by the Stehlik group. The same applies to the Schulterdobel population. It apperently either separated at the same time like the St. Blasien population or it was the same population like in St. Blasien that was isolated a long time ago. As St. Blasien is completely isolated in the southern part of the Black Forest one could not expect any gene flow with other populations. The limited gene flow by seed and high substitution variation support the glacial survival by the Stehlik group who found glacial survival of *R. nivalis* in northern peripheral refugia with a patchy distribution and ascribed the maintenance of their diversity to the limited gene flow by seed. However, it can not be ruled out that the Sägendobel and Löffeltal populations might have gene flow by wind or water dispersed seed. In the present study, the majority of the investigated populations was

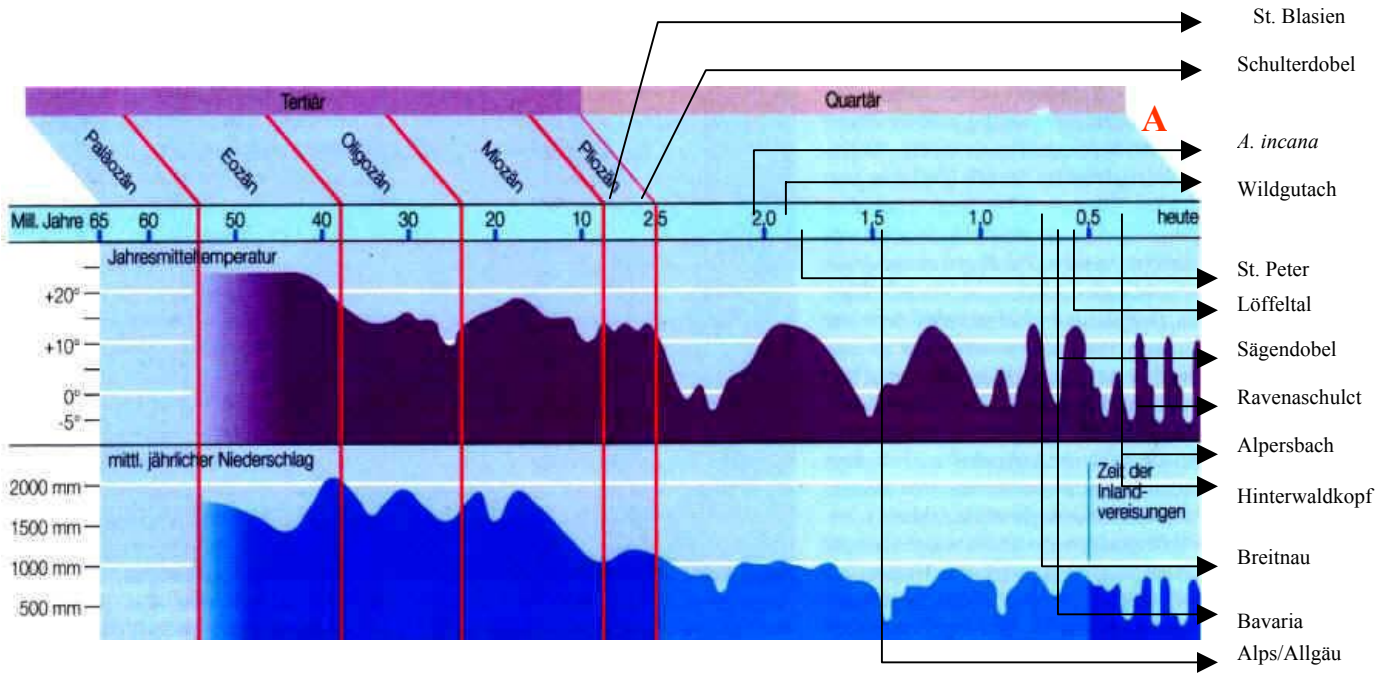
sampled within an area of approximately 150 km, and genetically variable populations were geographically structured. It is not surprising that closely related populations were detected at distances of less than 50 km. Additionally several glacial relict populations were documented within this distance. The populations from Allgäu and Bavaria formed a separate group. However, divergence time values indicated that both populations immigrated either from the Alps or they are the same population anyhow they survive with isolation and few nucleotide changes. *A. glutinosa* which showed strong differences in isozyme variation between populations (Prat et al., 1992) stands in contrast to the results of the Bousquet group (1990) who investigated North American *A. sinuata* and *A. crispa* populations which showed very little difference. However, King and Ferris (1998) showed variation between haplotypes by using RFLP of cpDNA of different European *Alnus* populations. They also suggest that most of the populations in Northern and Central Europe were founded by colonizers coming from a refugium in the Carpathian Mountains.

The divergence time distance shows that St Blasien is completely isolated from all other populations and it separated long time ago around 7 Myr. The Schulterdobel population isolated around 5 Myr earlier than the others (**Figure 18**). Maybe Schulterdobel is the intermediate population. Wildgutach and Sankt Peter formed one group and separated around 2.0 Myr ago from the others. This two populations were separated from others early in the Pleistocene ice age. All other populations from the Black Forest like Alpersbach, Hinterwaldkopf, Ravennaschlucht separated recently and coexisted very closely for some time with Wildgutach and Sankt Peter. We can thus distinguish at least four glacial relict populations in the Black Forest. For the relation of glacial relicts to a geological time scale we may think of the following hypothesis -

1. Progressive glacial relict
2. Lacking gene flow
3. *Nunatack* hypothesis

Our geological time scale relation to the glacial relict populations showed that most of the populations survived as a *Nunatack*. Earlier reports from Wilmanns (1977) suggest that in low valleys there has been only one population as glacial relict or founder. All other populations were established by migration from this founder. My results oppose her theory and show that several glacial relict populations exist in the Black Forest (**Figure 18**). I believe that they did not cross each other, nor exists gene flow among them and if only with very limited. However, gene flow can not be ruled out completely as the Alpersbach and Ravennaschluct populations

form one group the first one being situated in the upper valley and the second in the lower valley. From these two population one may suggest the possibility for seed dispersal by snow water flow from the upper valley to the lower valley. On the other hand the Hinterwaldkopf and Breitnau populations also formed one group and both population are situated in the upper valley. As Sägendobel and Löffeltal are far away with holding one group one should not think that gene flow could be maintained between them. Very little possibility exists to disperse seeds by wind in this area. Not only that I also believe all the *Alnus* population in the Black Forest area want to maintain their own trait without having any crossing. Therefore, it can be assumed that populations were maintained by vegetative propagation rather than sexual reproduction. As we know some polyploid plants exist in the polar regions that have only propagate vegetatively. However, since only a limited number of ten populations in this region has been investigated some possibility of gene flow between populations could have been overlooked.



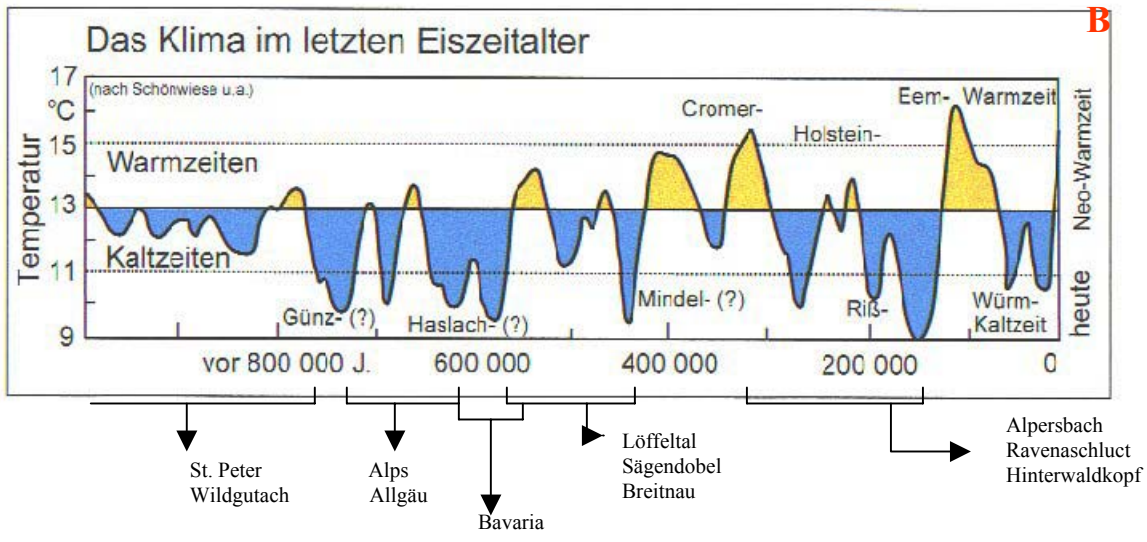


Figure 18: Course of climate changes in Europe (http://www.lothar-beckmann.de/Globalklima). (A) Progressive changes of climate during the last 65 million years in Europe and possible distribution of all ice age relicts *Alnus* population and (B) different ice age relicts *A. viridis* population and their possible distribution during the last several ice ages. Figure B possibly showed Alpersbach, Ravenaschluct and Hinterwaldkopf are the least glacial relicts population.

One can see that the divergence between the Alpine and the Black-Forest population has already resulted in adaptive changes related to the changed habitat conditions. In addition, the photomorphogenesis traits of *A. viridis,* which seems to be the most evolved adaptive feature, are altered due to habitat conditions. Light regulation for flavonoid and anthocyanin synthesis along with inhibition of hypocotyl elongation in two populations of *A. viridis* showed that these populations differ with respect to competence and sensitivity for certain photoreceptors. The result also indicates that during the separation of the populations, the role of the individual photoreceptors has been shifted from the UV-B receptor system (more pronounced in the Alpine population) in to the BL/UV-A receptors. The hypocotyl response of *A. viridis* was for all populations, most pronounced for UV-B, with the highest flavonoid content reached in the Alpine population as compared to the pre-Alpine or the Black-Forest population (Sägendobel) which is consistent with the high UV-B amount at hgher altitudes. In natural conditions the Black-Forest *Alnus* is situated typically in the dense canopy rather than in exposed sites (what could be seen for the Alpine population). Therefore, it is clear that the seed must be able to germinate even in the canopy shade. Thus, the dark germination would be a favourable adaptation for this new ecological niche of the Black Forest population. In this context the Allgäu population is somehow intermediate, because the flavonoid-synthesis in the hypocotyl is mostly induced by UV-B radiation (i.e. it is still like the Alpine population). The anthocyanin, however, is better induced by UV-A and B light (i.e. it already has acquired the Black-Forest trait).

6. Summary

Green Alder (*A. viridis*) is mainly confined to Europe and central Quebec in North America. In Europe, this species is mainly found in the sub-alpine region of the European Alps ranging between 1500 and 2000 m above sea level (also rarely found up to 2800 m), the Carpathian mountains, and the Dinarid mountains. Moreover, this plant is also distributed through the Alpine foreland, ranging from 500 m to 900 m above sea level and in isolated areas in the German *Mittelgebirge*, respectively. Interestingly, a *A. viridis* population is found in the central and Southern parts of the Black Forest ranging from 300 m to 1200 m above sea level.

Looking at the photomorphogenesis as an indicative trait to test, whether the divergence between the Alpine and the Black-Forest population of Green Alder has already resulted in adaptive changes related to the changed habitat conditions. Flavonoid induction in the hypocotyl is under control of short-wavelength light. The most important photoreceptor seems to be the UV-B receptor, whose action is complemented by UV-A/BL receptor(s). Phytochromes are not involved. A similar result is observed for the anthocyanins one of the end products of the flavonoid biosynthesis. In contrast, phytochromes are clearly involved in the regulation of flavonoid induction in the cotyledons. The significant differences in responsiveness and sensitivity between the Alpine, the Black-Forest, and the pre-Alpine population: Whereas flavonoid and anthocyanin synthesis in the hypocotyl are dominated by the UV-B receptor in the Alpine population, the Black-Forest population is characterized by a stronger influence of the UV-A/BL receptor system. The pre-Alpine population is somewhat intermediate, in terms of flavonoid induction it resembles the Alpine population, in terms of anthocyanin synthesis, it is closer to the Black-Forest population. Interestingly, the rate of dark germination is much higher in the Black-Forest population as compared to the other two populations.

To find the relationship among different populations of *A. viridis* and other *Alnus* species. I compared the sequences of the noncoding *trn*T-*trn*F (*trn*T-L and *trn*L-F-spacers and *trn*L-intron) region of the chloroplast genome. The overall length of the *trn*T-*trn*F region was 1968 bp in *Alnus*. From the *trn*L-*trn*F sequence data it is clear that this region of *Alnus* cpDNA consists of 456 bp. This part is the most variable part in *Alnus*. Although this region did not contain any inversion, it frequently exhibited transitions, transversions and insertions. From the sequence analysis major mutational variation was located between bp 1-22, 270-304 and 440-456.

Based on the base-pair variations, in the *trn*L-*trn*F region all individual population of *A. viridis* were cluster into 9 groups. However, within the Black Forest at least six different groups of *A. viridis* were identified. The St Blasien population was clearly distinct from all other populations and constituted a separate group (group 4). This is likewise for the Schulterdobel population that was also clearly distinct from the other populations but nonetheless there were also 21 substitutions between the `St. Blasien population`, so it had been grouped separately (group 5). The populations from the Alps, Allgäu and Bavaria population formed individual groups (group 1, 2 and 3, respectively). The populations from Breitnau and Hinterwaldkopf fall into one group (group 7). Group seven and eight (Alpersbach and Ravenaschlucht fall into group 8) were mutually closely related. Sägendobel and Löffeltal form group 9 and Sankt Peter and Wildgutach form group 6.

The substitution variations were used to estimate the divergence times when two given populations presumably separated. These data showed that the St. Blasien population separated a long time ago from all the other investigated *A. viridis* populations in the Black Forest area and even earlier than the two species *A. incana* and *A. viridis* separated. The same is true for the Schulterdobel population. The estimated divergence between St. Blasien and Schulterdobel populations occurred 4.9 Myr ago. However, the separation of the St. Blasien population from the other *A. viridis* populations happened much earlier (around 7 Myr ago). Therefore, the difference between the St. Blasien population and the remaining populations of A. viridis is considerably larger than that between the defined species *A. viridis* and *A. incana*. The Schulterdobel population became isolated between 4 and 5 Myr ago and thus seems to be an intermediate between the St. Blasien population and the other populations of *A. viridis*. A third wave of isolations seemed to have occurred around 1.5 Myr ago, when the Wildgutach and St. Peter populations diverged from most of the other populations in the Black Forest, and those, in turn, became isolated from the Alps population. Interestingly, among the pre-Alpine populations, the sample from Bavaria seemed to have separated later (around 0.4-0.6 Myr ago) from most of the Black-forest populations as compared to the sample from the Allgäu (more than 1 Myr ago). These results show that ancient (St. Blasien, Schulterdobel, Wildgutach/St. Peter) populations of *A. viridis* coexist with the relatively recently separated populations such as those from Löffeltal, Alpersbach, Ravennaschlucht, and Sägendobel. We can thus distinguish at least four glacial relict populations of *A. viridis* in the Black Forest that coexist in some times close vicinity.

7. References

Abbott, R.J., Chapman, R.H.M., Crawford, M.M. and Forbes D.G. (1995). Molecular diversity and derivations of populations of *Silene accuulis* and *Saxifraga oppositifolia* from the high Arctic and more southerly latitudes. *Mol. Ecology* **4**: 199-207.

Abbott, R.J., Smith, L., Milne, R., Crawford, R.M.M., Wolff, K. and Balfour, J. (2000). Molecular analysis of plants migration and refugia in the Artic. *Science* **289**: 1343-1346.

Ahmad, M., Cashmore, A. R. (1993). *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* **366**: 162-166.

Ahmad, M., Cashmore, A. R. (1996a). The *pef* mutants of *Arabidopsis thaliana* define lesions early in the phytochrome signaling pathway. *Plant. J.* **10**: 1103-1110.

Ahmad, M., Jarillo, J. and Cashmore, A. R. (1998). Chimeric proteins between *cry1* and *cry2 Arabidopsis* blue light photoreceptors indicate overlapping functions and varying protein stability. *Plant cell* **10**: 197-207.

Ahmad, M.,Lin, C. and Cashmore, A.R. (1995). Mutations throughout an *Arabidopsis* blue-light photoreceptor impair blue-light-responsive anthocyanin accumulation and inhibition of hypocotyl elongation. *Plant J.* **8**: 653-658.

Aldrich, J., Cherney, B.W., Merlin, E. and Christopherson, L. (1988). The role of insertions/deletions in the evolution of the intergenic region between *psb*A and *trn*H in the chloroplast genome. *Curr. Genet*. **14**: 137-146.

Avise, J.C. (1994). Molecular markers. In: *Natural History and Evolution.* Chapman and Hall, New York.

Bakker, F. T., Culham, A., Gomez-Martinez, R., Carvalho, J., Compton, J., Dawtrey, R. and Gibby, M. (2000). Patterns of nucleotide substitution in angiosperm cpDNA *trn*L (UAA)- *trn*F (GAA) regions. *Mol. Biol. Evol.* **17**: 1146-1155.

Baker, F.T., Culham, A., Daugherty, L.C. and Gibby, M. (1999). A *trn*L-F based phylogeny for species of *Pelargonium* (Geraniaceae) with small chromosomes. *Plant Syst. Evol*. **216**; 309-324.

Ballare´, C.L., Barnes, P.W. and Kendrick, P.W. (1991). Photomorphogenesis effects of UV-B radiation on hypocotyl elongation in wild type and stable-phytochrome-deficient mutant seedlings of cucumber. *Physiol. Planta*. **83**: 652-658.

Ballare´, C.L., Barnes, P.W. and Flint, S.D. (1995). Inhibition of hypocotyl elongation by ultraviolet-B in the de-etiolated tomato seedlings. I The photoreceptor. *Physiol. Planta* **93**: 584-592.

Bauert, M.R., Kaelin, M., Baltisberger, M. and Edwards, P.J. (1998). No genetic variation detected within isolated relict populations of *Saxifraga cernua* in the Alps using RAPD markers. *Mol. Ecol.* **7**: 1519-1527.

Batschauer, A. (1993). A plant gene for photolyse: an enzyme catalysing the repair of UV-light-induced DNA damage. *Plant Journal*. **4**: 705-709.

Batschauer, A. (1998). Photoreceptors of higher plants. *Planta* **206**: 479-492.

Batschauer, A., Rocholl, M., Kaiser, T., Nagatani, A, Furuya, M. and Schäfer, E. (1996). Blue and UV-A light-regulated CHS expression in *Arabidopsis*. *Genomics* **19:** 137-144.

Bayer, R.L. and Starr, J.R. (1998). Tribal phylogeny of the Asterraceae based on two non-coding chroloplast sequences, *trn*L intron and *trn*L/*trn*F intergenic spacer. *Ann. Missouri Bot. Gard*. **85:** 242-256**.**

Beggs, C.J. and Wellmann, E. (1985). Analysis of light-controlled anthocyanin formation in coleoptiles of *Zea mays* L.: The role of UV-B, Blue, Red and Far-Red light. *Biochem. & Photobiol*. **41(4)**: 481-486.

Beggs, C.J., Stoltzer-Jehl and Wellmann, E. (1985). Isoflavonoid formation as an indicator of UV stress in bean (*Phasiolus vulgaris* L.) leaves: the significance of photo-repair in assessing potential damage by increased solar UV-B radiation. *Plant Physiol*. **79**: 630-634.

Beggs, C.J., Holmes, M.G., Jabben, M. and Schäfer, E. (1980). Action spectra for the inhibition of hypocotyl growth by continuous irradiation in light and dark-grown *Sinapis alba* L. seedlings. *Plant Psysiol*. **66**: 615-618.

Bennett, K.D. (1990). Milankovitch cycles and their effects on species in ecological and evolutionary time. *Paleobiology* **16**: 11-21.

Bennett, K.D., Tzedakis, P.C. and Wills K.J. (1991). Quarternary refugia of North European trees. *J. Biogeog*. **5(18)**: 103-115.

Bennett, K.D.; Tzedakis, P.C. and Willis, K.J. (1991). Quaternary refugia of north European trees. *J. Biogeography* **16**: 503-540.

Bennett, K.D. (1997). Evolution and ecology. In: *The pace of life*. (**ed**.), Cambridge University press, Cambridge, UK.

Boccalandro, H.E., Mazza, C. A., Mazzella, M.A., Casal, J.J. and Ballare´C.L. (2001). Ultraviolete B radiation enhances a phytochrome-B mediated photomorphogenic responce in *Arabidopsis*. *Plant Physiol*. **126**: 780-788.

Böhle, U., Hilger, H.H., Cerff, R. and Martin, W.F. (1994). Noncoding chloroplast DNA for plant molecular systematics at the intragenetic level. In: *Molecular ecology and evolution: approach and application*, (Schierwater, B. Streit, G. Wagner P. and Salle, R. **eds.**), Birkhäuser, Basel, Switzerland, pp 391-403.

Böhle, U., Hilger, H.H. and Martin, W.F. (1996). Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). *Proc. Natl. Acad. Sci. USA* **93**: 11740-11745.

Bonnard, G., Michel, F., Weil, J.H. and Steinmetz, A. (1984). Nucleotide sequence of the split tRNA Leu (UAA) gene from *Vicia faba* chloroplasts: evidence from structural homologies of the chloroplast tRNA Leu intron with the intron from the autosplicable Tetrahymena ribosomal RNA precursor. *Mol. Gen. Genet.* **194**: 330-336.

Borsch, T. (2000). Phylogeny and Evolution of the genus Nymphyea (Nymphaeaceae). Ph.D. Thesis, University of Boon, Bonn.

Borsch, T., Hilu, W. K., Quandt, D., Wilde, V., Neinhuis, C and Barthlott, W. (2003). Noncoding plastid *trn*T-*trn*F sequences reveal a well resolved phylogeny of basal angiosperm. *J. Evol. Biol*. **16**: 558-576.

Bousquet, J.; Cheliak, W.M.; Wang, J. and Lalonde, M. (1990). Genetic divergence and introgressive hybridiztion between *Alnus sinuata* and *A. crispa* (Betulaceae). *Plant System. Evol*. **170**: 107-124.

Bousquet, J., Cheliak, W.M. and Lalonde, M. (1988). Allozyme variation within and among mature populations of speckled alder (*Alnus rugosa*) and relationship with green alder (*A. crispa*). *Am. J. Bot.* **75**: 1678-1686.

Brandt, K., Giannini, A., Lercari, B. (1995). Photomorphogenic responce to UV radiation III: a comparative study of UV-B effects on anthocyanin and flavonoid accumulation in wild-type and aurea mutant of tomato (*Lycopersicum esculentum* Mill.). *Photochem. & Photobiol*. **62**: 1081-1087.

Bratzeva, G.M. (1967). The problem of the Tsagaiansk flora with regard to spore and pollen analytical data. *Rev. Paleob. Palenol.* **2**: 119-126.

Braun, G. (1975). Zur Verbreitung und Soziologie von Alnus viridis im Schwarzwald- (Staatexamensarbeit am Lehrstuhl Geobotanik), Freiburg.

Braun-Blanquet, J. (1973). Zur Kenntnis der Vegetation alpiner Lawinenbahnen. *Mitt. Florist.-soziol. Arb. Gem*. **15/16**: 146-152.

Briggs, W.R. and Liscum, E. (1997b). Blue light-activated signal transduction in higher plants. In: *Signal transduction in plants,* (Aducci, P. **ed**.), Birkhäuser, Basel, Switzerland, pp. 107-135.

Briggs, W.R. and Huala, E. (1999). Blue-light photoreceptors in higher plants. *Annu. Rev. Cell Dev. Biol*. **15**: 33-62.

Bresinsky, A., (1965). Zur Kenntnis des circumpolaren Floren-elementes im Vorland nördlich der Alpen. *Berichte der Bayrischen Botanischen Gesellschaft zur Erforschung der heimischen Flora*, Bd. XXXVIII, 5-67.

Brüchert, F., Bogenrieder, A., Speck, T. (1994). Anatomischer und biomechanischer Vergleich der Sproßachsen von *Alnus viridis* (Chaix) DC. aus dem Schwarzwald und den Lechtaler Alpen mit Stockausschlägen von *Alnus glutinosa* (L) Gaertn. aus dem Schwarzwald im Hinblick auf die Standortsökologie beider Arten. Berichte der Natur-forschenden Gesellschaft Freiburg i.Br. **82/83,** 19-45.

Buchholz, G., Ehmann, B., Wellmann, E. (1995). Ultraviolet light inhibition of phytochrome-induced flavonoid biosynthesis and DNA photolyase formation in mustard cotyledons (*Sinapis alba* L.). *Plant Physiol.* **108**: 227-234.

Bush, M.B. and Colinvaux, P.A. (1990). A pollen record of a complete glacial cycle in lowland Panama. *J. Veg. Scin.* **5**: 105-118.

Caldwell, M. M. (1971). Solar UV irradiation and the growth and development of higher plants. In: *Photophysiology,* (Giese, AC, **ed**.) vol. **6**, Academic Press, New York, London, pp**.** 131-177

Caldwell, M. M. (1977). The effect of solar UV-B radiation (280-315 nm) on higher plants: Implications of stratospheric ozone redaction. In: *Research in Photobiology,* (Castellani, A **ed**.), Plenum Publishing Corp., New York, pp. 597-607.

Caldwell, M. M., Teramura, A.H. and Tevini, M. (1989). The changing solar ultraviolet climate and the ecological consequences for higher plants. *Trends Ecol. Evol*. **4**: 363-366.

Caldwell, M. M., Robberecht, R. and Billings, W.D. (1980). A steep latitudinal gradient of solar ultraviolet-B radiation in the arctic alpine life zone. *Ecology* **61**: 600-611.

Caldwell, M. M. (1981). Plant response to solar ultraviolet radiation. In: *Physiological plant Ecology I, Encyclopedia of Plant Physiology* (Lange, OL, Nobel, PS, Osmaon, CB and Ziegler, H, **eds**.), New Series, 12A**,** pp. 169-197.

Camm, E.L., McCallum, J. Leaf, E., and Koupai-Abyazani, M.R. (1993). Cold induced purpling of *Pinus controta* seedlings depends on previous daylight treatment. *Plant. Environ.* **16**: 761-764.

Cashmore, A.R., Jarillo, J.A., Wu, Y.J., and Liu, D. (1999). Cryptochromes: Blue light receptors for plants and animals. *Science* **284**: 760-765.

Chase, M.W, Soltis, D.E., Olmstead, R.G., Morgan, D., Les, D.H., Mishler, B.D., Duvall, M.R., Price, R.A., Hills, H.G., Qiu, Y-L. (1993). Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. *Ann. Missouri Bot. Gard.* **80**: 528-580.

Chen, D-D., Manchester, S.R. and Sun, H-Y. (1999), Phylogeny and evolution of the Betulaceae as inferred from DNA sequence, Morphology, and Paleobotany. *Am. J. Bot.* **86(6):** 1168-1181.

Christie, J. M. and Jenkins, G. I. (1996). Distinct UV-B and UV-A/blue light signal transduction pathways induce chalcone synthas gene expression in *Arabidopsis* cells. *Plant cell* **8**:1555-1567.

Christie, J. M., Reymond, P., Powell, G. K., Bernasconi, P., Raibekas, A. A., Liscum, E., and Briggs, W. R. (1998). Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. *Science* **282**: 1698-701.

Chory, J., Chatterjee, M., Cook, RK., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A., Poole, D., Reed, J. and Vitart, V. (1996). From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proc. Natl. Acad. Sci. USA* **93**: 12066-12071.

Chory, J. (1997). Light modulation of vegetative development. *Plant Cell* **9**: 1225-1234.

Chory, J. and Wu, D.Y. (2001). Weaving the complex web of signal transduction. *Plant Physiol*. **125**: 77-80.

Chung, T.F. and Huang, T.C. (1972). Paleoecological study of Taipei Basin. *Taiwania* **17**: 117-141 & 239-247.

Clack, T., Mathews, S. and Sharrock, R.A. (1994). The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: the sequences and expression of *PHYD* and *PHYE*. *Plant. Mol. Biol.* **25**: 413-427.

Clarke, G.C.S. and Jones, M.R. (1987). Plantaginaceae. In: *The Northwest European pollen flora II,* (Punt, W. and Clarke, GCS, **eds**.), Elsvier, Amsterdam, The Netherlands, pp. 129-154.

Clegg, M.T and and Zurawski, G. (1991). Chloroplast DNA and the study of plant phylogeny: present status and future prospects. In: *Molecular systematics of plants*, (Soltis DE and Doyle, JJ, **eds.**), Chapman and Hall, New York. pp 1-13.

Clegg, M.T., Learn, G.H. and Golenberg, E.M. (1991). Molecular evolution of chloroplast DNA. In: *Evolution at the molecuar level*, (Seleander, RK, Clark, AG and Whittam, TS **eds**.), Sinauer Sunderland Mass, pp. 135-149.

Clegg, M. T., Gaut, B. S., Learn, G. H. and Morton, B. R. (1994). Rates and patterns of chloroplast DNA evolution. *Pros. Natl. Acad. Sci. USA* **91**: 6795-6801.

Comes, H.P. and Kadereit, J.W. (1998). The effect of Quarternary climatic changes on plant distribution and evolution. *Trends Plant Sci.* **3**: 432-438.

Crane, P.R. (1989). Early fossil history and evolution of the Betulaceae. In: *Evolution Systematics and fossil history of the Hamamelidae*, Higher Hamamelidae, (Crane, PR and Blackmore, S **eds.),** Clarendon Press, Oxford, pp- 87-116.

Cronquist, A. (1981). An intigrated system of classification of flowering plants. Columbia University Press, New York.

Croll, J. (1875). Climate and time, Appleton, New York,

Curtis, S.E. and Clegg, M.T. (1984). Molecular evolution of chloroplast DNA sequences. *Mol. Biol. Evol.* **1**: 291-301.

Dahlgren, R. (1983). General aspects of angiosperm evolution and macrosystematics. *Nord. J. Bot.* **3**: 119-149.

Demesure, B. , Comps, B. and Petit R.J. (1996). Choroplast DNA phylogeography of the common Beach (*Fagus syslvatica* L.) in Europe. *Evolution* **50**: 2515-2520.

Deng, X.-W. and Quail, P.H. (1999). Signaling in light-controlled development. *Semin. Cell Dev. Biol.* **10**: 121-129.

Devlin, P.F., Patel, S.R. and Whitelan, G.C. (1998). Phytochrome E influences internode elongation and flowering time in *Arabidopsis. Plant Cell* **10**: 1479-1487.

Dixon, R. J. and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**: 1085-1097.

Dixon, R.A. and Christopher, L.S. (1999). Flavonoids and isoflavonoids- a gold mine for metabolic engineering. *Trends Plant Sci*. **4(10)**: 394-400.

Doebley, J., Durbin, M., Golenberg, E.M., Clegg, M.T. and Ma, D.P. (1990). Evolutionary analysis of the large subunit of carboxylase (*rbc*L) nucleotide sequence among the grasses (Gramineae). *Evolution* **44**: 1097-1108.

Doebley, J.F., Ma, D.P and Renfroe, W.T. (1987). Insertion/deletion mutations in the *Zea* chloplast genome. *Curr. Genet*. **11**: 617.624.

Dong, Y.H., Beuning, L., Davies, K., Mitra, D., Morris, B and Kootstra, A. (1998). Expression of pigmentation genes and photo-regulation of anthocyanin biosynthesis in developing Royal Gala apple flowers. *Aust. J. Plant Physiol*. **25**: 245-252.

Dörr, E. and Müller, L. (1982). Flora des Allgäus. Berichte der Bayerischen Botanischen Gesellschaft zur Erforschung der heimischen Flora. Selbstverlag der Gelesllschaft, München, 1964.

Downie, S.R. and Palmer, J.D. (1991). Use of chloroplast DNA rearrangements in reconstructing plant phylogeny. In: *Molecular Systemmatics of plants*, (Soltis, PS, Soltis, DE and Doyle, JJ. **eds**.), Chapman and Hall. New York, pp 14-35.

Doyle, J.J. (1992). Gene trees and species trees: Molecular systematics as one-character taxonomy. *Systematic Bot*. **17**: 144-163.

Drumm-Herrel, H. (1984). Blue/UV light effects on anthocyanin synthesis. In: *Blue light effects in biological systems,* (Senger, H, **ed**.), Springer-Verlag, Berlin, pp. 375-383.

Drumm-Herrel, H. and Mohr, H. (1982). Effect of blue/UV light on anthocyanin synthesis in tomato seedlings in the absence of bulk carotenoids. *Photochem. & Photobiol.* **36**: 229-233.

Drumm-Herrel, H. and Mohr, H. (1981). A novel effect of UV-B in a higher plant (*Sorghum vulgare*). *Photoche. Photobiol*. **33**: 391-398.

Dumolin L.S., Demesure, B., Finneschi, S. Le Corre, V. and Petit, R.J. (1997). Phylogeographic structure of white oaks throughout the European continent. *Genetics* **146**: 1475-1487.

Duplessy, J.C. (1978). Climatic Change, (Gribbin, J, **ed**.), Cambridge University Press, pp. 46-67.

Dynesius, M. and Jansson, R. (2000). Evulotionary consequence of changes in species: geographical distributions driven by Milankovitch climate oscillations. *Proc. Nat. Acad. Sci.* **97**: 9115-9120.

Easteal, S., Collet, C. and Betty, D. (1995). The mammalian molecular clock, (Landes, RG, **ed**.), Austin, Texas.

Engelsma, G. (1970). Photoinduction of phenylalanine deaminase in growing seedlings IV. The role of the temperature. *Planta* **90**: 133-141.

Fankhauser, C. (2001). The phytochromes, a family of red/far-red absorbing photoreceptors. *J. Biol. Chem*. **276**: 11453-11456.

Faragher, J.D. (1983). Temperature regulation of anthocyanin accumulation in apple skin. *J. Exp. Bot.* **43**: 1291-1298.

Ferris, C., King, R.A., Vainola, R. and Hewitt, G.M. (1998). Chloroplast DNA recognises three refugial sources of European oaks and shows independent eastern and western immigrations to Finland. *Heredity*. **80**: 584-593.

Fumiyasu, Y., Masayuki, N., Hitoshi, Y. and Hiroyoshi, K. (2000). Effects of temperature on the pattern of anthocyanin accumulation in seedlings of *Polygonum cuspidatum*. *J. Plant Research* **113**: 71-77.

Furuya, M. (1993). Phytochromes: their molecular species, gene families, and function. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **44**: 617-645.

Gaba, V., Black, M. and Attride, T.H. (1984). Photocontrol of hypocotyl elongation in etiolated *Cucumis sativus* L. long term, fluence rate responses to blue light. *Plant Physiol*. **74**: 897-900.

Gabrielsen, T.M., Bachmann, K., Jakobsen, K.S. and Brochmann, C. (1997). Glacial survival does not matter: RAPD phylogeography of nordic *Saxifraga oppositifolia. Mol. Ecol.* **6**: 831-842.

Gallenmüller, F., Bogenrieder, A., Speck, T. (1999). Biomechanische und ökologische Untersuchungen an *Alnus viridis* (Chaix) DC. in verschiedenen Höhenlagen der Schweizer Alpen. *Berichte der Eidgenössischen Forschungsanstalt für Wald, Schnee und Landschaft*, **347**: 1-31.

Gärtner, W., Hill, C., Worm, K., Braslavsky, S.E. and Schaffner, K. (1996). Influence of expression system on chromophore binding and preservation of spectral properties in recombinant phytochrome. *Eur. J. Biochem*. **236**: 978-983.

Gaut, B.S., Muse, S.V., Clarke, W.D. and Clegg, M.T. (1992). Relative rates of nucleotide substitution at the *rbc*L locus of monocotyledonous plants. *J. Mol. Evol.* **35**: 292-303.

Gielly, L. and Taberlet, P. (1994). The use of chloroplasts DNA to resolve plant phylogenies: noncoding versus *rbc*L sequences. *Mol. Biol. Evol.* **11**: 769-777.

Gielly, L. and Taberlet, P. (1996). A phylogeny of the European gentians inferred from chloroplast *trn*L (UAA) intron sequences. *Bot. J. Linn. Soc.* **120**: 57-75.

Gielly, L., Yuan, Y.M., Küpfer, P. and Taberlet, P. (1996). Phylogenetic use of noncoding regions in the genus *Gentiana* L.: chloroplast *trn*L (UAA) intron versus nuclear ribosomal internal transcribed spacer sequences. *Mol. Phyl. Evol.* **5**: 460-466.

Gitz, D.D., Liu, L., and McClure, J.W. (1998). Phenolic metabolism, growth, and UV-B tolerance in phenylalanine ammonia-lyase-inhibited red cabbage seedlings. *Phytochemistry* **49**: 377-386.

Godwin, H. (1975). History of the British flora: a factual basis for phytogeography, 2nd editin, Cambridge University Press, Cambridge, UK.

Graham, T.L. (1991). Flavinoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. *Plant Physiol*. **95**: 594-603.

Graham, S.W. and Olmstead, R.G. (2000). Utility of 17 chloroplast genes for inferring the phylogeny of the basal angiosperms. *Am. J. Bot.* **87**: 1712-1730.

Graham, A. (1973). Literature on vegetational history in Latin America. In: *Vegetation and vegetational history of northern Latin America*, (Graham, A. **ed.**), Elsevier, Amsterdam, The Netherlands, pp. 315-360.

Gressel, J. (1979). Blue light photoreception. *Photochem. Photobiol*. **30**: 749-754.

Hada, M., Tsurumi, S., Suzuki, M., Wellmann, E. and Hashimoto, T. (1996). Involvement and non-involvement of pyrimidine dimer formation in UV-B effects on Sorghum bicolor Moench seedlings. *J. Plant Physiol.* **148**: 92-99.

Hagen, A.R., Giese, H. and Brochmann, C. (2001). Trans-Atlantic dispersal and phylogeography of *Cerastium arcticum* (Caryophyllacese) inferred from RAPD and SCAR markers. *Am. J. Bot.* **88**: 103-112.

Hallam, A. (1994). An outline of Phanerozoic Biogeography. Oxford University Press, New York.

Hamby, R.K. and Zimmer, E.A. (1992). Ribosomal RNA as a phylogenetic tool in plant systematics. In: *Molecular Sytematics of Plants* (Soltis, PS, Solis, DS and Doyele, JJ, **eds**.), Chapman and Hall, New York, pp. 50-91.

Harrison, S.P., Kutzbach, J.E., Prentice, I.C., Behling, P.J., and Sykes, M.T. (1995). The response of northern hemisphere extratropical climate and vegetation to orbitally induced changes in insolation during the last interglaciation. *Quater*. *Res*. **43**, 174-184.

Hegi, G., (1957). Flora von Mitteleuropa. Bd. III/1. Teil. 2. Aufl. München, Hanser.

Hennig, L., Poppe, C., Unger, S and Schafer, E. (1999). Control of hypocotyle elongation in *Arabidopsis thaliana* by photoreceptor interaction. *Planta* **208**: 257-263.

Hewitt, G.M. (2000). The genetic legacy of the Quaternary ice ages. *Nature*. **405**: 907-913.

Hill, C., Gärtner, W., Towner, P., Braslavsky, S.E. and Schaffner, K. (1994). Expression of phytochrome apoprotein from *Avena sativa* in *E. coli* and formation of photoactive chromoproteins by assembly with phycocyanobilin. *Eur. J. Biochem*. **223**: 69-77.

Hiratsuka, J., Shimada, H., Whittier, R., Isibashi, T., Sakamoto, M., Kondo, C., Honji, Y., Sun, C.R., Meng, B.Y., Li, Y.Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K., Sugiura, M. (1989). The complete sequence of rice (*Oryza sativa*) chloroplast genome: Intermolecular recombination between distinct tRNA genes accounts for a major plastid DANN inversion during the evolution of the cereals. *Mol. Gen. Genet*. **217**: 185-194.

Hoffman, P. D., Batschauer, A. and Hays, J. B. (1996). *ATPHH1*, a novel gene from *Arabidopsis thaliana* related to microbial photolyases and plant blue light photoreceptors. *Mol. Gen. Genet*. **253**: 259-265.

Holdereger, R., Stehlik, I. and Abbott, R.J. (2002). Molecular analysis of the pleistocene history of *Saxifraga oppositifolia* in the Alps. *Mol. Ecol.* **11**: 1409-1418.

Huala, E. Oeller, P.W., Liscum, E., Han, I-S., larsen, E. and Briggs, W.R. (1997). *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. *Science* **278**: 2120-2123

Huh, M.K. & Huh, H.W. (1999). Genetic diversity and population structure of *Alnus hirsuta* (Betulaceae) in Korea. *Plant res.* **112**: 437-442.

Huntley, B. & Birks, H.J.B. (1983). An atlas of past and present pollen maps for Europe: 0-13 000 years ago. Cambridge University Press, Cambridge**.**

Hutchinson, J. (1967). The genera of flowering plants. **Vol.** 2: 124-126; 132-134, Clarendon Press, Oxford.

Jakson, J. A. and Jenkins, G. I. (1995). Extension growth responses and flavonoid biosynthesis gene expression in the *Arabidopsis hy4* mutant. *Planta* **197**: 233-239.

Jain, V.K and Guruprasad, K.N. (1990). Involvement of riboflavin in UV-A and white light induced synthesis of anthocyanin in Sorghum bicolor. *J. Exp. Bot.* **41**: 53-58.

Jansen, R.K. and Palmer, J.D. (1987). A chloroplast DNA inversion marks an ancient evolutionary split in the sunflower family (Asteraceae). *Proc. Nat. Acad. Sci*. *USA* **84**: 5818-5822.

Jenkins, G. I., Christie, J. M., Fuglevand, G., Long, J. C. and Jakson, J. A. (1995). Plant responses to UV and blue light: biochemical and genetic approaches. *Plant Sci*. **112**: 117-138.

Jordan, B.R., James, P.E, Strid, A. and Anthony, R.G. (1994). The effect of ultraviolet-B radiation on gene expression and pigment composition in etiolated and green pea leaf tissue: UV-B induced changes are gene-specific and dependent upon the developmental stage. *Plant Cell Environ*. **17**: 45-54.

Jordan, E.T., Cherry, J.R., Walker, J. M. and Vierstra, R.D. (1995). The aminoterminus of phytochrome A contains two distinct functional domains. *Plant J*. **9**: 243-257.

Kaliamoorthy, S. and Rao, A.S. (1994). Effect of salinity on anthocyanin accumulation in the root of maize. *Int. J. Plant. Physiol*. **37**: 169-170.

Kakegawa, K., Kaneko, Y., Hattori, K.K. and Takeda, K. (1987). Cell cultures of *Centaurea cyanus* produce malonated anthocyanin in UV light. *Phytochemistry* **26**. 2261-2263.

Kandrick, RE. and Kronenberg GHM (1994). **In:** *Photomorphogenesis in plants,* 2nd edition. Kulwer Academic Publishers, Dordrecht, The Netherlands, Boston.

Kedves, M. and Uri-Kiss, I. (1968). tudies comparatives sur les pollens du gerne *Alnus* de Tertiaire de Hongrie. *Acta Botanica Acad. Sci. Hung.* **14**: 315-321.

Kellogg, E.A., Appels, R. Mason-Gamer, R.J. (1996). When genes tell different stories: the diploid genera of Triticeae (Gramineae). *Syst. Bot*. **21**: 321-347.

Khurana, J. P. and Poff, K. L. (1999). Blue light perception and signal transduction in higher plants. In: *Concepts in Photobiology: Photosythesis and hotomorphogenesis*. (Singhal, GS, Renger, SK, Sopory, K-D and Govindjee **eds**.), Narosa Pulishing Houase, New Delhi, India, pp. 796-832.

King, R.A. and Ferris, C. (1998). Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. *Mol. Ecol*. **7**: 1151-1161.

Koes, R. E., Spelt, C. E. and Mol, J. N.M. (1989). The chalcone synthase multigene family of *Petunia hybrida* (V30) differential, light-regulated expression during flower development and UV light induction. *Plant Mol. Biol.* **12**: 231-225.

Koes, R. E., Quattrocchio, R. and Mol, J.N.M. (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *Bio Essays* **16**: 123-132.

Koornneef, M., Rolf, E. and Spruit, C.J.P. (1980). Genetic control of light inhibited hypocotyl elongation in *Arabidopsis thaliana* (L). HEYNH. Z *Pflanzenphysiol*. **100**: 147-160.

Krizek, D.T., Britz, S.J. and Mirecki, R.M. (1998). Inhibitory effects of ambient levels of solar UV-A and UV-B radiation on growth cv. new leaf fire Lettuce. *Physiol. Planta* **103**: 1-7.

Kubasek, W.L., Shirley, B.W., McKillop, A., Goodman, H.M., Briggs, W. and Ausubel, F.M. (1992). Regulation of flavonoid biosynthesis genes in germinating *Arabidopsis* seedlings. *Plant Cell* **4**: 1229-1236.

Kubasek, W.L., Ausubel, F.M. and Shirley, B.W. (1998). A light-independent developmental mechanism potentialities flavonoid gene expression in *Arabidopsis* seedlings. *Plant. Mol. Biol*. **37**: 217-223.

Kutzbach, J.E., Gallimore, R.G., and Guetter, P.J. (1991). Sensitivity experiments on the effect of orbitally-caused insolation changes on the interglacial climate of high northern latitudes. *Quaternary International*, **10-12**, 223-229.

Kuhsel, M.G., Strickland, R. and Palmer, J.D. (1990). An ancient group I intron shared by eubacteria and chloroplast. *Science* **250**: 1570-1573.

Kumer, S. and Hedges, B. (1998). A molecular time scale for vertibrate evolution. *Nature* **392**: 917-920.

Larson, R.A., Garrison, W.J. and Carlson, R.W. (1990). Differential responses of alpine and non-alpine *Aquilegia* species to increased ultraviolte-B radiation. *Plant Cell Environ*. **13**: 983-987.

Leyva, A., Jarillo, J.A., Salinas, J., and Martinez-Zapater, J.M. (1995). Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis thaliana* in a light-dependent manner. *Plant physiol*. **108**: 39-46.

Lercari, B., Sodi, F. and Sbrana, C. (1989). Comparison of photomorphogenetic responses to UV light in red and white cabbage (*Brassica oleracea* L.). *Plant Physiol*. **90**: 345-350.

Li, J., Ou-Lee, T., Raba, R., Amundson, R.G. and Last, R.L. (1993). *Arabidopsis* flavonoid mutants are hypersensitive to UV-B irradiation. *Plant Cell* **5**: 171-179.

Lin, C., Ahmad, M. and Cashmore, A.R. (1996a). *Arabidopsis* cyptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. *Plant J*. **10**: 893-902.

Lin, C., Ahmad, M., Chan, J. and Cashmore, A.R. (1995). Expression of an *Arabidopsis* cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV-A, and green light. *Proc. Natl. Acad. Sci. USA* **92**: 8423-8427.

Lin, C., Ahmad, M., Chan, J. and Cashmore, A.R. (1996b). *CRY2*: a second member of the *Arabidopsis* cryptochrome gene family. *Plant. Physiol*. **110**: 1047.

Lin, C. (2000b). Plant blue light receptors. *Trends plant Sci*. **5**: 337-42.

Liscum, E. and Briggs, W.R. (1996). Mutations of *Arabidopsis* in potential transduction and response components of phototropic signaling pathway. *Plant physiol*. **112**: 291-296.

Liscum, E. and Hangarter, R.P. (1993). Phototropic mutants of *Arabidopsis thaliana* reveal activities of multiple photosensory systems during light-stimulated apical-hook opening. *Planta* **191**: 214-221.

Lois R. (1994). Accumulation of UV-absorbing flavonoids induced by UV-B radiation in *Arabidopsis thaliana* L. *Planta* **194**: 498-503.

Madeyaska, T. (1990). The distribution of human setlement in the extra-tropical old world 24,000-15,000 BP. In: *The world at 18,000 BP*. (Softer, O. and Gamble, G. **eds**.), Unwin Hyman, London, pp. 24-37.

Maier, R.M., Neckermann, K., Igloi, G.L. and Kössel, H. (1995). Complete sequence of the Maize chloroplasts genome: Gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *J. Mol. Biol*. **251**: 614-628.

Malaviya, B., Laloraya, M.M. (1966). Anthocyanin biosynthesis in *Celosia* seedlings. I. Locus of anthocyanin formation and effect of seedling age. *Arch. Biochem. Biophys.* **114**: 56-60.

Mancinelli, A.L. (1994). The physiology of phytochrome action. In: *Photomorphogenesis in plants,* (Kendrick, RE and Kronenberg, GHM, **eds**.), Kulwer Academic Publishers, Dordrecht, The Netherlands, pp. 211-269.

Markham, K.R. (1982). Techniques of flavonoid identification. Academic Press, London, New York.

Mathews, S. and Donoghue, M.J. (1999). The root of Angiosperm Phylogeny inferred from duplicat phytochrome Genes. *Science* **286**: 947-950.

Martin, P.S. and Harrell, B.E. (1957). The Pleistocene history of temperate biotas in Mexico and eastern United States. *Ecology* **38**: 468-480.

McCauley, D.E. (1994). Contrasting the distribution of chloroplast DNA and allozyme polymorphism among local populations of *Silena alba*: implications for the study of gene flow in plants. *Proc. Natl. Acad. Sci. USA* **91**: 8127-8131.

McDade, L. and Moody, M.L. (1999). Phylogenetic relationships among Acantheceae: evidence from noncoding *trn*L-*trn*F chloroplast DNA sequences. *Am. J. Bot.* **86**: 70-80.

McNellis, TW. And Deng, X-W. (1995). Light control of seedling morphogenetic pattern. *Plant Cell* **7**: 1749-1761.

Meißner, K., Frahm, J.P., Stech, M. and Fey, W. (1998). Molecular divergence patterns and infrageric relationships of Monoclea (Monocleaceae, Hepaticeae). *Nova Hedwigia* **67**: 289-302.

Mes, T.H.M. and Hart, H. (1994). *Sedum surculosum* and *S. jaccardianum* (Crassulaceae) share a unique 70 bp deletion in the chloroplast DNA *trn*L (UAA)/*trn*F (GAA) intergenic spacer. *Pl. Syst. Evol.* **193**: 213-221.

Millar, AJ., McGrath, RB. And Chua, NH. (1994). Phytochrome phototransduction pathways. *Annu. Rev. Genet*. **28**: 325-349.

Miki, A. (1977). Late cretaceous pollen and spore floras of northern Japan: composition and interpretation. *J. Fcult. Scin. Hokkaido Univ. Ser. IV. Geol. and Minarl*. **17**: 399-436.

Milankovitch, M. (1930). In: *Handbuch der klimatologie*, (Koppen, W and Geiger, R, **eds**.), Gebruder Borntraeger, Berlin, pp. 1-176.

Mohr, H., and Drumm-Herrel, H. (1981). Interaction between blue/UV light and light operating through phytochrome in higher plants. In: *Plants and the Daylight Spectrum,* (Smith, H, **ed**.), Academic Press, London and New York, pp. 423-441.

Mohr, H. (1983). Pattern specification and realization in photomorphogenesis. In: *Photomorphogenesis Encyclopedia of plant physiology,* (Shropshire, JW and Mohr, H **eds**.), Springer-Verlag, Berlin, Heidelberg, New York, Tokyo. pp. 336-357.

Mohr, H. (1984). Criteria for photoreceptor involvement. In: *Methods in photomorphogenesis. Biologiocal techniques series*, (Smith, H and Holmes, MG, **eds**.), Academic Press, London, pp. 13-42.

Mohr, H. (1994). Coaction between pigment systems. In: *Photomorphogenesis in Plants*, (Kendrick, RE and Kronenberg, GHM, **eds**.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 353-373.

Mohr, H and Shropshire, W. Jr. (1983). An introduction to photomorphogenesis for the general reader. In: *Photomorphogenesis, Vol. 16, Encyclopedia of Plant Physiology, N.S.,* (Shropshire, JW and Mohr, H, **eds**.), Springer-Verlag, Berlin, pp. 24-38.

Mohr, H. (1994). Coaction between pigment systems. In: *Photomorphogenesis in Plants,* (Kendrick, RE and Kronenberg, GHM, **eds**.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 353-373.

Mol, J., Jenkins, G., Schäfer, E. and Weiss, D. (1996). Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. *Crit. Rev. Plant Sci*. **15**: 525-557.

Morton, B.R. & Clegg, M.T. (1993). A chloroplast DNA mutational hotspots and gene conversion in a non-coding region near *rbc*L in the grass family (Poaceae). *Curr. Genet.* **24**: 357-365.

Müller, J. (1981). Fossil records of extent angiosparms. *Botanical Review* **47**: 1-142.

Mumford, P.M. (1990). Dormancy break in seeds of *Impatitiens glandulifera* Royle. *New Phytol*. **115**: 171-175.

Neff, M.M., Fankhauser, C. and Chory, J. (2000). Light: An indicator of time and place. *Genes Dev*. **14**: 257-271.

Neff, M.M. and Chory, J. (1998). Genetic interactions between phytochrome A, and phytochrom B, and cryptochrome 1 during *Arabidopsis* development. *Plant Physiol*. **118**: 27-35.

Newton, A.C., Allnutt, T.R., Gillies, C.M., Lowe, A.J. and Ennos, R.A. (1999). Molecular phylogeography, intraspecific variation and the conservarion of tree species. *Trends in Ecol. & Evol.* **14**: 140-145.

Nimzyk, R. Schondorf, T. and Hachtel. W. (1993). In frame length mutations associated with short tandem repeats are located in unasigned open reading frames of *Oenothera* chloroplast DNA. *Curr. Genet*. **23**: 265-270.

Normand, P. and Lalonde, M. (1986). The genetics of *Frankia*: A review. *Plant & Soil*. **90**: 429-453.

Normand, P. and Bousquet, J. (1989). Phylogeny of *nif*H sequences in *Frankia* and in other nitrogen fixing microorganisms. *J. Mol. Evol.* **29**: 436-447.

Novacek, M.J. (1992). Mammalian phylogeny: Shaking the tree. *Nature* **356**:121-125.

Oberdorfer, E. (1957). Süddeutche Pflanzengesellschaften. Jena, **S** 564.

Ohl, S., Hahlbrock, K. and Schafer, E. (1989). A stable blue light-derived signal modulates ultraviolet-light-induced activation of the chalcone synthase gene in cultured parseley cells. *Planta* **177**: 1275-1282.

Ogihara, Y., Terachi, T. and Sasakuma, T. (1988). Intramolecular recombination of chloroplast genome mediated by short direct repeat sequences in wheat species. *Proc. Natl. Acad. Sci. USA* **85**: 8573-8577.

Ogihara, Y., Terachi, T. and Sasakuma, T. (1991). Molecular analysis of the hot-spot region related to length mutations in wheat chloroplast DNAs I. nucleotide divergence of genes and intergenic spacer regions located in the hot-spot region. *Genetics* **129**: 873-884.

Palmer, J.D. (1987). Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *Am. Natu*ralist **130**: S6-S29.

Palmer, J.D. (1985). Comparative organization of chloroplast genomes. *Ann. Rev. Genet.* **19**: 325-354.

Palmer, J.D. (1991). Plastid chromosomes structure and evolution. In: *Molecular biology of plastids and the photosynthetic apparatus,* (Vasil I.K, **ed.**), Academic Press. New York. pp. 5-53.

Palmer, J.D., Jansen, R.K., Michaels, H.J. Chase, M.W. and Manhart, J.W. (1988). Chloroplast DNA variation and plant phylogeny. *Anna. Missou. Bota. Gard*. **75** : 1180-1206.

Parks, B.M. and Quail, P.H. (1993). *Hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. *Plant Cell* **5**: 39-48.

Petit, R.J., Kremer, A. and Wagner, D.B. (1993). Finite island model for organelle and nuclear genes in plants. *Heredity* **71**: 630-641.

Peters, J.L., Kendrick, R.E. and Mohr, H. (1991). Phytochrome content and hypocotyl growth of long hypocotyl mutant and wild type cucumber seedlings during de-etiolation. *J. Plant. Physiol*. **137**: 291-296.

Prat, D., Leger, C. and Bojovic, S. (1992). Genetic diversity among *Alnus glutinosa* (L.) Garten. Populations. *Acta Oecol.* **13**: 469-477.

Qie,Y-L., Lee, J., Bernasconi-Ouadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolanien, V. and Chase, M.W. (1999). The earliest angiosperms : evidence from mitochondrial, plastid and nuclear genomes. *Nature* **402**: 404-407.

Qie,Y-L., Lee, J., Bernasconi-Ouadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainen, V. and Chase, M.W. (2000). Phylogeny of basal angiosperms: analyses of five genes from three genomes. *Int. J. Plant Sci.* **161**: 3-27.

Qiu, Y-L., Chase, M.W., Les, D.H. and Parks, C.R. (1993). Molecular phylogenetics of the Magnoliidae: Cladistic analyses of nucleotide sequences of the plastid gene *rbc*L. *Ann. Missouri Bot. Grad*. **80**: 587-606.

Quail, P.H. (2002a). Phytochrome photosensory signaling net works. *Nat. Rev. Mol. Cell Biol*. **14**: 180-188.

Quail, P.H. (1994a). Photosensory perseption and signal transduction in plants. *Curr. Opin. Genet. Dev*. **4**: 652-661.

Quail, P.H. (2002b). Photosensory perception and signaling in plant cells : new paradigms. *Curr. Opin. Cell Biol*. **14**: 180-188.

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y. and Wagner, D. (1995). Phytochromes: photosensory perception and signal transduction. *Science* **268**: 675-680.

Quandt, D. (1999). Molekulare Divergenzmuster neuseeländischer meteoriaceae und lembophyllaceae basierend auf dem intern transkribiertern Spacer 2 *(ITS2*) ribosomaler Kern Dann (nrDNA) und nichtcodierenden Bereichen der Chloroplasten-Dann. Unveröffentichte Diplomarbeit, Rheinische Friedrich-Wilhelms-Universität Bonn.

Rapparini, F., Rotondi, A. and Baraldi, R. (1999). Blue light regulation of the growth of *Pinus persica* plants in a long term experiment: morphological and histological observations. *Trees* **14**: 169-176.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M. and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. *Plant Physiol*. **104**: 1139-1149.

Renner, S.S. (1999). Circumscription and phylogeny of the Laurales: evidence from molecular and morphological data. *Am. J. Bot.* **86**: 1301-1315.

Reddy, V.S., Goud, K.V., Sharma, R. and Reddy, A.R. (1994). Ultraviolate-B responsive anthocyanin production in a rice cultivar is associated with a specific phase of phenylalanine ammonia lyase biosynthesis. *Plant Physiol.* **105**: 1059-1066.

Robberecht, R. and Caldwell, M.M. (1983). Protective mechanisms and acclimation to solar ultraviolet-B radiation in *Oenothera stricta*. *Plant Cell Environ*. **6**: 477-485.

Rhodes, M.J.C. and Wooltorton, L.S.C. (1978). Changes in the activity of enzymes of phenylpropanoid metabolism in tomatoes stored at low temperatures. *Phytochemistry* **16**: 655-659.

Richerd, L. (1967). L'aire de repartition de l' Aune vert (*Alnus viridis* Chaix). Facteurs climatiques et édaphiques. Grenoble, Documents pour la carte de la végétation des Alpes **VI**. 110-158.

Rice-Evans, C.A. and Miller, N.J. (1998). Structure-antioxidant activity relationships of flavonoids and isoflavonoids. In: *Flavonoids in Health and Diseases,* (Rice-Evans, CA and Packer, L, **eds**.), Marcel Dekker Inc., New York, pp. 199-220.

Rieseberg, L.H and Soltis, D.E. (1991). Phylogenetic consequences of cytoplasmatic gene flow in plants. *Evol. Tren. Plants* **5**: 65-84.

Reuber, S., Bornman, J.F. and weissenböck, G. (1996). Phenylpropanoid compounds in primary leaf tissues of rye (*Secale cereale*). Light response of their metabolism and the possible role in UV-B protection. *Physiol. Plant*. **97**: 160-168.

Rubli, D., (1974). Waldbauliche Untersuchungen in Grünerlen-beständen. Diss. Nr. 5326, ETH, Zürich. **S** 82.

Rozema, J., Staaij, J. van de, Björn, L.O. and Caldwell, M. (1997). UV-B as an environmental factor in plant life: Stress and Regulation. *Trees* **12(1)**: 22-28.

Saiki, R.K., Gelfand, K.B., Stoffel, S., Scharf, S., Higuchi, R.H., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer-directed enzymatic amplifications of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Sang, T., Crawford, D.J. and Stuessy, T.F. (1997). Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *Am. J. Bot.* **84**: 1120-1136.

Schäfer, E. and Haupt, W. (1983). Blue light effects in phytochrome mediated responses. In: *Encyclopedia of Plant Physiology N.S Vol. 16B. Photomorphogenesis,* (Shropshire, W and Mohr, H, **eds**.) pp. 723-747.

Schnitzler, J.P., Jungblut, T.P., Feicht, C., Köfferlein, M., Langebartels, C., Heller, W. and Sandermann, H.J. (1997). UV-B induction of flavonoid biosynthesis in scots pine (*Pinus sylvestris* L.) seedlings. *Trees* **11**: 162-168.

Schaal, B.A., Hayworth, D.A., Olsen, K.M., Rauscher, J.T. and Smith, W.A. (1998). Phylogeographic studies in plants: problems and prospects. *Mol. Ecol*. **7**: 465-474.

Sewell, M.M., Parks, C.R. and Chase, M.W. (1996). Intraspecific chloroplast DNA variation and biogeography of North American Liriodendron L. (Magnoliaceae). *Evolution* **50:** 1147-1154.

Shirley, B. W. (1996). Flavonoid biosynthesis: 'new' functions for an 'old' pathway. *Trends plant Sci.* **1**: 377-382.

Shirley, B.W., Kubasek, W.L., Storz, G., Bruggemann, E., Koornneef, M., Ausubel, F.M. and Goodman, H.M. (1995). Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J*. **8**: 659-671.

Sebald, O., Seybold, S., Philippi, G. and Wörz, A. (1998). Die Farn- und Blütenpflazen Baden-Württembers, Verlag Eugen Ulmer, Stuttgart, S 540.

Sherwin, H.W. and Farrant, J.M. (1998). Protection mechanisms against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*. *Plant Growth Reg*. **24**: 203-210.

Shinomura, T., Nagatani, A., Hanzawa, H., Kubota, M., Watanabe, M. and Furuya, M. (1996). Action spectra for phytochrome A- and B- specific photoinduction of seed germination in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **93**: 8129-8133.

Shimada, H. and Sugiura, M. (1991). Fine structural features of the chloroplasts genome: comparison of the sequenced chloroplast genomes. *Nuclic Acid Res.* **19**: 983-995.

Small, R. L., Ryburn, J.A., Cronn, R.C., Seelanan, T and Wendel, J.F. (1998). The tortois and the hare: choosing between noncoding plastome and nuclear ADH sequences for phylogeny reconstruction in a recently diverged plant group. *Am. J. Bot.* **85**: 1301-1315.

Smith, H. (1995). Physiological and ecological function within the phytochrome family. *Annu. Rev. Plant. Physiol. Mol. Biol*. **46**: 289-315.

Smith, H. (2000). Phytochromes and light signal perception by plants-an emerging synthesis. *Nature* **407**: 585-591.

Smith, J.F., Kress, W.J. and Zimmer, E.A. (1993). Phylogenetic analysis of the Zingiberales based on *rbc*L sequences. *Ann. Missouri. Bot. Gard*. **80**: 620-630.

Soltis, D.E. and Kuzolf, R.K. (1995). Discordance between nuclear and chloroplast phylogenies in the Heuchera Group (Saxifragaceae). *Evolution* **49**: 727-742.

Soltis, D.E., Soltis, P.S. and Milligan, B.G. (1992). Intraspecific chloroplast DNA variation: systematic and phylogenetic implications. In: *Molecular Plant Systematics.* (Soltis, DE, Soltis, PS and Doyle, JJ, **eds.**), Chapman and Hall, New York, pp. 117-150.

Soltis, D.E., Gitzendanner, M.A., Strenge, D.D. and Soltis, P.S. (1997). Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant syst. Evol.* **206**: 353-373.

Soltis, P.S., Soltis, D.E. and Chase M.W. (1999a). Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature* **402**: 402-404.

Speck, T., Rowe, N.P., Brüchert, F., Haberer, W., Gallenmüller, F., Spatz, H.C. (1996). How plants adjust the "material properties" of their stems according to differing mechanical constraints during growth: an example of smart design in nature. In: *Bioengineering, Engineering Systems Design and Analysis Conference, The american society of mechanical engineer,s* (Engin AE, **ed**.), PD-Vol 77, New York, pp. 233-241.

Stafford, H.A. (1990). Flavonoid Metabolism**,** Boca Raton, FL: CRC Press, Inc**.**

Stehlik, I., Scneller, J. and Bachmann, K. (2001a). Resistance or emigration: response of the high-alpine plant *Eritrichum nanum* (L.) Gaudin to the ice age within the central Alps. *Mol. Ecol*. **10**: 357-370.

Stehlik, I. (2002). Glacial history of the Alpine *Rumex nivalis* (Polygonaceae): A comparison of traditional phylogeograpfic methods with nested clade analysis. *Am. J. Bot.* **89(12)**: 2007-2016.

Stehlik, I. (2000). Nunataks and peripheral refugia for alpine plants during Quarternary glaciation in the midlle parts of the Alps. *Bot. Helvetica* **110**: 25-30.

Stehlik, I., Tribsch, A. and Schönswetter, P. (2001b). First joint botanical mountain phylogeography meeting. *Boauhinia* **15**: 69-90.

Stehlik, I., Schneller, J. J. and Bachmann, K. (2002). Immigration and in situ glacial survival of the low-alpine *Erinus alpinus* (Scrophulariaceae). *Biol. J. Linn. Soc*. **77**: 87-103.

Strid, A. (1993). Alteration in expression of defence genes in *Pisum sativum* after exposure to supplementary ultraviolet-B radiation. *Plant Cell Physiol*. **34**: 949-953.

Strid, A., Chow, W.S. and Anderson, J.M. (1994). UV-B damage and protection at the molecular level in plants. *Photosyn. Res*. **39**: 475-489.

Sugiura, M. (1989). The chloroplast chromosomes in land plants. *Annu. Rev. Cell Biol*. **5**: 51-70.

Sun, X-J., Zhang, D.H. and Hou, J.-S. (1979). Maastrichtian pollen and spore flora of northern Inner Mongolia. *Acta Botanica* **21**: 285-393.

Sun, X-J., Kong, Z.C., Li, P. and Li, M-X. (1981). Early Tertiary pollen and spore flora of northern south Sea in China. *Acta Phyt. Sinica.* **19**: 186-193.

Sytsma, K.J., Morawetz, J., Pires, J.C., Nepokroeff, M., Conti, E., Zjhra, M., Hall, J.C. and Chase, M.W. (2002). Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on *rbc*L, *trn*L-F, and *ndh*F sequences. *Am. J. Bot*. **89**: 1531-1546.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G and Cousson, J.F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol*. **7**: 453-464.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. (1991). Universal primers for the amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol*. **17**: 1105-1109.

Takhtajan, A. (1980). Outline of the classification of flowering plants (Magnoliophyta). *Bot. Review* **46**: 225-359.

Takaiwa, F. and Sugiura, M. (1982). Nucleotide sequence of the 16s-23s spacer region in a rRNA gene cluster from tobacco chloroplast DNA. *Nuclic Acid. Res*. **10**: 2665-2676.

Tallantier, P.A. (1974). The paleohistrory of the grey alder (*Alnus incana*) L. (Moench) and black alder (*A. glutinosa*) L. (Geartn) in Fennoscandia. *New Phytolosist* **73**: 529-546.

Taylor, L. P. and Briggs, W.R. (1990). Genetic regulation and photocontrol of anthocyanin accumulation in maize seedlings. *Plant Cell*, **2**: 115-127.

Terry, R.G., Nowak, R.S. and Tausch, R.J. (2000). Genetic variation in chloroplast and nuclear ribosomal DNA in Utah Juniper (*Juniperus osteosperma*, Cupressaceae): Evidence for interspecific gene flow. *Am. J. bot*. **87**: 250-258.

Tiffney, B.H. (1985). The Eocene North Atlantic land bridge: its importance in Tertiary and mordern phytogeography of the Northern Hemisphere. *J. Arn. Arb*. **66**: 243-273.

Tollefsrud, M.M., Bachmann, K., Jakobsen, K.S. and Brochmann, C. (1998). Glacial survival does not matter-II: RAPD phylogeogryphy of Nordic *Saxifragia cespitosa*. *Mol. Ecology* **7**: 1217-1232.

Travis, S.E.,Maschinski, J. and Keim, P. (1996). An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant using AFLP markers. *Mol. Ecol*. **5**: 735-745.

Van Ham, R.C.H.G, Thart, H., Mes, T .H.M. and Sandbrink, J.M. (1994). Molecular evolution of non-coding regions of the chloroplast genome in the Crasulaceae and related species. *Curr. Genet*. **25**: 558-566.

Van der Hammen, J. (1989). History of the Montane forests of the northern Andes. *Plant Syst. Evol*. **162**: 109-140.

van Tunen, A. J. and Mol, J.N.M. (1991). Control of flavonoid synthesis and manipulation of flower colour. In: *Plant Biotechnology,* Vol. 2, (Grierson, D, **ed**.), Blackie, Glasgow, pp. 94-125.

Vierheilig, H., Bago, B., Albrecht C., Poulin, M.J. and Piche´, Y. (1998). Flavonoids and arbuscular-mycorjyzal fungi. In: *Flavonoids in the living system,* (Manthey, JA and Buslig, BS, **eds.**), Plenum Press, New York, pp. 9-33.

Vom Stein, J. and Hachtel, W. (1988). Deletions/insertions: Short inverted repeats sequences resembling att-lambda and frame-shift-mutated open reading frames are involved in chloroplast DNA differences in the genus *Oenothera* subsection *Munzia*. *Mol. Gen. Genet*. **213**: 513-518.

Wakasugi, T., Sugita, M., Tsudzuki, T. and Sugiura, M. (1998). Updated gene map of tobacco chloroplast DNA. *Plant Mol. Biol. Rep*. **16**: 231-241.

Wilmanns, O. (1977). Verbreitung, Soziologie und Geschichte der Grün-Erle (*Alnus viridis*. Chaix) DC. im Schwarzwald. *Mitt. Florist.-soziol. Arb.Gem*. **19/20**: 323-341.

Wellmann, E. (1971). Phytochrome mediated flavon glycoside synthesis in cell suspension cultures of *Petroselinum hortense* after preirradiation with ultravoilet light. *Planta* **101**: 283-286.

Wellmann, E., Hrazdina, G., and Grisebach, H. (1976). Induction of anthocyanin formation and of enzymes related to its biosynthesis by UV light in cell cultures of *Haplopappus gracilis*. *Phytochemistry* **15**: 913-915.

West, R. (1978). Quarternary Geology and Biology. Cambridge University Press, Cambridge, UK.

Wetzel, A., M. Parniske and Werner, D. (1995). Pleiotropic effect of fluoranthene on anthocyanin synthesis and nodulation of *Medicago sativa* is reversed by the plant flavone luteolin. *Bull. Environ. Contam. Toxicol*. **54**: 633-639.

Whitelam, G.C. and Devlin, P.F. (1997). Roles of different phytochromes in *Arabidopsis* photomorphogenesis. *Plant Cell Environ*. **20**: 752-758.

Wolfe, K.H., Li, W.H. and Sharp, P.M. (1987). Rates of nucleotide substitution vary greatly amoung plant mitochondrial, chloroplast, and nuclear DNAs. *Proc. Natl. Acad. Sci. USA* **84**: 9054-9058.

Wolfson, R., Higgins, K.G. and Sears, B.B (1991). Evidence for replication slippage in the evolution of *Oenothera* chloroplast DNA. *Mol. Biol. Evol*. **8**: 709-720.

Woodland, D.W. (1991). Contemporary plant Systematics. Chapman and Hall, Inc., Englewood Cliffs, N.J.

Yamaguchi, F., Nozue, M., Yasuda, H. and Kubo, H. (2000). Effects of temperature on the pattern of anthocyanin accumulation in seedlings of *Polygonum cuspidatum*. *J. Plant Res.* **113**: 71-77.

Ylstra, B., Touraev, A., Benito Moreno, R.M., Stöger, E., van Tunen, A.J., Vicente, O., Mol, J.N.M. and Heberle-Bors, E. (1992). Flavonols stimulate development, germination and tube growth of tobacco pollen. *Plant Physiol*. **100**: 902-907.

Yoshitama, K. (2000). Recent advances in secondary metabolism research: Regulation of biosynthesis and physiological functions of flavonoids and some phenolics. *J. Plant Res. Sym*. 123.

Zanis, M., Soltis, D.E., Soltis, P.S., Mathews, S. and Donoghue, M.J. (2002). The root of angiosperms revisited. *Proc. Natl. Acad. Sci. USA* **99**: 6848-6853.

Zurawski, G. and Clegg, M.T. (1987). Evolution of higher-plant chroloplast DNA encoded genes: implications for structure function and phylogenetic studies. *Ann. Rev. Plant Physiol*. **38**: 391-418.

Zurawski, G., Clegg, M.T. and Brown, A.H.D (1984). The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. *Genetics* **106**: 735-749.