Biology and evolution of the European mistletoe (*Viscum album*)

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Biology and evolution of the European mistletoe (*Viscum album*)

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Summary

Parasitic interactions among organisms are highly diverse and play important roles in all ecosystems. These interactions span all group of organisms, from phages and viruses to bacteria, animals (mainly insects) and fungi to flowering plants. A well-known group of flowering plant parasites are mistletoes.

The European mistletoe *Viscum album* (Viscaceae) is a known pathogen, a pharmaceutical plant, and a symbol in mythology. The aim of this thesis was to provide new insights into the biology and evolution of this mistletoe with the help of molecular markers.

Viscum album is an evergreen hemiparasitic shrub, growing on different woody hosts. *Viscum* is dioecious, insect pollinated and has fleshy fruits that are bird dispersed. Based on host specificity, three host races are distinguished in Europe: *V. album album* grows on a wide variety of deciduous trees, *V. a. abietis* is restricted to fir (*Abies* spp.), and *V. a. austriacum* occurs mainly on pine (*Pinus* spp.). A fourth host race, *V. a. creticum*, is associated with a sole pine host, *Pinus halepensis* ssp. *brutia,* and occurs exclusively on the island of Crete (CHAPTER I & APPENDIX).

Nuclear ribosomal DNA (nrDNA) ITS sequences and partial sequences of three non-coding chloroplast DNA (cpDNA) introns and spacers were used to assess genetic variation within and among these presumed host races. The molecular analysis of DNA variation supported the distinction of these four taxa, although sequence variation was low. We interpret the small genetic distances among host races as evidence for their recent formation. The results further suggest that *V. a. album* and *V. a. abietis* are more closely related to each other than to *V. a. austriacum* and that hybridization is more likely between *V. a. album* and *V. a. abietis.* Indeed, putative hybrids found so far are the result of crosses between *V. a. album* and *V. a. abietis* (CHAPTER II).

Chloroplast DNA fragment length polymorphisms were used to study genetic differentiation among the host races, population genetic structure, and to elucidate the postglacial migration history of *Viscum album* across the entire natural distribution range in Europe. The populations sampled belonged to the four closely related European *Viscum* races. The molecular analysis of cpDNA variation further supported the distinction of four taxa on a much larger geographic and sampling scale. We further found evidence for phylogeographic structure in each of the three widely distributed host races. The parasitic life form and the scattered distribution of hosts may have favoured the high degree of population differentiation found in *Viscum*. Possible glacial refugia and postglacial immigration routes of mistletoe host

races were found to be only roughly comparable with those of their host trees, because each mistletoe host race is able to parasitize several tree species and may have switched among closely related hosts during glacial survival and subsequent recolonization.

Independent of host race, mistletoe haplotypes from Turkey were distinct and distant from those found elsewhere in Europe, suggesting that highly differentiated populations, and possibly new taxa, exist at the range limit of the species (CHAPTER III).

Zusammenfassung

Parasitische Interaktionen zwischen Organismen sind vielfältig und spielen eine wichtige Rolle in allen Ökosystemen. Solche Interaktionen sind in allen Organismusgruppen vertreten von Phagen und Viren über Bakterien, Tiere (v.a. Insekten) und Pilzen bis hin zu Blütenpflanzen. Die Gruppe von Misteln ist ein bekanntes Beispiel für parasitische Blütenpflanzen.

Die Europäische Mistel *Viscum album* (Viscaceae) ist als Schädling, als Arzneipflanze und als ein Symbol in der Mythologie bekannt. Das Ziel der vorliegenden Arbeit ist es, mit Hilfe von molekularen Markern einen neuen Einblick in die Biologie und Evolution dieser speziellen Mistel zu geben.

Viscum album wächst als immergrüner halb-parasitischer Strauch auf zahlreichen verschiedenen Wirts-Holzpflanzen. *Viscum* ist zweihäusig, wird von Insekten bestäubt und bildet fleischige Beeren, die von Vögeln verbreitet werden. Aufgrund der Wirtsspezifität werden innerhalb Europas drei Wirtsrassen unterschieden: *V. album album* kommt auf verschiedenen Laubbäumen vor, *V. a. abietis* wächst auf Tannen (*Abies* spp.), und *V. a. austriacum* ist vor allem von Kiefern (*Pinus* spp.) bekannt. Eine vierte Wirtsrasse, *V. a. creticum*, kommt nur auf einer bestimmten Kiefernart (*Pinus halepensis* ssp. *brutia*) auf Kreta vor (KAPITEL I & APPENDIX).

Um die genetische Variation sowohl innerhalb als auch zwischen den möglichen Wirtsrassen zu untersuchen, wurden ITS Sequenzen der ribosomale Kern DNA (nrDNA) und Teilsequenzen von drei nicht kodierenden Chloroplast DNA (cpDNA) Abschnitten benutzt. Die molekuare Analyse der Sequenzunterschiede unterstützt die Unterscheidung der vier Taxa, obwohl die Unterschiede gering waren. Wir nehmen an, daß die geringen genetischen Unterschiede ein Zeichen dafür sind, daß die Wirtsrassen erst kürzlich entstanden sind. Die Ergebnisse lassen weiter den Schluß zu, daß *V. a. album* und *V. a. abietis* näher miteinander verwandt sind als mit *V. a. austriacum* und daher eine Hybridisierung zwischen *V. a. album* und *V. a. abietis* am wahrscheinlichsten ist. Tatsächlich sind mögliche Hybride, die bisher gefunden wurden, ein Ergebnis einer Kreuzung zwischen *V. a. album* und *V. a. abietis* (KAPITEL II).

Chloroplast-DNA-Abschnitt-Längen-Polymorphismen wurden benutzt, um die genetische Differenzierung zwischen den Wirtsrassen, die genetische Populationsstruktur und die Migrationsgeschichte von *V. album* in ihrem natürlichen Verbreitungsgebiet in Europa nach

den Eiszeiten zu untersuchen. Die gesammelten Populationen gehören zu den vier nah verwandten europäischen *Viscum* Rassen. Die molekulare Analyse der Chloroplasten DNA Variation unterstützt wiederum die Trennung der vier Taxa in einem größeren Rahmen, da ein größeres Verbreitungsgebiet und eine größere Anzahl Proben untersucht wurden.

Außerdem wurden Anzeichen für eine phylogeographische Struktur in jeder der drei weit verbreiteten Wirtsrassen gefunden. Der hohe Grad der Populationsdifferenzierung der Mistel wird vermutlich durch die parasitische Lebensweise und die zerstückelte Verbreitung der Wirte hervorgerufen. Mögliche Eiszeitrefugien und die Migrationsrouten der verschiedenen Mistel Wirtsrassen nach der Eiszeit können nur bedingt mit denen der Wirtsbäume verglichen werden, da jede Wirtsrasse mehrere Baumarten befallen kann und so in den Refugien und während der Rekolonisation zwischen nah verwandten Wirten gewechselt haben kann.

Die Mistel Haplotypen aus der Türkei passen nicht in die bisherigen Wirtsrassen und sind auch anders als alle in Europa gefundenen Haplotypen. Es kommen also stark differenzierte Populationen und eventuell neue Taxa am Rand des Verbreitungsgebietes vor (KAPITEL III).

General introduction

Mistletoes

Parasitic interactions between organisms play a fundamental role in ecosystems. Parasites are organisms that obtain sustenance from another organism and complete at least part of their life cycle on their host (Hawksworth & Wiens 1996). The interacting partners often belong to different families or even kingdoms. Most scientific literature on parasites is dominated by examples of animal, fungal or bacterial parasites. But there are numerous examples of parasitism in higher plants (Weber 1993). Based on the degree of chlorophyll content and nutrient uptake, two major types of plant parasites can be distinguished. Hemiparasites with green leaves contain chlorophyll and perform photosynthesis, but are dependent upon host plants for water and nutrient uptake. Holoparasites have low chlorophyll content or lack chlorophyll altogether and are therefore often conspicuously pale green to brown. These plants take all nutrients from their host plants.

One well-known group of parasitic angiosperms are mistletoes. The majority of mistletoes belong to families Loranthaceae and Viscaceae within the order Santalales (Kuijt 1969; Barlow 1987). In the past, Viscaceae have often been placed inside Loranthaceae, but because of differences in floral structure, embryology and chromosomal traits, the distinction of the two families is mostly accepted (Calder 1983). Molecular phylogenetic studies further support this separation by resolving both families as independent monophyletic groups (Nickrent & Duff 1996; Nickrent 2002). According to Barlow (1983), the morphological similarities between Viscaceae and Loranthaceae are a good example for convergent evolution.

The term mistletoe was first applied to the European mistletoe *Viscum album*. In the seventeenth and eighteenth centuries when new species were discovered all over the world, all plants that showed similarities in morphology and life-history to *Viscum album* where classified as mistletoes (Calder 1983; Kuijt 1969; Barlow 1987). Nowadays, mistletoes are flowering plants that show some degree of parasitism. Mistletoe types include root-parasitic, terrestrial shrubs (e.g. *Nuytsia floribunda*), common epiphytic stem parasites (e.g. *Viscum album*) and even endophytic species that produce only flowers and fruits on the surface of the host (e.g. *Viscum minimum*). All mistletoes are shrubs and develop a haustorium to contact the host xylem for water and nutrient uptake. They are classified as hemiparasites but are also

called water parasites, partial parasites, aerial parasites or epiparasites. Here the functional term hemiparasite is used.

The pattern of host specificity in mistletoes covers a wide spectrum from highly host-specific species to host generalists (Barlow & Wiens 1977). Mistletoes have developed specialized seed dispersal and pollination mechanisms generally in association with animals. Most mistletoes are dispersed by birds and often have a close association with particular bird groups (Reid *et al.* 1995). An exception are dwarf mistletoes (*Arceuthobium* sp.), that have an explosive mechanism of seed dispersal.

Pollination of mistletoes is dependent either on insects or birds. Mistletoes of family Loranthaceae are generally pollinated by birds (Calder 1983). Members of the family Viscaceae are mainly pollinated by insects. Some species (*Viscum album, Arceuthobium* sp.) show floral characteristics of both insect-pollinated and wind-pollinated flowers. It has been found that wind pollination is possible, but entomophily is more common (Hawksworth & Wiens 1996).

Mistletoes can affect their host trees in many ways. Known effects include that mistletoe infections lower the vigor of the host, induce premature mortality, reduce the quality and quantity of wood produced, reduce fruiting of infected trees and predispose trees to be attacked by other agents, such as insects or decay fungi (Hawksworth 1983). In particular dwarf mistletoes (*Arceuthobium* sp.) are of substantial economic importance because they are destructive pathogens of commercially valuable coniferous timber trees (Hawksworth & Wiens 1996). The European mistletoe (*Viscum album*) is also known as forest pathogen because it can induce water stress in the host, especially to the host branches above the infection site (Fisher 1983). *Viscum album* is of further economic interest because it contains a diversity of pharmacological substances.

Host race evolution and speciation

Speciation via host race formation is an important evolutionary process in parasites (Dres & Mallet 2002; Norton & Carpenter 1998). Host race evolution has been studied in numerous parasites, e.g. phytophagous insects (Dres & Mallet 2002) and fungi (Brem & Leuchtmann 2003; Shykoff *et al.* 1999) but also in mistletoes (Glazner *et al.* 1988; Jerome & Ford 2002).

Genetically-based adaptation to hosts is important for the evolution of parasites, as parasite populations may eventually become differentiated into races and subsequently speciate as they adapt to the divergent selection pressures exerted by different host genotypes or taxa

(Thompson 1994; Glazner *et al.* 1986). Once host races are established, the development of isolation mechanisms (prezygotic or postzygotic isolation barriers; reviewed in Coyne & Orr (1998)) eventually leads to the formation of new species.

Gene flow is known to be as important force influencing genetic variation and affecting evolution (Slatkin 1987). Limited gene flow can lead to increasing local adaptation and further to formation of host races (Price 1980; Norton & Carpenter 1998; Coyne & Orr 1998). A model developed by Gandon *et al.* (1996) on local adaptation predicted that no local adaptation evolves in parasites when hosts and parasites have similar migration rates. Parasites, however, become locally adapted when parasite migration rate is higher than the host migration rate, provided that host migration rate is not very high. Hosts become locally adapted when conditions are reversed. Additionally, independent of its hosts, parasite populations may differentiate into races when gene flow between parasite populations is diminished by factors such as distance, limited dispersal, and patchy host populations (Norton & Carpenter 1998). On the other hand, high gene flow may act against race formation by inhibiting genetic adaptation. But high gene flow may also promote evolutionary processes by introducing novel and potentially advantageous alleles into the population (Slatkin 1987).

In general, host races are taxa that associate with different principal hosts, showing no consistent morphological or physiological differences, but genetic differentiation. (Hawksworth & Wiens 1996; Dres & Mallet 2002).

Study system

The European mistletoe, *Viscum album* L. (Viscaceae), is widely distributed across Europe. It is a perennial evergreen shrub growing as hemiparasite on woody plant species. *Viscum album* is dioecious and insect pollinated. Female and male flowers are yellowish-green and inconspicuous. The white berries are dispersed by a variety of birds, the most important being the mistle thrush (*Turdus viscivorus*), fieldfare (*Turdus pilaris*), waxwing (*Bombycilla garrula*) and blackcap (*Sylvia atricapilla*). The birds mainly feed on the berries, but intestinal passage is not necessary for germination. A peculiarity of the mistletoe berry is the mucilaginous substance viscin, which is able to stick strongly onto tree bark. Unusual for a European plant, the berries start ripening from November to December and are dispersed between February and May, mainly when the migratory birds fly northwards (Fig. 1).
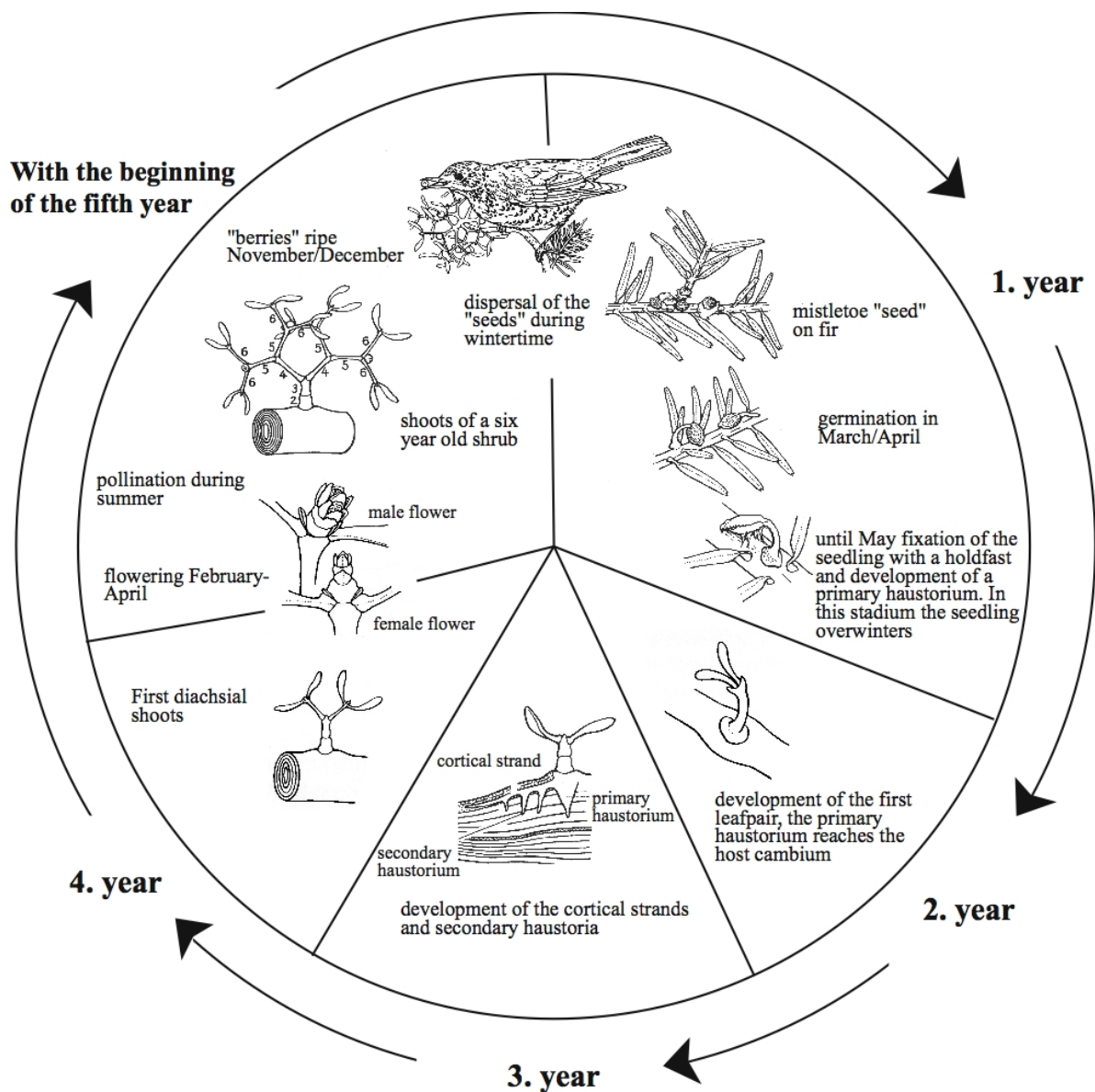
Figure 1 Life cycle of *Viscum album* on fir (Nierhaus-Wunderwald & Lawrenz 1997)

V. album is able to infect a large number of host plants. A compilation of the host range by Barney (1998) has shown that 384 taxa of shrubs and trees may become infected under natural conditions. Three widely distributed subspecies of *V. album* that differ in host specificity have been recognised in Europe (Stopp 1961, Ball 1993,):

V. album subsp. *album* L. on dicotyledonous trees,

V. album subsp. *abietis* (WIESB.) ABROMEIT on *Abies* spp.

V. album subsp. *austriacum* (WIESB.) VOLLMANN on *Pinus* spp and rarely *Larix* sp. and *Picea* sp.

The subspecies of *V. album* are variously treated as forms, varieties, subspecies or species in floras and no general agreement concerning the taxonomic status of the taxa has emerged to date (Ball 1993), therefore here the term host race is used. Evidence supporting the distinction of three host races of *V. album* comes from biochemical investigations. Schaller *et al.* (1998) have shown that host races differ in viscotoxin composition. Morphological differentiation of the three host races can only be done through characteristics of the ripe berry (Grazi & Urech 1981). However, female plants without berries and male plants cannot be distinguished morphologically. Therefore, identification of the three host races relies on the identification of their hosts.

A second *Viscum* species in Europe is *Viscum cruciatum*. It grows on deciduous trees in southern Spain, Israel and Morocco. *V. cruciatum* differs from *V. a. album* by red berries and developing all leaf pairs decussate. Further east than 70° E occurs a taxon on deciduous trees which is either treated as a *V. album* ssp. *coloratum* KOM. or as distinct species, *V. coloratum* (KOM.) NAKAI and differs from *V. a. album* by the more eastern distribution and yellow or orange berries.

Main methods

Identification of host races

The large host range and the ability to readily infect a variety of potential new hosts may suggest that *V. album* is not host-specific. On the other hand, the three recognised host races presumably differ in their host specificities. Cross-infection experiments are useful to analyse host-specificity (Clay *et al.* 1985), but because of the hemiparasitic life form and the long life cycle of *V. album,* such experiments are time consuming (Fig.1). It may take up to three years before successful seedling establishment can be observed, and onset of flowering as a reliable indicator of successful infection may take four more years (Langbehn & Weber 1995). As an alternative to cross-infection experiments, molecular markers may allow to assess host specificity of presumed *V. album* host races. For phylogenetic analyses among mistletoe species, sequence information from the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA and non-coding sequences of the chloroplast genome have been used successfully (Nickrent *et al.* 1994; Molvray *et al.* 1999). ITS and chloroplast DNA sequences, however, may not only be useful for phylogenetic studies among taxa, but may also uncover substantial intraspecific variation, such as e.g. in black alder *Alnus glutinosa* (King & Ferris 1998), *Coreopsis grandiflora* (Mason-Gamer *et al.* 1995) and *Draba aizoides* (Widmer &

Baltisberger 1999) and may thus be ideal markers to unravel the presence of host races in *V. album*.

Phylogeography

The contemporary distribution of many plant species in Europe is influenced by the climate changes between glacial and interglacial periods (Taberlet *et al.* 1998; Hewitt 1999). During maximal glaciation, distribution areas of most species were reduced and fragmented. The plants were restricted to ice-free refugia, from which they frequently expanded during interglacial periods. Major European glacial refugia have been postulated for the peninsulas of Iberia, Italy, and Greece. Additional refugia may have been in Turkey and in the east near the Caspian Sea (Hewitt 1999). The postglacial history of numerous European tree and shrub species has been investigated in recent years including many host trees of *Viscum album*. To examine postglacial colonization routes, genetic markers have been used together with fossil pollen data (Vendramin *et al.* 2000; Petit *et al.* 2002; Demesure *et al.* 1996) mainly in wind-pollinated trees or shrubs (Demesure *et al.* 1996). Because *Viscum* is insect pollinated and a sparse pollen producer, its pollen record is of little value for reconstructing the phylogeographic history of the species. Furthermore, it is impossible to distinguish the pollen of the different *V. album* races. As a consequence, the study of the postglacial history of *Viscum* has to rely on genetic markers. The marker of choice for most phylogeographic studies in angiosperms is chloroplast DNA because it is non recombining and is in the majority of species maternally inherited. The smaller effective population size, compared to nuclear markers, reduces the level of genetic diversity in the chloroplast genome and increases genetic differentiation among populations (Petit *et al.* 2005). This effect is even more pronounced in dioecious species such as *V. album*, because the chloroplast genome has a four times smaller effective population size compared to nuclear genes. An efficient method to assess intraspecific variation in a large number of individuals is the analysis of cpDNA fragment length polymorphisms (Horning & Cronn 2006). This method is thus ideally suited for phylogeographic analyses of plants.

Thesis outline

The main objective of this thesis was to study host race differentiation in the European mistletoe with molecular methods. While *Viscum album* has been thoroughly investigated with respect to its physiology, morphology and pharmacology, little is known about its

population structure, phylogeographic history and evolution. Molecular data therefore have the potential to add a new perspective to research on *V. album*.

The main research questions were the following:

i) *What is known about V. album biology?*
To gain an overview and to compile the available knowledge about *V. album* biology, a thorough literature review has been performed and the results are presented in a review paper (CHAPTER I).

ii) *How genetically diverged are Viscum album populations that grow on different host species?*
To address this question, nuclear DNA and chloroplast DNA sequences were used. *Viscum album* samples were classified on the basis of their host-trees. We tested if patterns of genetic differentiation support the recognition of host races and if so, whether these host races correspond to subspecies recognized in the literature (CHAPTER II). In addition, the phylogenetic relationship among host races was investigated.

iii) *Is there evidence for host race differentiation and phylogeographic structure in Viscum album*?
Based on the results presented in CHAPTER II, the test whether host races of *V. album* correspond to differentiated gene pools is here examined in a much larger scale. Mistletoe populations from across the entire distribution area were sampled to compare population genetic structure between mistletoe host races in relation to host diversity and distribution. Furthermore we asked whether the population genetic structure in the mistletoe contains a phylogeographic signal that allows to identify possible glacial refugia and postglacial recolonization routes (CHAPTER III).

As part of a collaborative project I further investigated the following question:
What is the taxonomic status of Viscum album from Crete
Based on host tree identification, *V. album* individuals from pine in Crete should belong to host race *V. album austriacum*. But the Cretan mistletoes show morphological features of *Viscum* from fir. Genetic analyses helped to answer whether mistletoes from Crete belong to one of the known three host races or not (APPENDIX).

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Biological flora of Central Europe: *Viscum album* L.

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Summary

Viscum album is an evergreen, perennial, epiphytic, hemiparasitic shrub that lives on a wide range of woody plant species. It is native to Europe and is a known pathogen, a pharmaceutical plant, and a symbol in mythology. This article reviews the taxonomy, morphology, ecology, population biology, genetics, chemistry and physiology of this taxon.

Keywords: Subspecies, Central Europe, hemiparasite, host-race, dioecious, mistletoe

1. Taxonomy and morphology

1.1. Name of species

Viscum album L. - mistletoe - Mistel

Three widely distributed subspecies (or races, see 3.12) that differ in host specificity and a fourth subspecies only known from Crete have been recognised in Europe (STOPP 1961; BALL 1993; BÖHLING et al. 2002):

V. album subsp. *album* L. on dicotyledonous trees

> (synonyms: *V. album* L. var. *platyspermum* KELLER
>
>   *V. album* L. var. *mali* TUBEUF),

V. album subsp. *abietis* (WIESB.) ABROMEIT on *Abies* spp.

> (synonyms: *V. laxum* var. *abietis* (WIESB.) HAYEK
>
>   *V. austriacum* WIESB. var. *abietis* WIESB.
>
>   *V. abietis* (WIESB.) FRITSCH),

V. album subsp. *austriacum* (WIESB.) VOLLMANN on *Pinus* spp. and rarely *Larix* sp. and *Picea* sp. (synonyms: *V. austriacum* WIESB.

>   *V. laxum* BOISS. & REUT.
>
>   *V. laxum* BOISS. & REUT. var. *pini* (WIESB.) HAYEK
>
>   *V. album* L. var. *laxum* (B. & R.) FIEK)

V. album subsp. *creticum* N. BÖHLING, GREUTER, RAUS, B. SNOGERUP, SNOGERUP & ZUBER on *Pinus halepensis* subsp. *brutia* exclusive from Crete.

Viscum includes approximately 100 species most of them in Africa and Madagascar and a smaller number in southern Asia. Only a few species are known from Europe, temperate Asia, Malesia and eastern Australia. The genus *Viscum* is nowadays generally assigned to family Viscaceae (BARLOW 1983; BARLOW & MARTIN 1984; NICKRENT & FRANCHINA 1990; NICKRENT 2002). In the past it has often been placed in family Loranthaceae. Viscaceae (as well as Loranthaceae) belong to Santalales. Within the Santalales Viscaceae is more closely allied to Santalaceae, and Loranthaceae to the other families of the order. Viscaceae is even sometimes included in Santalaceae (BREMER et. al. 1998).

1.2. Morphology

Viscum album is a mostly globose perennial evergreen shrub with persistent haustoria in the host. Globe diameter may reach up to 150 cm (WANGERIN 1937) with diachsial branching pattern first forming a fan and with increasing growth forming a globe. Foliage leaves are opposite, rarely 3 (- 4 - 5) whorled, sessile, obovate-oblong, obtuse, leathery and (yellowish-) green (BALL 1993). In general leaf length ranges between (1.3-) 2 - 8 (-10.7) cm, with a minimum width of 0.3 cm and a maximum of 4.3 cm (TUBEUF 1923, BÖHLING et al. 2002). Foliage leaf internodes are 1 - 9 cm long. The length of leaves and internodes increases during the first five years after germination and decreases slowly thereafter (see 3.1. & Fig. 6)

(MONTFORT & MÜLLER 1951; LANGBEHN & WEBER 1995). Shape and size of leaves may vary considerably, not only within an individual, but between different individuals of the same host tree or of different host trees (WANGERIN 1937).

The inflorescence is a dichasium subtended by a pair of usually fused bracts (bracteal cup) (POLHILL & WIENS 1998). The flowers are inconspicuous, sessile, small and yellowish-green. *Viscum album* is dioecious. Staminate and pistillate flowers are usually 3 (-5) in triads with one terminal and two lateral flowers. Flowers of both sexes produce nectar, with females producing more nectar than males (WALLDÉN 1961; LUTHER & BECKER 1986). Male flowers are larger than female flowers; males have four tepals arranged in two whorles, transverse section of the two outer tepals is trapeziform, of the two internal cuneate (STEINDL 1935). On the inside of the tepals 6 - 20 (-50) adnate pollen chambers are situated (WANGERIN 1937). The pollen chambers are globose voids with a diameter of 0.3 - 0.5 mm (STEINDL 1935). The anthers dehisce through numerous pores. Female flowers have four tepals, too; the epigynous ovary is formed by two carpels, the style is short or absent, the stigma is rotund and emerges directly from the summit of the ovary. An ovule is absent. There is neither a funicle, nor a nucellus, nor an integument. The development of the embryo sac is equivalent to the Bisporic-Allium type (RUTISHAUSER 1969). The mature female gametophyte is straight and vertically placed (KUIJT 1969; BHANDARI & VOHRA 1983). Although there are normally four tepals, aberrations may occur in flowers of both sexes.

In adult plants, one diachsial shoot with one short and one long internode and one pair of scale leaves and one of foliage leaves per leaf axil is formed each year (TROLL 1937). Leaf age ranges between 17 months and 3 years (WANGERIN 1937; LUTHER & BECKER 1986). Leaves have three to five clearly visible, nearly parallel veins and a diffuse reticulate venation. *Viscum album* has equifacial leaves. In contrast to leaves from other flowering plants, epidermal cells contain chlorophyll. The cuticula is about 9 μm in cross-section (LUTHER & BECKER 1986). Stomata are distributed on both sides of the leaves; their density may vary. Baillon et al. (1988) found about 5,500 stomata per cm^2 on each side of the leaf, and TUBEUF (1923) reported about 100 stomata per mm^2 (= $10,000 cm^{-2}$) in a one-year-old leaf and about 60 per mm^2 (= $6,000 cm^{-2}$) in a two-year-old leaf. The viscous fruit is a berry, often called a pseudoberry, because it consists of carpel and stemborne tissue. Three such berries are typically formed in a crotch on a shoot apex from the previous year and sometimes in anterior branching points (WANGERIN 1937). Berries are 6 - 10 mm in diameter, globose or pyriform. The epicarp is white or occasionally yellow with a ring of 4 short dark lines representing the tepalar scars and a central point caused by the stigma (WANGERIN 1937;

BALL 1993). The thick mesocarp consists of viscin, a mucilaginous substance. There are two viscid layers: an outer one apparently digestible, cellulosic slimy layer, and an inner, indigestible pectin layer. The very thin endocarp adheres to the "seed" (KUIJT 1969; SALLÉ 1983). In the strict sense, the "seed" is an embryo, enclosed in the endocarp (GRAZI & URECH 1981). For more details about embryology see KUIJT (1969) and BHANDARI & VOHRA (1983).

There are two parts of the endophytic system. Firstly the haustoria or sinkers which grow radically and reach the host cambium to absorb water and mineral salts. Haustoria do not penetrate the host xylem; they are embedded in host xylem tissue. Secondly, the cortical strands run through parenchymatous or phloem tissue and as a result cause lateral spread (TROLL 1941; SALLÉ 1983) (Fig. 1). Cortical strands are chlorophyllous and on average have a length of 4 - 6 cm (THODAY 1951; WANGERIN 1937). Cortical strands have phloem and xylem, but no connection between host and parasite phloem parenchyma is known (LUTHER & BECKER 1986). Secondary haustoria or sinkers, originated from cortical strands grow like the primary haustoria to the host cambium. As a consequence, additional shoots (adventitious-shoots) may be formed (Fig. 1).
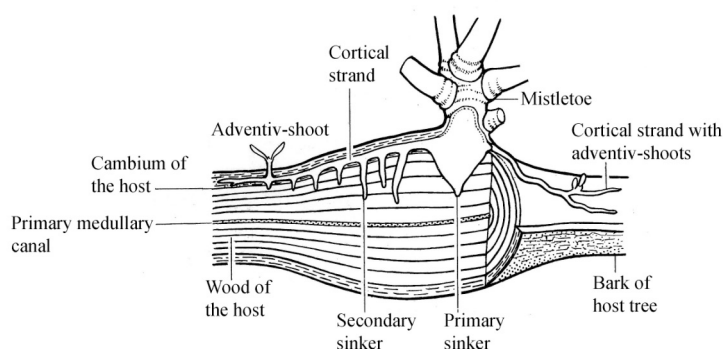

Fig. 1. Perennial mistletoe on a host shoot. Left part shows longitudinal section, right part topview with partly removed bark (NIERHAUS-WUNDERWALD 1997).

Morphological differentiation of the three main subspecies of *V. album* can only be done through characteristics of the ripe berry. For distinction between mistletoe plants from hardwood and from conifers, the formation of long mucilaginous threads between the inner and the outer layer is used. These threads are representative for subsp. *album*, whereas no such threads occur in the berries of subsp. *austriacum* and *abietis*. To distinguish between the two subspecies growing on conifers, the shape of the free tip of the hypocotyl is used. In subsp. *abietis* the hypocotyl is cylindrical with a swollen meristematic tip caused by a constriction below. The hypocotyl of subsp. *austriacum* is thin without any constriction

(GRAZI & URECH 1981). The hypocotyl of subsp. *creticum* does not match to this shape criterion, but it is nearest to subsp. *austriacum* (BÖHLING et al. 2002). Male plants can not be distinguished morphologically.

2. Distribution and habitat requirements

2.1. Geographical distribution

Mistletoes have been known for a very long time. The occurrence of *V. album* in southern Europe was first reported by Theophrast (371-287 BC) and was later mentioned by others such as Aristoteles and Plinius (23-79 AD) (TUBEUF 1923; JANSSEN 2001). *V. album* is native to most parts of Europe. Southern and Western borders are the Mediterranean Sea and the Atlantic Ocean, respectively. It also occurs in the south of Great Britain, but it is not native in Ireland. In the North, *V. album* reaches southern Sweden, and in the East it occurs around the Black Sea and in the mountains south of the Caspian Sea. The entire distribution area extends from 10° W to 80° E and from about 60° N (max. 59°38' N) to 35° S. (Fig. 2, 3). *Viscum album* has been reported on the following islands: Great Britain (to max. 55° N), Corse, Sicily, and Crete, as well as small islands in the Swedish Mälaren Sea (WALLDÉN 1961; HEGI 1981; PIGNATTI 1982; CATALÁN & APARICIO 1997). An old record from Mallorca has not been confirmed (BOLÒS & VIGO 1990). Further east than 80° E, occurs a taxon which is either treated as subspecies, *V. album* subsp. *coloratum* KOM. (synonym *V. album* var. *coloratum* (KOM.) OHWI), or as distinct species, *V. coloratum* (KOM.) NAKAI. Further members of aggregate *V. album* s. l. see 3.12. and comments to Fig. 2. *V. album* subsp. *abietis* (Fig. 2b) and *V. album* subsp. *austriacum* (Fig. 2c) go beyond the distribution range of *V. album* subsp. *album* (Fig. 2a) only in Spain, Corse, the Balcan and S-Turkey. The maximal extension of the current distribution limits is not the same as those of the host trees. Hosts clearly have a wider distribution (WANGERIN 1937). The limiting factor for the northern and eastern distribution limit of *V. album* is temperature (LUTHER & BECKER 1986). A thermosphere as a function of the corresponding mean January and July temperatures is shown in figure 4. Conditions in which *V. album* can grow are shown below the curve (SKRE 1979) according to IVERSEN (1944). *Viscum album* is classified as adapted to temperate climate, outside of extreme continental regions. Normally it occurs in the colline and submontane region not above 1000m altitude, but at sites exposed to the sun, such as in southern Spain, it may also be found at higher altitudes (WANGERIN 1937; LANDOLT 1977; HOFSTETTER 1988). It has been reported that cold summers can be compensated by mild winters, and that areas with
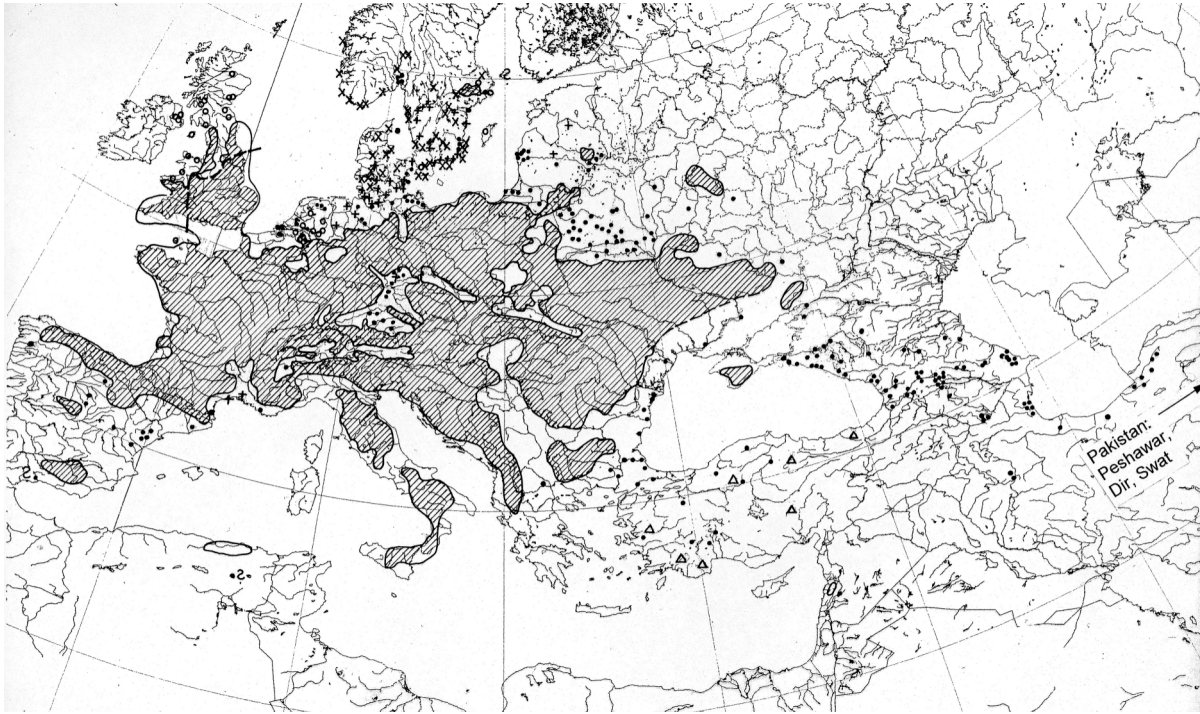
Fig. 2a. Geographic distribution of *Viscum album* subsp. *album* (see comments *). - triangles in Turkey: subspecies uncertain, possibly belonging to subsp. *abietis* or subsp. *austriacum*. - small crosses: extinct. - small circles: introduced. - lying crosses in Scandinavia: postglacial fossil record. - broken line in England: approximate northern limit of native distribution. - Map prepared by E. J. JÄGER (Halle).
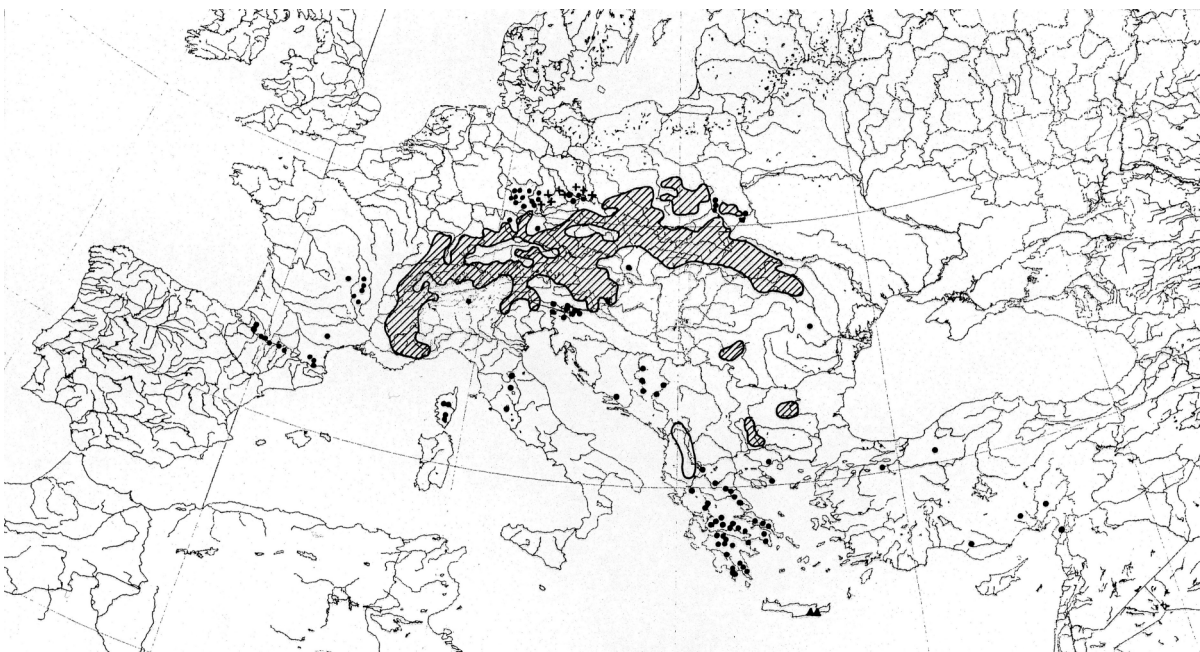

Fig. 2b. Geographic distribution of *Viscum album* subsp. *abietis*. (hatched area and dots, see also comments*) and subsp. *creticum* (triangles). - Map prepared by E. J. JÄGER (Halle).
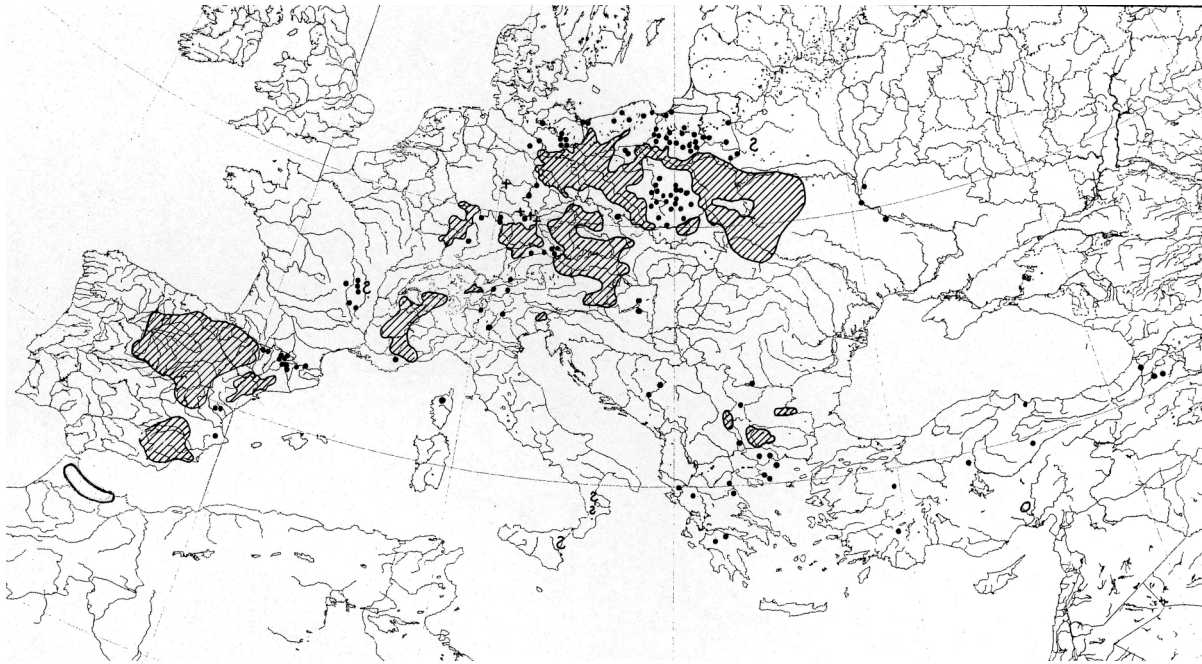
Fig. 2c. Geographic distribution of *Viscum album* subsp. *austriacum* (see comments*). - Map prepared by E. J. JÄGER (Halle).

*comments:

East- Afghanistan and Pakistan: According to RECHNINGER (FLORA IRANICA 1976) *V. album* occurs in Pakistan in addition to the small, endemic *Viscum dryophilum* RECH. F., on *Quercus baloot* GRIFFITH. In Iran the distribution area of *V. album* reaches east to Khorassan (Birjand-Qayen). The race of the West-Himalayas is not separated from *V. album* (but there is a given synonym: *V. stellatum* D. DON) in the floras of Pakistan, India and Nepal: possibly it represents indeed a separate subspecies. *V. meridianum* (DANSER) LONG (synonym: *V. costatum* GAMBLE) occurs from the East- Himalayas to Yunnan.

A related taxon is known from Taiwan (approximately 2000m): *V. alniformosanae* HAYATA (synonym: *V. coloratum var. alniformosanae* (HAYATA) IWATA).

In Far East Russia, Japan, Korea, China (Manchuria, Inner Mongolia, Central and West-China to Gansu, Sichuan, Yunnan, Jiangsu and Shandong), North-Vietnam and Myanmar occurs *V. coloratum* (KOM.) NAKAI. (syn.: *V. album* subsp. *coloratum* KOM.; *V. album* var. *coloratum* (KOM.) OHWI; *V. album* var. *lutescens* MAKINO; *V. album* var. *rubroaurantiacum* MAKINO; *V. magnificum* KOIDZ.)

Occurrence of *V. album* subsp. *austriacum* in Morocco according to GREUTER et al. (1989).

The distribution maps of *V. a.* subsp. *austriacum* and *V. a.* subsp. *abietis* in Atlas Flora Europaeae (AFE) (JALAS & SUOMINEN 1976) are incomplete and *V. a.* subsp. *album* is not separated: AFE does not show occurrence of the species on the Balearic Islands. According to Flora Iberica (1997) there is an old, unconfirmed record of *V. a.* subsp. *album* is known from Mallorca, not included in the new map of Catalonia by BOLÒS (2000) and therefore omitted in the map. AFE does not show *V. a.* subsp. *abietis* in Russia (inclusive the Ukraine), but it occurs in the Ukrainian Carpathians and their foothills. AFE does not show *V. a.* subsp. *austriacum* in Russia, but it is known from the West-Ukraine (east to Kiew and Kanew) according to Flora URSR (FOMIN 1952); additional occurrence is known from White Russia (PARFENOV 1983, map without differentiation among the subspecies, but "on *Pinus*"). The occurrence in Malaga (Spain) in AFE is not supported by the Flora Iberica (CATALÁN & APARICIO 1997). France: (DUPONT 1990) combines *V. a.* subsp. *abietis* and *V. a.* subsp. *austriacum*. (AFE and GREUTER et al. (1989) mentioned only *V. a.* subsp. *abietis*). In Central-France only subsp. *abietis* has been confirmed until now.
According to CHAS (1994), *V .a.* subsp. *austriacum* is common in Hautes Alpes (France) (not mentioned in AFE), and *V. a.* subsp. *abietis* is rare (AFE: frequent). In Corsica occur *V. a.* subsp. *abietis* and *V. a.* subsp. *austriacum* (PIGNATTI 1982) (AFE: not *austriacum*).

According to HARTL et al. (1992) *V. a.* subsp. *austriacum* was not found in Kärnten (Austria). According to MAURER (1996) it is frequent in Southeast Steiermark (Austria). Subspecies *abietis* likely has a wider distribution in former Yugoslavia than shown in the map. It is also known from Albania (MITRUSHI 1955) (AFE: not mentioned). The statement of the occurrence of subsp. *abietis* in Caucasus (SEBALD et al. 1992) is not verified in any Russian flora. Some records from Turkey without determination of the subspecies (DAVIS 1982) are shown on the map of *V. a.* subsp. *album*.
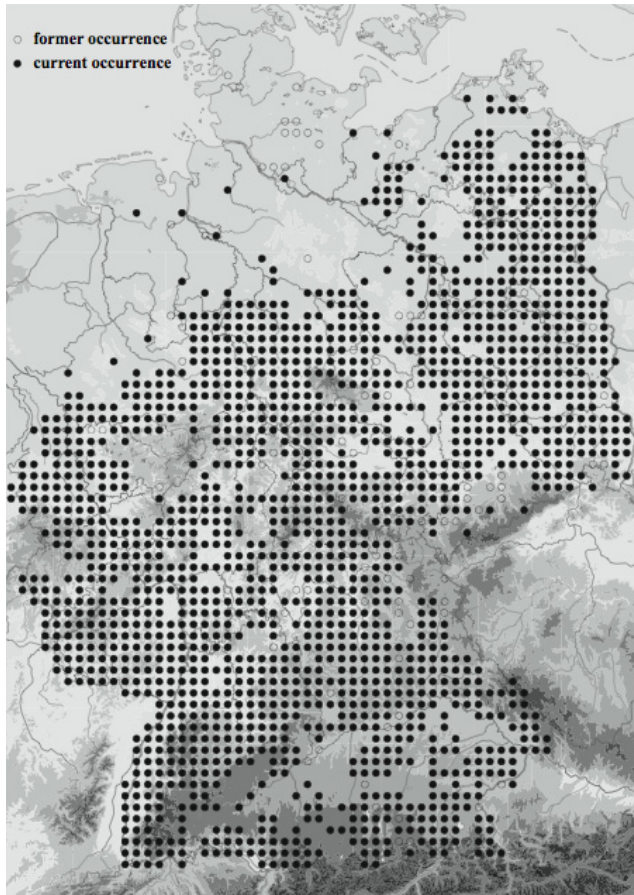
Fig. 3. Distribution of *Viscum album*-group in Germany. The map was kindly provided by the Bundesamt für Naturschutz/ Zentralstelle für Phytodiversität in Deutschland (ZePhyDe). Additional distribution maps of the three subspecies in Germany can be found at http://www.floraweb.de

strong winters can be inhabited if the temperature in summers is proportionately higher (IVERSEN 1944; WALLDÉN 1961). JEFFREE & JEFFREE (1996) used *V. album* for modelling range changes of plants in Europe as consequences of temperature changes due to doubling of the atmospheric CO_2. The model predicts a range expansion to the north and east if climate warming occurs, and may result in the extinction of *V. album* in Britain.

Fossil evidence shows that the distribution of *V. album* extended further north (Sweden) during the postglacial heat period, compared to the present distribution (IVERSEN 1944; TROELS-SMITH 1960) (Fig. 2a). The two occurrences in Sweden are relics of this postglacial warmth period (WALLDÉN 1961). During the glacial period, mistletoes most probably survived in southern and eastern Europe and recolonised Europe after the retreat of the glaciers from the South and East, together with their host trees.

Few fossil pollen samples from Switzerland and Denmark are known. Apart from a slight increase at a single locality (Dyrholmen, Denmark) an abrupt decline of mistletoes is inferred simultaneously with the introduction of agriculture. Two possible explanations have been proposed. First, logging for pastures in areas rich in mistletoes; second, and more plausibly, shepherds cut down and used trees with mistletoes as reserve food in late winter. In

Dyrholmen (Denmark), where the number of mistletoes increased simultaneously with early agriculture, the more copious light available after forest cleaning may have improved conditions for mistletoes (TROELS-SMITH 1960).

Around 1900, *V. album* was introduced by humans for the first time in North America (Sonoma County, California, USA). Since then the population has spread and nowadays covers a range of approximately 114 km^2 (HAWKSWORTH & SCHARPF 1986; HAWKSWORTH et al. 1991). Four individuals have been reported in Victoria, British Columbia, Canada, but this population did not spread out (DORWORTH 1989).

2.2. Habitat

The habitat is topographically and ecologically defined by the host tree (WANGERIN 1937). Host size and canopy characteristics determine where mistletoes can grow (DAWSON et al. 1990). *Viscum album* takes water and dissolved inorganic compounds directly from the xylem of its host. Therefore precipitation and soil chemistry affect the mistletoe only indirectly through the host (see 3.8.).

BARNEY et al. (1998) compiled a list of known hosts of *V. album.* 384 hardwood taxa have been reported as host of *V. album* subsp. *album* in Europe. Of those, 190 hosts are introduced taxa. For *V. album* subsp. *abietis* 10 hosts (including 2 introduced taxa) have been identified, and for *V. album* subsp. *austriacum,* 16 (including 3 introduced) hosts are known. A single dicotyledonous host, *Genista cinerea*, is known that is a shared host for the two subspecies *V. album* subsp. *austriacum* and *V. album* subsp. *album* under natural conditions (GRAZI & ZEMP 1986). Other host switches are only known from artificial inoculation experiments.

V. album can grow in a temperature-sphere with an average temperature above 15°C in the warmest month, and an average above -8°C in the coldest month (SKRE 1979) (Fig. 4). Flowers tolerate frost down to -8°C (WALLDÉN 1961).

V. album is a light-demanding species, especially for germination. According to the indicator values of LANDOLT (1977) and ELLENBERG (1996) it is classified as growing chiefly in full light, but sometimes surviving in slight shade. One examination of the orientation of the seedlings on the host shows that on pine, *V. album* infects mainly the not corked long-shoots, where 34% of seedlings are situated on the upper surface, 57% lateral, and 9% on the lower side of the twig (HARTMANN 1990). Although infection of stems is possible, mistletoes are generally situated in the crown of the host trees on relatively young, thin-barked shoots (SHOWLER 1974).
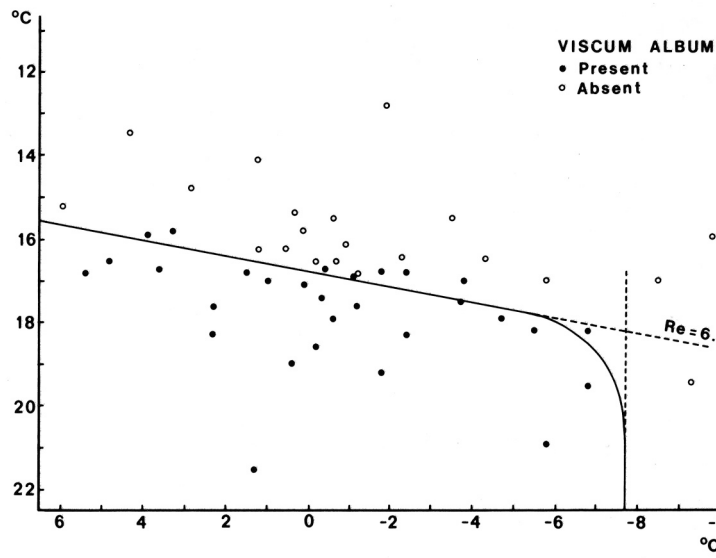
Fig. 4. The thermosphere of *Viscum album*. Mean temperatures for July (y-axis) and January (x-axis) were plotted against each other. Re is the respiration equivalent necessary to produce viable seeds and winter buds. *V. album* is found at all localities where mean January temperature is higher than -7.7°C (SKRE 1979).

2.3. Communities

Because of the epiphytic life form, mistletoes often are not considered in descriptions of vegetation types and only the hosts are known as the typical accompanying species. The other plants of the herbaceous layer are not of much interest, because there is no concurrence or interaction with the mistletoes (WANGERIN 1937).

One specification on *V. album* subsp. *austriacum* is mentioned by ELLENBERG (1996) who stated that it occurs on *Pinus* sp. in a Dicrano-Pinion. In Switzerland, *V. album* subsp. *austriacum* belongs to Erico-Pinion sylvestris and Ononido-Pinion communities (DELARZE et al. 1999).

The subspecies *abietis* lives on *Abies alba* in Switzerland, where it grows in the Abieti-Fagenion (DELARZE et al. 1999).

The subspecies *album* occurs on deciduous trees in many different kinds of plant communities. It grows mainly in old apple-orchards, in forests with hardwood like *Tilia* spp. and on hedges, shrubbery and forest-edges that harbour different hosts, such as several Rosaceae, *Acer* spp., *Populus* spp. and *Salix* spp. *Viscum album* subsp. *album* also occurs on various ornamental trees in parks, on parkway trees along the roads and urban areas. It is interesting to note that despite a large host range, some tree species, such as *Fagus sylvatica,* one of the dominant forest trees in central Europe, are never infected by *V. album*.

The endemic subspecies *creticum* is found in Cretan pine forests on *Pinus halepensis* subsp. *brutia*.

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2.4. Response to abiotic factors

A host is adequate for *V. album* if it can provide sufficient nutrients. If nutrients are in short supply, it is the host rather than the mistletoe that shows signs of nutrient deficiency. Common factors such as precipitation are mediated through the host, but other factors such as duration of vegetation period affect the mistletoe directly (WANGERIN 1937). There is no proof of the often made statement that the mistletoe needs host trees growing on calcareous soil or sites with high water availability (TUBEUF 1923; WANGERIN 1937). A correlation exists between mistletoe infection and damaged host trees: chlorotic pines for example are more heavily infected than healthy pines and harbour more fruiting female mistletoes (HARTMANN 1990).

Viscum album responses to extremely cold winters by forming adventitious shoots, if exposed parts were frozen. Berry-production does not fluctuate over years (WALLDÉN 1961).

The infestation dynamics of *V. album* was investigated on 120-year-old Austrian black pine (*Pinus nigra* ssp. *nigra*) trees in France. Thirty highly infested, but otherwise healthy trees, were examined in 1995 (VALLAURI et al. 2002). The mean number of mistletoes per tree was 287.4 (range from 64 - 720); the mean age was 6.7 years (range from 4.9 - 9.9). The mean first mistletoe infection took place in 1975 (range 1962 - 1982). The mean maximum number of individuals per tree and year was 46.1 (range 8 - 110). The annual infestation maximum occured 10 to 15 years after the start of infestation.

Data on population growth in Sweden have shown that in a first population, the number of mistletoes increased from 11047 to 17867 over five years, whereas the number of infected trees increased from 618 to 838. Another population that consisted of 7 mistletoes on 2 trees in 1923 increased to 230 mistletoes on 42 trees in 1945. In 1951, 572 mistletoes grew there on 60 trees. Moreover, in a third population the number of mistletoes increases by about 400% over fifteen years (WALLDÉN 1961).

2.5. Abundance

The patchy distribution depends on available hosts, birds and humans. The distribution partly agrees with the migration routes of birds, but this correlation is not a strict one (WANGERIN 1937). Furthermore there are sometimes single mistletoe shrubs, because these individuals are either only female or only male plants (WALLDÉN 1961). The abundance of *V. album* subsp. *album* has increased as consequence of apple tree cultivation in orchards, and planting parks or parkways with suitable hosts (WANGERIN 1937). On the other hand, humans

reduce *V. album* by collecting them for Christmas decoration and by trying to control them as parasites in orchards. Nowadays, human impact strongly affects the dynamics of *Viscum* populations (WANGERIN 1937).

3. Life cycle and biology

3.1. Life cycle

Viscum album is an evergreen epiphytic phanerophyt (RAUNKIAER 1934), respectively an epiphytic hemi-parasitic shoot parasite (sensu (WEBER 1993a)). The maximum age of a shrub is about 27 - 30 years (WANGERIN 1937; NIERHAUS-WUNDERWALD 1997). An overview of the life cycle of *Viscum album* subsp. *abietis* is presented in figure 5.

During the first 4-5 years after germination, *V. album* produces only internodes of decussate leaves, one pair each year. WEBER (1993b) found that mistletoes can grow within the host for three years after germination. After about 4-5 years it develops diachsial shoots and starts flowering. Mature plants flower and fruit once every year. *Viscum album* reproduces predominantly sexually, and as a consequence of dioecy it is an obligate outcrosser. Vegetative propagation is rare. Birds are the main vectors of mistletoes. The "seeds" survive the passage through the digestive tract unharmed and are released with the faeces. Some bird species, however, damage the "seeds" while foraging on the outer, digestible layer. Mortality of *V. album* "seeds" as a consequence of titmouses has been shown. Three common European titmouses (*Parus coeruleus*, *Parus ater* and *Parus palustris*) pick up *V. album* "seeds", mainly to forage on the endosperm (WEBER 1993b; GRAZI & URECH 1996; GRAZI & URECH 2000). GRAZI & URECH (2000) have shown that titmouses learn to accept *V. album* "seeds" as food. "Seed" mortality may be up to 100% in experiments, because the birds destroy the embryos. The titmouses prefer "seeds" of *V. album* subsp. *austriacum* over *V. album* subsp. *album* if they can choose. A few other songbirds, as well as snails (FROCHOT & SALLÉ 1980) and insects and last but not least martens are mentioned as rare mistletoe "seed" feeders that do not disperse "seeds" (WANGERIN 1937).

Upon seedling establishment, *V. album* has no real enemies except humans. Host death or branch atropy, however, causes mortality of mature mistletoes (REID et al. 1995).

As mentioned in 1.2., the length of leaves as well as the length of internodes increases sharply over the first five years and decreases slowly afterwards. This means that leaf size does not depend on nutrition or abiotic factors but rather on the age of the plant (Fig. 6) (MONTFORT & MÜLLER 1951). May and June are the months with the highest growth rate and
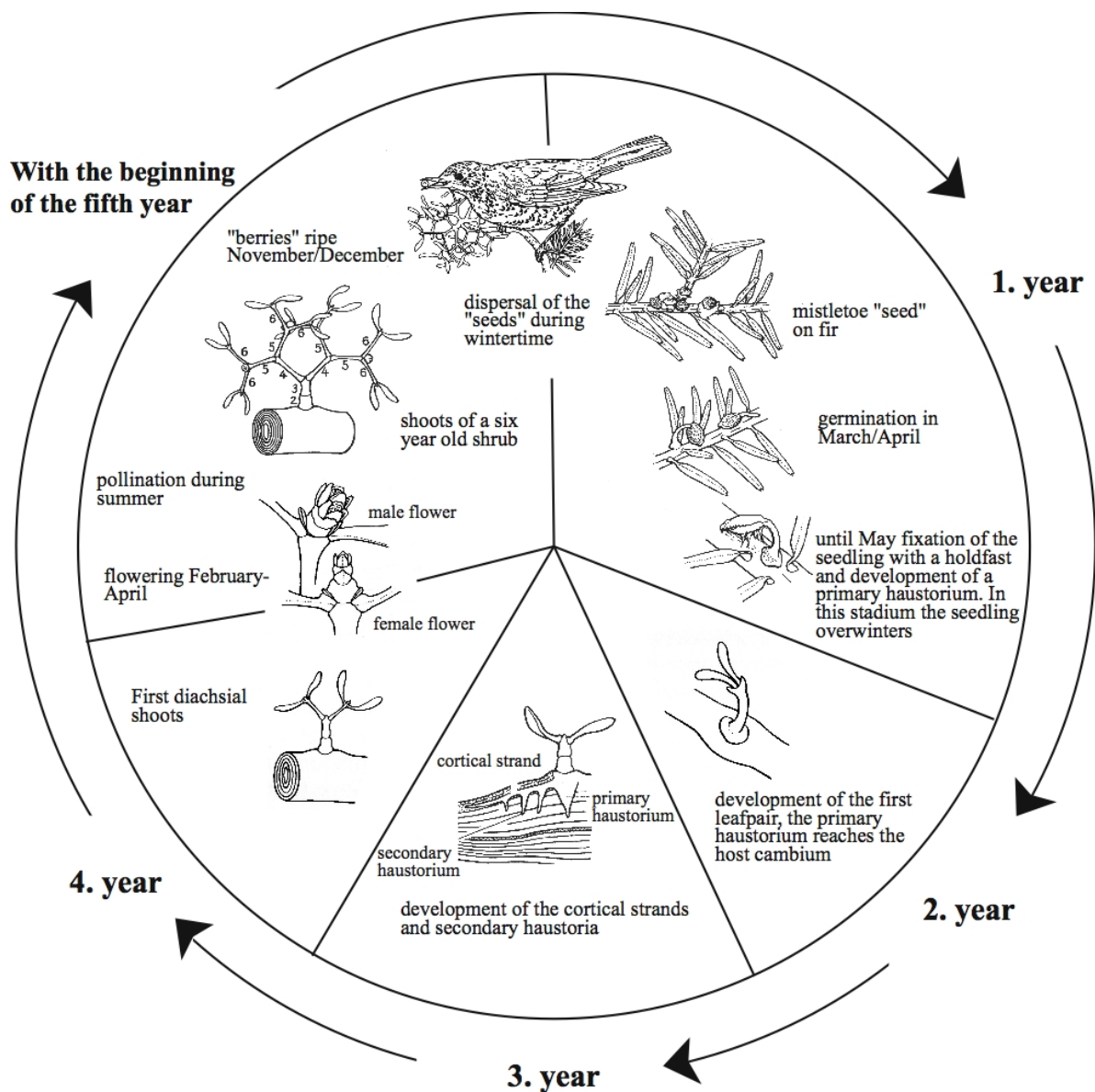
Fig. 5. Life cycle of *Viscum album* subsp. *abietis* (NIERHAUS-WUNDERWALD 1997).

growth form can vary considerably (WEBER 1993b, LANGBEHN & WEBER 1995).

Cortical strands have a constant growth, whereas haustoria, which reach the host cambial zone show a correlated growth with this region (SALLÉ 1983). According to HARTIG (1876 quoted in WANGERIN 1937) the average accrescence of cortical strands is 0.75 cm each year.

3.2. Spatial distribution of plants within populations

V. album mostly grows as a solitary individual, but up to four individuals can form a shrub, if they originate from "polyembryonal seeds" (LUTHER & BECKER 1986). Mistletoe berries can contain 1 - 4 "seeds". Different *V. album* individuals can also parasitize each other. All

reports on plants carrying male and female flowers are due to two or more *Viscum* plants of both sexes growing on each other.

Fig. 6. Leaf and internode growth (data from MONTFORT & MÜLLER (1951)).

3.3. Phenology

The growth of the endophytic parts of the plant differs. Whereas the cortical strands grow continuously and independently of the host, the 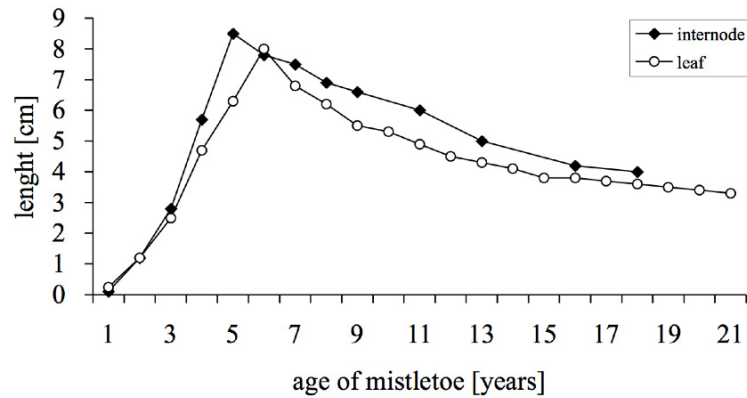division of the cambium cells of the haustorium differs seasonally. The cambium activity increases from February to May and decreases until October. During wintertime it is dormant, which corresponds to that of the host cambium (SALLÉ 1979 quoted in LUTHER & BECKER 1986). This coincided growth of host and parasite cambium prevents the two tissues from separating. The main period of "above ground" growth of the mature plant is May and June. The flowers are already established in early summer, therefore they overwinter with fully developed anthers and embryosacs, before they open up the following springtime (STOPP 1961). The flowering time is from March to April. In a year with warm climatic conditions, flowering starts in February (TUBEUF 1923; STOPP 1961). The flowering time of *V. album* is always the same and independent of the host, no matter whether the host is flowering early (e.g. *Corylus* sp.) or late (e.g. *Tilia* sp.) (WALLDÉN 1961). Under equal conditions the female flowers open earlier than the male flowers (TUBEUF 1923). Normally mistletoes flower for several weeks, but this can depend on climate and weather (WANGERIN 1937). Flowers are usually pollinated during summer and berries starts ripening November to December (NIERHAUS-WUNDERWALD 1997). Berries are dispersed between February and May; STOPP (1961) observed that dispersal takes place from February to March, when the migratory birds (mainly thrushes) fly back

northwards, whereas WANGERIN (1937) found that dispersal may be continued until April or May, especially in northern regions (Fig. 5). Germination occurs between March and April, again in northern regions as late as May (see 3.5). The first leaves appear at the earliest the following year, but it may take up to three years before the first leaf pair is formed (FROCHOT & SALLÉ 1980; WEBER 1993b).

First year: Germination takes place in March - April. Building of the holdfast from the hypocotyl, the epicotyl stays in the rest of the "seed". With the fixation of the holdfast the short non-parasitic life phase of the mistletoe ends. The parasitic phase begins with the development of a complex haustorial system or endophytic system consisting of a primary haustorium, which is derived from the holdfast and cortical strands (WEBER 1993a). There is no further growth of leaves or haustoria during wintertime.

Second year: The hypocotyl raises, the cotyledons die and, decussate to them, the first pair of leaves appears. The primary haustorium grows longitudinally towards the host cambium to reach the secondary xylem (SALLÉ 1983).

Third year: The second leaf-pair develops decussate to the first. Secondary haustoria, or sinkers, are developed; these originate from the cortical strands which run through the superficial host tissues. Haustoria grow radically and absorb water and mineral salts from the host xylem, whereas cortical strands or sinkers spread the endophyte laterally (SALLÉ 1983).

Fourth year: Again the third leave-pair develops decussate to the second.

Fifth year: The first diachsial shoots, with tiny scale-leaves on their bases, are formed. From then on all foliage leaves no longer grow decussate, because they are decussate to the scale-leaves. As a result the leaves appear in the same level. At the beginning the mistletoe shrub grows fan-like. A few years later it forms an almost perfect sphere because of formation of shoot bearing leaves from the axils of the scale-leaves (LUTHER & BECKER 1986; LANGBEHN & WEBER 1995).

From approximately the fifth year on, the mistletoes start flowering (WALLDÉN 1961).

3.4. Reproduction

Sexual reproduction usually begins when the plant reaches an age of 4-5 years. *Viscum album* is strictly dioecious and as a result self-fertilisation is impossible. Until the plants flower there is no possibility to distinguish morphologically female from male plants. Sex-ratio in dioecious mistletoes often deviates significantly from a 1:1 ratio and are typically female-biased (APARICIO et al. 1995; WIENS et al. 1996), although male-biased sex ratios

have been reported (DAWSON et al. 1990). WALLDÉN (1961) reported a *V. album* population with 71.6% females. The sex-ratio in an experiment, where plants were raised from "seeds", was clearly female-skewed as well, with 67.3% females at first reproduction (SHOWLER 1974; WIENS et al. 1996). BARLOW et al. (1978) found 69.9% females in populations in England, 72.9% females in Luxembourg and 67.3% females in California. The sex ratio of *V. album* is likely to be under genetic control and not influenced by the environment or the host (WIENS et al. 1996).

Although female flowers produce more nectar than males, the male flowers emit a more intensive odour than the females. So it is accepted that honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*) only visit the male flowers (WALLDÉN 1961). However the "real" pollinators are flies (order: Diptera), recorded by (HATTON 1963-64):

Muscidae: *Dasyphora cyanella* (MG.) (KAY 1986), *Musca autumnalis* DEG., *Opsolasia* spp.

Calliphoridae: *Pollenia rudis* F., *P.vespilio* MG. (PLATEAU 1908)

Helomyzidae: *Heteromyza rotundicornis* ZETT.

Milichiidae: *Madiza glabra* FALL.

Sepsidae: *Sepsis* spp.

Bibionidae: *Scatopse pulicaria* LÖW. (PLATEAU 1908)

Rare pollinators are *Spilogaster duplicata* MG. (TUBEUF 1923), *S. cynipsea* L., *S. flavimana* MG. (HEINRICHER 1920) and *Callyphora erythrocephala* MEIG. (HEINRICHER 1925). According to HATTON (1963-64), mistletoe flowers show entomophilous features and are visited and pollinated by insects, but more pollination is brought about by the agency of wind than by insects, at least when male and female plants are in close proximity. This is the only known statement that *V. album* is mainly wind-pollinated. It seems that *Viscum* can use both wind and insects to achieve pollination (HEINRICHER 1920; WANGERIN 1937; WALLDÉN 1961), although it is likely that wind pollination plays a minor role in *V. album* (KUIJT 1969). WANGERIN (1937) observed that the pollen remains in the pollen chambers until an insect has visited the flower. APARICIO et al. (1995) found that *V. cruciatum*, a closely related and comparable mistletoe is entomophilous. (WANGERIN 1937) mentioned that *V. album* is insect-pollinated, because pollen shows pollenkitt, which is incompatible for transport by wind. Other evidence for insect pollination is that *V. album* has barbed pollen, which clumps together and the intense odour from the male flowers. The distance of pollen distribution is between several hundred meters and 2 kilometres (WALLDÉN 1961). The male flowers fall off after withering (LUTHER & BECKER 1986). Pollen grains are oval and measure 35 x 60 μm.

The tricolporate grain has numerous spinules with a length of 1 - 3 μm (LUTHER & BECKER 1986). Male and female determing pollen grains are produced in equal numbers (WIENS et al. 1996). The pollen grain is two-celled (STEINDL 1935; BHANDARI & VOHRA 1983).

The comparable *V. cruciatum* from Mediterranean, which only has a slightly larger male flower, was found to have a mean dry mass of male flowers of 14.4 mg (N=234) and a mean number of pollen grains of 58˙485 (APARICIO et al. 1995). Thus the relative staminate effort (RSE) of *V. cruciatum* is with 4.059 rather low, which indicates an insect pollination system. Furthermore not a single bagged flower ever set fruit.

Normally the disposition for 7 - 9 pollensac-mothercells is available but only 1 - 4 are finally developed (STEINDL 1935; KUIJT 1969). Common double fertilisation has been observed (STEINDL 1935; BHANDARI & VOHRA 1983). The endosperm is cellular and chlorophyllous. The embryo is located freely in the tissue. The mature embryo lacks a suspensor (KUIJT 1969; BHANDARI & VOHRA 1983). As a speciality and difference to autotrophic plants, the accompanying cells in the hypocotyl have starch-filled plastids (LUTHER & BECKER 1986).

The diaspore which becomes distributed is the white viscaceous berry. It contains between one and four chlorophyllous embryos (LUTHER & BECKER 1986; WEBER 1993a). The average size of the embryos for *V. album* subsp. *austriacum* are 5.47 x 3.98 mm, for *V. album* subsp. *abietis* 6.42 x 4.20 mm and for *V. album* subsp. *album* 5.20 x 4.30 mm (TUBEUF 1923). Number of berries per kg of mistletoe vegetation is on average 175 (max. 237, min. 96) depending on the host (PRESTON 1977). The diaspores are dispersed by birds. Most important birds for mistletoe dispersal are the mistle thrush (*Turdus viscivorus*), fieldfare (*Turdus pilaris*), waxwing (*Bombycilla garrula*) and blackcap (*Sylvia atricapilla*). While the first three feed on the whole berry and excrete the "seed" without the berry skin, the blackcap feeds only on the skin of the berry and leaves the "seed" on a shoot nearby the mistletoe shrub. While sitting with the berry on a branch, it maintains the berry against the bark with its leg. It withdraws and swallows the external part of the pulp adhering to the teguments. The "seed" stays on the bark intactly and ready to germinate. In this way a blackcap can disperse about 100 berries per day. It is an important "seed" disperser in southern France in winter, whereas in the north of France it may be observed on its back migration-route in March - April and in Switzerland only on a few cold days in April (FROCHOT & SALLÉ 1980). The thrushes swallow the whole berry and digest a part of the viscin, then it purges the "seed" surrounded by a thin layer of viscin, which is able to stick very well onto tree bark. It has been shown that intestinal passage is not necessary for germination. WANGERIN (1937) mentioned that the

nutritional benefit of the berries is very low because only a small part is digested. Therefore the birds have to feed a lot to get enough food. So the *V. album* berries are in fact not an attractive food like insects or snails, but because of its occurrence in wintertime, some specialised birds feed on it. The mucilaginous layer is necessary to stick the "seed" onto the shoot. The adhesive substance is viscin. If berries are not dispersed, they do not drop down but remain on the shrub. It has been estimated that "seed" dispersal by thrushes may be approximately 17 km (TUBEUF 1923). Similar results are mentioned by FROCHOT & SALLÉ (1980) who noted that a "meal" of 6 - 10 berries is dispersed to a maximum of 20 km by a flying speed of 35 - 45 km/h. Migration routes of the thrushes are listed by TUBEUF (1923). Thrushes disperse the mistletoes regularly in a south to north direction, when they fly back. In addition they cause a local lateral spread when they fly around after their return from the south. On the northern edge of the *V. album* distribution area, the main distributor is not the thrush but the waxwing (WALLDÉN 1961). Feeding experiments with waxwings have shown that the intestinal passage needs only 7 - 10 minutes, thus the dispersed distance is approximately 5 - 6 km and shorter than that of the thrushes (TUBEUF 1923). The statement of WALLDÉN (1961), who mainly found short distance dispersion, agrees with these measurements. Recent observation of longer distance disperse by mistle thrushes and shorter by blackcaps in France are mentioned by VALLAURI et al. (2002).

The long distance distribution has to take place in another way, such as being stuck on the plumage or bill of birds and stripped off somewhere else (WANGERIN 1937). Other songbirds are observed on mistletoe shrubs, but there is no evidence that these birds are acting as vectors (TUBEUF 1923; WANGERIN 1937; FROCHOT & SALLÉ 1980).

A well-known example for mistletoe dispersal is the population in the USA, where *V. album* is likewise dispersed by birds: by the cedar waxwing (*Bombycilla cedrorum*) and the American robin (*Turdus migratoris*) (HAWKSWORTH & SCHARPF 1986). The average dispersal distance from the point where *V. album* was introduced was 2.4 km in 1971, 6.2 km in 1986 and about 8 km in 1991 (based on measurements in 16 directions). The area has expanded more than 4 times from 27 km^2 in 1971 to 114 km^2 in 1984 and in the last measurement 1991 up to 182 km^2. Interestingly the rate of spread increased after 1971 dramatically in the next 13 years from 0.04 km per year to 0.24 km per year in 1984 and to 0.35 km per year in 1991 (HAWKSWORTH et al. 1991). But it is expected that the rate of spread will soon decline because *V. album* has reached its potential limit in this area, due to the lack of suitable hosts (HAWKSWORTH et al. 1991).

In addition to sexual reproduction, sinkers can build additional shoots (adventitious-shoots) near the mother plant. These mostly take place if the main-shrub is cut, broken away or frozen. There are examples from Sweden, where it was possible to cut off several years up to 100 kg of mistletoe from only one tree, because they were replaced by adventitious shoots (WALLDÉN 1961).

Even if not much is known about the sex-ratio, it is well investigated that *V. album* has a translocation heterozygosity but does not have sex chromosomes like some other dioecious plants (see 3.10.). There is little doubt that dioecy in *Viscum* is derived from monoecy (WIENS & BARLOW 1979).

3.5. Germination

The mistletoe "seeds" have a dormancy that normally lasts five to six months. This time corresponds to the winter months in Europe (WANGERIN 1937). In this time there is no cell division activity or DNA synthesis in the terminal meristem, the cells remain in the G_1 phase of the cell-cycle. Activity begins 3 - 4 days after germination (SALLÉ 1976). It is possible to shorten the dormancy with very high, intensive and artificial light, but not with increasing temperature (STOPP 1961). Other possibilities are treatment with warm water, ether, or the removal of the endocarp. Indoleacetic acid can initiate germination but does not improve germination rate (STOPP 1961; LUTHER & BECKER 1986). The development of the hypocotyl does not need any stimulus from a suitable host (FROCHOT & SALLÉ 1980). *Viscum album* can germinate on all types of surfaces like glass, stone, wood, paper or free hanging on a thread (STOPP 1961). Furthermore, the germination is totally independent of budding of the host. Germination requires light, which is necessary from the very beginning (TUBEUF 1923) and high enough temperature. The ability to germinate drops considerably after several days in darkness. The percentage of seedlings decreased from 84% after 8 days in darkness to 68% after 14 days, furthermore to 36% after three weeks, 8% after 4 weeks. "Seeds" that were longer then 5 weeks in darkness did not germinate (TUBEUF 1923). The light quality was also examined, 80 - 100% of "seeds" germinated by direct sunlight or diffuse light, under a yellow glass only 56% and under blue light as well as in darkness no germination occurred. The light is only necessary for germination, after germination the seedlings can grow in darkness (TUBEUF 1923). Light is not replaceable with indoleacetic acid, kitenin, gibberellic acid or with treatment with cold temperature (LUTHER & BECKER 1986). The germination can start at 8 - 10°C, which corresponds to the common temperature in March/April. The optimum

temperature for germination is 15°- 20°C (LAMONT 1983a), the maximum temperature is about 30°C (HEINRICHER 1912 cited by WANGERIN 1937).

The young seedling is influenced by phototropism and geotropism. It is necessary to mention that only the young seedlings are influenced, because the aerial parts of the mature plant show absence of geotropism, otherwise the spherical form would be impossible (TUBEUF 1923). The hypocotyl is negatively phototropic and weakly negatively geotropic. The seedling is resistant against frost down to −15°C (WANGERIN 1937).

Artificial germination is described by WEBER (1993a). He used fresh ripe berries (not dried or stored in the dark) collected during the winter months, separated the berry coat, put the "seed" with the mucilaginous viscin layer on a flat glass-dish with a layer cotton covered with a tight clear substratum (like blotting-paper) wet with little boiled water to reach a humidity of 60 - 80%. The cotton should stay humid whereas the substratum should stay almost dry. This arrangement was stored at light and at room temperature. After a few days a seedling grew.

To germinate *V. album* in nature, the "seed" is placed on a suitable host, such as an apple tree for *V. album* subsp. *album*, by sticking the "seed" with the viscin on a branch from the previous year (SHOWLER 1974; WEBER 1993a). It is not necessary to damage the host in any way (STOPP 1961).

Viscum album contains medicinal interesting agents, which differ heavily depending on the host tree, time of harvest and manufacturing process. Therefore it is a biotechnological interest to get homogenous material. There are experiments, which grow *V. album* in callus form in tissue culture to obtain comparable extracts (BECKER & SCHWARZ 1971; DEEKS et al. 1999). A detailed list of medium compositions for successful tissue culture and the accumulation of selected macronutrients is given in BARBERAKI & KINTZIOS (2002). The artificial germination is easy, but a successful establishment of seedlings is difficult and needs some compatibility between the host and the parasite. The "seeds" dispersed by birds during the winter germinate between March and April. Because of the viscid tissue the "seed" is fixed on the host surface. Normally the "seeds" are flat, dark green to greenish yellow, with one or more embryo initials at their widest end (SHOWLER 1974). When the "seeds" have good conditions, that is light and warm temperature, their hypocotyl starts to elongate (FROCHOT & SALLÉ 1980). The elongation takes as long as 60 days and it reaches on average a length of 5.6 mm (LUTHER & BECKER 1986) according to (SALLÉ 1975) respectively 4 - 8 mm mentioned by STOPP (1961). It bends towards the host and swells at the tip by developing a holdfast. The formation of a holdfast marks the end of the short non-parasitic life of the

mistletoe. If the mistletoe germinated on dead or wrong material the seedling then dies. On a suitable surface, the parasitic cells infiltrate the host with enzymatic support and develop the primary haustorium. It is possible that the infection is directly visible. In some cases the outer parts of the seedling dry up, but the successful infection can be seen for the first time up to three years later (WEBER 1993a, b).

3.6. Response to competition and management

Mistletoes affect their host trees in many ways, they adversely affect height and diameter growth, lower the vigor of the host, induce premature mortality, adversely affect the quality and quantity of wood produced, reduce fruiting of infecting trees, predispose trees to be attacked by other agents, such as insects or decay fungi (HAWKSWORTH 1983). Besides these effects, *V. album* is mostly known to induce water stress in the host, especially to the host branches above the infection site (FISHER 1983). It has long been known that *V. album* causes damage to many forest trees, street trees and orchards in southern and central Europe (TUBEUF 1923). PRESTON (1977), who examined the influence of *V. album* on apple trees on different rootstocks, found that nine years after mistletoe inoculation, host tree size was generally reduced and crop production was reduced up to 50%. However, *V. album* is no threat to ecological silviculture (HARTMANN 1997).

On host monocultures, mistletoes may reach large population sizes with high population densities (WEBER 1993a). A correlation exists between host tree condition and mistletoe infection: chlorotic pines are more heavily infected than healthy ones (HARTMANN 1990), and there is an increase of mistletoe populations in areas where trees are affected by air pollution (HOFSTETTER 1988). However, it remains to be tested whether mistletoe infection is the cause or consequence of host damage (WEBER 1993a). *Viscum album* is of further interest because it contains a diversity of pharmacological substances, and is also harvested as a decoration plant. As a consequence, conflicts between conservationists and foresters may occur, and *V. album* is a protected species in several European countries (WALLDÉN 1961).

Management

Several means of controlling mistletoes have been tested, but direct methods, such as pruning infected branches or removing infected trees are still the only practical methods (HAWKSWORTH 1983). However, they are applicable only in small areas, such as parks, orchards or cities (WEBER 1993a).

The systemic herbicides 2.4-D, 2.4-5 T, 2.4-MCPB, and di-chloro ethane were found to kill *Viscum album* shoots on *Abies,* with little host damage, and tests with herbicides on *V. album* growing on various deciduous trees looked promising (HAWKSWORTH 1983). BAILLON et al. (1988) reported detailed experiments with 2.4-DB and glyphosate. They observed that no herbicide is found in the host, but that maximum effects occur only 4 - 6 months after treatment. Other possible herbicides are listed by WEBER (1993a). The application of ethephon ((2-chloroethyl)-phosphonic acid, Florel®,) has been discussed by ADAMS et al. (1993). Ethephon releases ethylene during absorption by the plant, which enhances the natural ripening process and leads to abscission of mature mistletoe shoots, but the endophytic system is not affected and thus only leads to the defoliation of the mistletoe. However, the use of herbicides is expensive and ecologically questionable (ADAMS et al 1993; WEBER 1993a). Other possibilities to restrict the mistletoe damage is to avoid monocultures of host trees and to divide large area forests and manage smaller areas instead (WEBER 1993a).

Also, the development of resistant host cultivars should be taken into consideration. A study by GRAZI & URECH (1983) with *V. album* on rarely infected hosts (*Quercus*, *Larix* and *Ulmus*) revealed that the resistance of hosts is genetically determined. Resistant cultivars are known from poplars (*Populus*) (SALLÉ et al. 1993). The resistance of poplar cultivars to mistletoe is dependent on the production of defensive mechanisms against the pathogen (HARIRI et al. 1991): The resistance involves pre-existing anatomical and chemical features in the host, i.e. thickness of phellem, number of fibre clusters, cells with polyphenolic contents and induced barriers developed during parasitic attack. The most resistant cultivar showed the greatest number of polyphenolic cells per unit surface area. A clear correlation was found between flavonoid content and degree of resistance: a large amount of flavonoids in the host provides good resistance to mistletoes. Flavonoids could act in two different ways; they could act as toxic substances, or work as a chemical barrier. The flavonoids were rapidly and strongly synthesised in resistant cultivars, at the very beginning of haustorium penetration. This means that the degree of resistance depends on the rate of reaction; if the host reacts too slowly, it is sensitive (HARIRI et al. 1991). More details (taniferous and flavonoidic nature of the polyphenols) about the possible defence mechanism of poplar against *V. album* are provided in SALLÉ et al. (1993). Another study with three oak species (*Quercus* sp.) revealed the same pre-existing anatomical and chemical features of resistance as those of poplars: thickness of cortex, density of polyphenol-containing cells, thickness of the first layer of fibers, and thickness of collenchyma (HARIRI et al. 1992). HARIRI et al. (1992) further

developed a resistance coefficient that can for example be used by foresters to identify resistant cultivars.

Biological control methods that target pollinators or "seed" dispersers are unacceptable, because they are neither practical nor do they make sense (WEBER 1993a). An innovative control strategy is hyperparasitism. *Viscum album* has been used to reduce *Loranthus europaeus* (another common mistletoe in Europe), but this approach presumably works only in small-scale experiments (GRAZI & URECH 1986). Other possibilities including pathogenic fungi may be possible, but have not been evaluated to date (WEBER 1993a).

Altogether, it is difficult to efficiently control *V. album* because the host trees should be not damaged, and because greenhouse experiments are not feasible.

3.7. Herbivores and pathogens

Insects

SCHUMACHER (1918) found 21 insect species on *V. album.* Six of them are exclusive mistletoe parasites, including two beetles (1 & 2), three bugs (3 - 5), and one flea (6):

1.) *Apion variegatum* WENCK. (fam.: Curculionidae/order: Coleoptera), lives in the fresh green part of the plant, lays its eggs into the mistletoe stem below the branching of the leafpair, has been reported from France (including Corse), Germany and Austria.

2.) *Liparthrum bartschi* MÜHL. (fam.: Colydiidae/Coleoptera) lives in the dead parts, likewise laying eggs into the mistletoe stem, known from Austria

3.) *Lygus viscicola* PUT. (fam.: Geocorisae/Heteroptera) known from France, England, Germany and Hungary, but has certainly a larger distribution area.

4.) *Hypseloecus visci* PUT. (Geocorisae/Heteroptera) known from France, Germany, Austria and Hungary.

5.) *Anthocoris visci* DLG. (Geocorisae/Heteroptera) known from England, France and Germany.

6.) *Psylla visci* CURT. (Psyllidae/Sternorrhyncha) develops two generations per year, known from England, France, Austria, Germany and Hungary (SCHUMACHER 1918).

The afore-mentioned family names of insects are according to JAKOBS & RENNER (1988). Also common on *V. album* is the scale insect *Diaspis visci* SCHR. (=*D. juniperi* BCHÉ) (fam.: Diaspididae/Coccina), which is a primary parasite of conifers, mainly *Juniperus* sp. It is known from Austria, Germany, Turkey and Hungary. It switches from conifers to mistletoes, in areas where *Viscum* is common, but where conifers are absent, *D. visci* is absent too

(SCHUMACHER 1918). The other 15 insect species known from *V. album* are all secondary pathogens, many of which switch between *Viscum* and its host trees. The subspecies *V. a.* subsp. *album* harbours more insect species than the conifer mistletoes and it is worth mentioning that the six main parasites, are all known specifically from *V. a.* subsp. *album*. There are no menognathous insects or aphids known to feed on *V. album*. All insects that can damage mistletoes are listed in SCHUMACHER (1918) and TUBEUF (1923).

Herbivore

Due to the epiphytic life form, mistletoes usually elude herbivores, although they are eaten when herbivores can reach them. Known herbivores are: rabbits, roe deer, deer, goats, sheep, livestock and likely even mice (TUBEUF 1923).

Fungi

Several fungi can parasitize *V. album*: *Botryosphaerostroma viscii* (WEBER 1993a), *Colletotrichum gloeosporoides* (SACC.) PENZ. (STOJANOVIC 1989), *Plectophomella visci* MOESZ (=*Phyllosticta visci* SACC.), *Septoria visci* BRES. and *Sphaeropsis visci* (FR.) SACC. (BRANDENBURGER 1985). Nevertheless, *V. album* is known to harbour relatively few fungal pathogens, presumably because of an effective defence system (HOLTORF et al. 1998). A study with transgenic *Arabidopsis thaliana* that contains the gene for viscotoxin A3, which acts against phytopathogens has shown that transgenic *A. thaliana* contains a high level of viscotoxins and showed increased resistance against a primary infection by the clubroot pathogen *Plasmodiphora brassicae* (HOLTORF et al. 1998).

3.8. (Eco-)Physiological data

Viscum album contains chlorophyll *a* and *b* and is able to photosynthesise. Because of the method of CO_2 fixation it belongs to the C_3-plants, but the power of photosynthesis is within the lower limits of this group (WEBER 1993a). *Viscum album* has a high transpiration rate, similar to many other parasitic plants (WEBER 1993a), and a low photosynthetic rate. SCHULZE et al. (1984) showed that the major difference between *Viscum* and its hosts is, that *Viscum* has a much higher rate of transpiration and stomatal conductance and a much lower leaf water potential than the host *Pinus sylvestris*. This leads to threefold lower water-use efficiency and a higher internal CO_2 concentration in the leaf mesophyll of the parasite. The cumulative CO_2 uptake in *Viscum* is similar to that in the host, whereas the daily cumulative

transpiration of the parasites is threefold higher than in the host. Mistletoe transpiration on deciduous trees is also high if the host foliage is present; it is reduced during the leafless period of the host (Fig. 7; see also PFIZ et al. 1998). An unexplained phenomenon leading to high rates of transpiration is the unability of mistletoe stomata to respond to water stress. One possible explanation is that hemiparasites lack absisic acid (ABA), which is known to control transpiration (FISHER 1983). Stomatal responses of *V. album* to meteorological influences do exist. This is illustrated (Fig.8) by two-peaked time courses of leaf resistance and transpiration ("mid-day depression") of mistletoe and host (*Malus transitoria*, in the particular case) under the influence of a dome-shaped daily course of air temperature and vapour pressure deficit.
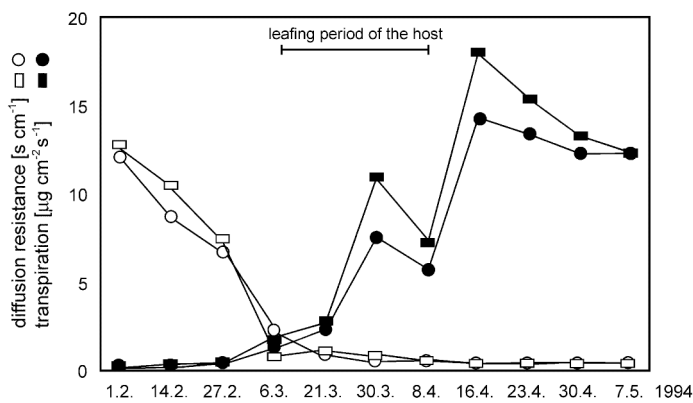

Fig. 7. Seasonal course of daily means of transpiration (closed symbols) and diurnal minima of diffusion resistances (open symbols) of male (■, □) and female (●, ○) *Viscum album* subsp. *album* on *Malus transitoria* (LÖSCH & BIENERT, unpubl.)

The study of the nutrient content of the xylem and seasonal measurements of the biomass and the tissue nutrient content of *Viscum* and *Pinus* has led to the hypothesis that the high rate of transpiration may be necessary for the parasites to take up sufficient nitrogen from the xylem of the host. The nitrogen is important for the production of biomass (SCHULZE et al. 1984). Other studies of growth rate and the accumulation of nutrients like N, P, K and Ca as well as values for carbon isotope ratios of mistletoe tissue further support the hypothesis that the higher transpiration rates of mistletoes represent a nitrogen gathering mechanism. Nitrogen potentially is the nutrient most limiting to mistletoe growth (FISHER 1983; LAMONT 1983b; SCHULZE & EHLERINGER 1984; EHLERINGER & SCHULZE 1985; EHLERINGER et al. 1985; SCHULZE et al. 1991). There is evidence, that besides the gathering mechanism *V.*

album is able to store nitrogen in the form of arginine accumulation (URECH 1997). Generally the solutes in the xylem sap of the host reach the mistletoe through the transpiration stream. And according to this, the amount of nutrients like N, K, P, S, Ca and Mg are considerably higher in *Viscum* than in the host, especially when compared to infected host branches (LUTHER & BECKER 1986; WEBER 1993a). The accumulation of macronutrients is likewise shown in tissue cultures (BARBERAKI & KINTZIOS 2002). Calcium is the only exception, because it is not always higher; a potential explanation for this is the binding of Ca in the Donnan free space of the host (FISHER 1983). In an example with *Phoradendron* sp., a mistletoe similar to *V. album*, the nutrients K and Ca seem to be passively accumulated and the high K enrichment is the consequence of a lack of a retranslocation link (i.e. phloem) between the mistletoe and its host (SCHULZE & EHLERINGER 1984). In difference to this the absorption of organic substances seems to require an active mechanism (FISHER 1983), but the nature of the absorption mechanism of minerals and organic compounds in contact between host and parasite is not exactly known (WEBER 1993a).

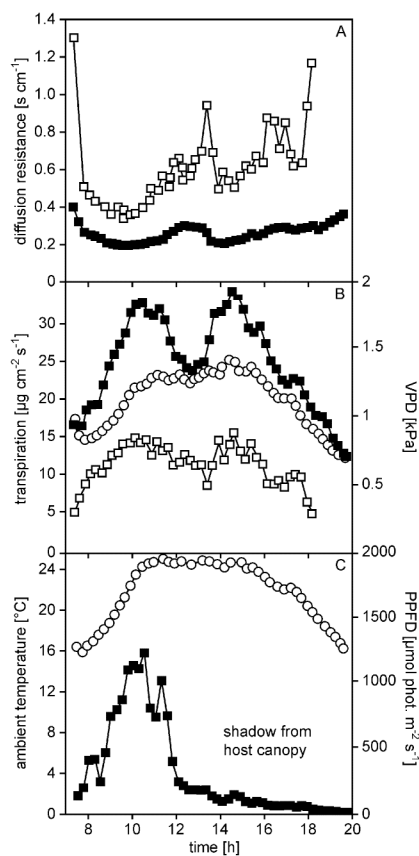Fig. 8. Diurnal course from April 30, 1994 of (A) leaf diffusion resistance, (B) transpiration (■, □) and vapour pressure deficit (O), as well as (C) air temperature (O) and photosynthetically active radiation reaching the mistletoe leaves (■) of *Viscum album* subsp. *album* (closed quadrats in A and B) on *Malus transitoria* (open quadrats in A and B); Botanical Garden Düsseldorf, LÖSCH & BIENERT, unpubl.

Chloroplast characteristics were studied by TUQUET & SALLÉ (1996) to explain the low photosynthetic rate of *V. album*. They found that the structure of chloroplasts is close to that reported for autotrophic higher plants. In green leaves there are no striking differences in the ultrastructure of *Viscum* chloroplasts in winter and summer. Both possess well-developed thylakoid systems. Only in yellowish leaves in winter does degradation of the thylakoids occur (HUDÁK & LUX 1986). The peculiarity is, that the *Viscum* chloroplasts contain low amounts of chlorophyll Chl *a* as well as Chl *b* and that their protein/chlorophyll ratio is higher than that of autotrophic plants (HUDÁK & LUX 1986), and the lipid content is in the lower range of chloroplasts of non-parasitic plants. Measurements of photosystems PS I and PS II activity show low activities in both systems and a low PS I/PS II ratio. Altogether, the chloroplasts of *V. album* present large deficiencies in their photosystems and this supports the hypothesis, that the mistletoe is not only a water and mineral parasite, but it partly takes carbon from the host to complement its own nutrition (HUDÁK & LUX 1986). In addition to the low PS activity, a low activity of the phosphoenolpyruvate (PEP)-carboxylating enzyme, found in Viscaceae, supports the partial heterotrophy of hemiparasitic mistletoes (WEBER 1993a). Thus it seems that the mistletoe is partially heterotrophic and the high transpiration rate is explained not only by the nitrogen demand but also by the carbon demand (MARSHALL & EHLERINGER 1990). RICHTER & POPP (1992) found a high carbon input from the host into *V. album*, ranging from 22.6 to 45%, no transport of phloem-mobile compounds occurs in the opposite direction, from the mistletoe to the host (BAILLON et al. 1988).

The seasonal changes in physiological activity of *V. album* are similar to that of woody evergreen species and it depends on the development stages of the parasite (BAILLON 1988). Studied parameters were respiration rate, sucrose phloem transport and carbohydrate content. In summary, the highest physiological activity takes place in spring with the development of young shoots, low activity during the summer and a little increase during autumn for flower bud formation and berry development and a second increase in early spring during flowering (BAILLON 1988).

Parasites always have more negative values of water potential than the host does, with daily and seasonal variations. The difference of suction potential between host and parasite increases during the day and decreases in the evenings (FISHER 1983; WEBER 1993a; LÖSCH & GANSERT 2002). Besides the high transpiration rate a low osmotic potential is responsible for the negative water potential. Cyclitols and K-ions mainly control the osmotic potential. Minimum accumulation of cyclitols in summer (10% of the osmotic potential) and maximum during the winter (24% of the osmotic potential) might be explained by the high input in

summer respectively low input of inorganic ions like potassium in the cold season. Cyclitols are qualified osmotica, because they are nonpolar and do not influence enzyme activity. Altogether, cyclitols could be regarded as multifunctional compounds, acting as organic osmotica, cryoprotectants and carbon storage (RICHTER & POPP 1992; WEBER 1993a). In addition, concentrations of the compatible solute proline undergo seasonal changes, depending on the prevailing night temperatures (Fig. 9), with highest values only in spring when (probably) by higher transpiration rates nitrogen supply is improved as compared with the situation in winter (LÖSCH, unpubl.)

The main agents likely responsible for cryoprotection are lectins (MLI and MLIII see 3.9), which are in general known to act as toxic defence molecules (HINCHA et al. 1997), but interaction between the lectins and membrane lipids may lead to cryoprotection. Frost hardiness of mistletoe leaves is seasonally regulated. They tolerate clearly colder temperature (down to -20°C) during wintertime than during summer (down to -5°C). Likewise, the amount of the cryoproctective lectins varies during the year, with the highest amounts in wintertime and the lowest during summer (HINCHA et al. 1997). Likewise, protoplasmatic drought tolerance of leaves is very high in winter (critical water saturation deficit producing necroses, "crit. WSD", in January: 70-80%) and is substantially lowered in spring and summer (crit. WSD in May: 40-50%): LÖSCH & BIENERT (unpubl.).
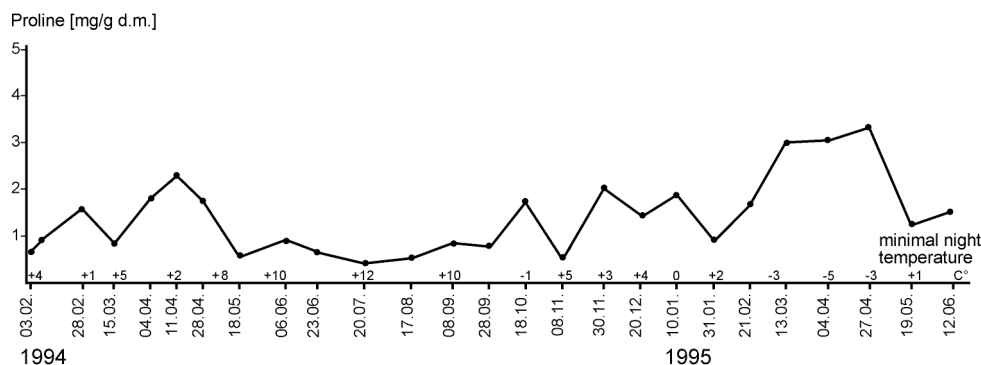
Fig. 9. Proline concentrations of female mistletoe leaves during the years 1994 and 1995, correlated with air temperature minima during night (*Viscum album* subsp. *album* growing on *Malus transitoria*, Botanical Garden Düsseldorf, LÖSCH, BIENERT & KIEFER, unpubl.).

A study of the thiol composition of *V. album* showed that *Viscum* has the highest thiol contents so far reported for green plants. Thiols are most likely removed from the xylem sap of the host and accumulated in the mistletoe. It is not known yet, how far leaves of mistletoes are capable of assimilatory sulphate reduction (RENNENBERG et al. 1994). The results of

SCHRADER-FISCHER & APEL (1993) suggest that the amount of sulphur offered by the host may not be sufficient for the growth. They studied another characteristic of *V. album* physiology: the anticyclic timing of leaf senescence. Leaf senescence of the mistletoe occurs in summer, at the maximum metabolic activity of the host. In contrast to non-parasitic plants mistletoes selectively degrade only one leaf protein, the sulphur rich viscotoxins, whereas most of the remaining proteins appear to be lost during abscission. The selective degradation of viscotoxins may represent a sulphur and nitrogen source, which can be utilised during new leaf formation (SCHRADER-FISCHER & APEL 1993; HOLTORF et al. 1998).

3.9. Biochemical data

Viscum album has been known for a long time for its pharmaceutical use. In former times there were a lot of possible uses, listed by (MACHAIDZE et al. 1996; TUBEUF 1923). Biologically active substances are mainly lectins and viscotoxins, but also special alkaloids, flavonoids, amines, acids and terpenoids (FRANZ 1985; LUTHER & BECKER 1986; OCHOCKA & PIOTROWSKI 2002).

The most interesting fraction of the proteins are the lectins VAA I and VAA II (respectively former ML I, ML II). There is confusion about the nomenclature of the compounds. Common synonyms and the chronology of the description of the compounds can be found in LUTHER & BECKER (1986) and OCHOCKA & PIOTROWSKI (2002). The other important active group of compounds synthesised by the mistletoe are the viscotoxins, the most important are A2, A3 and B (LAMONT 1983b). Compounds of *V. album* are discussed among other things to be cardiac-effective and to lower blood pressure (FRANZ 1985; LUTHER & BECKER 1986; URECH et al. 1996). Interestingly, viscotoxins are closely related to the group of cardiotoxins found in poisonous snakes (cobra). A comparison of these two substances can be found in URECH & RAMM (1997). In recent times the main interest has been in compounds with a possible effect against cancer. The toxic effect of *V. album* on tumour cells was first tested with plant tumours and than with animal tumours (FRANZ 1985; URECH 1987). For a general detailed description of medicinal interesting substances see FRANZ (1985), LUTHER & BECKER (1986) and OCHOCKA & PIOTROWSKI (2002), for viscotoxins see ORRÙ et al. (1997), and for lectins SCHINK et al. (1992). The first anti-cancer medication was developed by anthroposophical scientists, the well-known Iscador®, but in the meantime there are a few more listed by LUTHER & BECKER (1986). For production it is important to consider the time of harvesting the mistletoe, the host tree and the used part of the plant. In

the case of viscotoxins, the host influences only the total amount of the viscotoxins and not the composition (SCHALLER et al. 1998, 2000). PARK et al. (1999) showed that the cytotoxic effect of the components decreases after heat treatment, which is important for a few medicaments in traditional therapy.

Besides the medically interesting compounds a summary about the mineral content of mistletoes is shown in LAMONT (1983b). No cyanogenic (CN) glycosides common to deter herbivores have been found in mistletoes.

Aside from the influence on plant tumours, extracts of *V. album* are used to cause induced resistance. Two susceptible host plants were treated with *V. album* extracts and afterward showed a reduction of bacterial development and retarded disease symptoms of the fireblight bacterium (*Erwinia amylovora*) (MENDE et al. 1994). Furthermore there is evidence of a higher resistance of transgenic *Arabidopsis* plants, which have expressed viscotoxins (HOLTORF et al. 1998) (see 3.7.).

3.10. Genetic data

Viscum album is diploid (2n=20) and dioecious. Dioecy is correlated with a specific translocation-heterozygosity-complex (MECHELKE 1976). Permanent translocation heterozygosity has been confirmed for *V. album* in Europe, Japan, and California (BARLOW et al. 1978). The chromosomes of female plants are "normal" structurally homozygous and form 10 regular bivalents in meiosis. In contrast, in male plants four chromosome pairs are complex-heterozygous, which always form a zig-zag-octovalent. During the first meiotic division, two structurally different complexes are developed. Accordingly, the resulting male gametes are of the standard type and of the translocation type. With respect to the chromosome complexes, female plants are homogametic, whereas male plants are heterogametic. Occasionally, supernumerary chromosomes occur (MECHELKE 1976; WIENS & BARLOW 1979). BARLOW et al. (1978) confirmed these results from European populations, but they found translocation polymorphisms in *V. album* populations from Japan (BARLOW 1981). In contrast to some other dioecious plants like *Silene latifolia* (GRANT et al. 1994) or *Rumex acetosa* (SHIBATA et al. 1999), *V. album* has no sex chromosomes. SCHINK & MECHELKE (1989) found two proteins that are present only in male plants and can thus be regarded as sex-specific markers. This protein pattern is not influenced by the species or geographical location of the host and may be used to determine the sex of mistletoe plants at any developmental stage. *Viscum album* is the diploid angiosperm with the highest (2C)*

nuclear DNA content reported so far, i.e. 107 pg on average, but the DNA is much less repetitive (65%) than expected from this genome size. The GC content as calculated from the melting temperature is 30% (NAGL et al. 1983). DNA content varies only little among the three main subspecies (NAGL & STEIN 1989).

Genetic evidence for host specificity has been reported in an analysis of nuclear ribosomal DNA ITS sequences and non-coding chloroplast DNA (ZUBER & WIDMER 2000).

* C = DNA content of the haploid genome

3.11. Hybrids and host switches

Hybrids

Little is known about hybrids among the subspecies. There have been many attempts to raise hybrids, but all failed. Artificial pollination of *V. a. album* with *V. a. abietis* led to berry formation. Hybrid berries were planted on fir and apple trees, but although many berries germinated, all plantlets died soon thereafter (HEINRICHER 1919). Other crossing experiments with closely related *Viscum cruciatum* and *V. album* produced no viable offspring (HEINRICHER 1922, 1926). Crossing experiments are time-consuming and extremely difficult because of the parasitic growth form. Possibly, molecular studies could alleviate this problem. Chloroplast and nuclear DNA variation revealed evidence for a potential hybrid origin between *V. a. album* and *V. a. abietis* for one individual (ZUBER & WIDMER 2000).

Host switches

TUBEUF was the first to differentiate between host races. His findings were confirmed by HEINRICHER (1911) and GÄUMANN & PÉTER-CONTESSE (1951). So far only one host switch example has been reported for *V. album* subsp. *austriacum* growing on a dicotyledonous host: *Genista cinerea* (GRAZI & ZEMP 1986). The experiments of HEINRICHER (1911) and TUBEUF (1923) revealed that germination on wrong hosts may occur but plants fail to establish (WANGERIN 1937).

3.12. Status of the species

Viscum album has a special place in legends and folklore in Europe. However, *V. album* on oaks had a mystical status for Druids in Great Britain and is still of interest in recent literature (TUBEUF 1923; KUIJT 1969; CALDER 1983; BARLOW 1987; BOX 2000). From the seventeenth century on, mistletoes became a part of the English Christmas tradition. Furthermore it is

known as medical plant used traditionally against high blood pressure, arthritis, epilepsy and as a narcoticum, and as a "cure-all plant". Now it is mostly mentioned in context with therapy of cancer (TUBEUF 1923; CALDER 1983; BARLOW 1987; BARBERAKI & KINTZIOS 2002). Use of mistletoes as fodder plants for livestock has been common through the ages (TUBEUF 1923; TROELS-SMITH 1960). The biology of *V. album* is of further interest because of the threefold dependence or co-evolution with host trees, birds and pollinators (BARLOW 1987).

Currently, *V. album* is widespread in Europe and in its peripheral distribution areas like in Norway or Sweden it can not be regarded as threatened, as it has been protected by law (WALLDÉN 1961; JOSEPHSEN 1993). But there are areas in Germany where *Viscum* is threatened, in parts because of the decline of the hosts: *V. a.* subsp. *abietis* in Sachsen, Thüringen and Bayern and *V. a.* subsp. *austriacum* in Mecklenburg-Vorpommern and Sachsen-Anhalt. Evidence further suggests that *V. album* has benefited and is spreading out in areas where hosts (mainly fir) have been damaged by air pollution (HOFSTETTER 1988).

Taxonomy

The subspecies of *V.album* are variously treated as forms, varieties, subspecies or species in floras and no general agreement concerning the taxonomic status of the taxa has emerged to date (BALL 1993). The term "host races" instead of subspecies may to be more appropriate. Evidence for the existence of these host races exists - morphologically (GRAZI & URECH 1981), physiologically (SCHALLER et al. 1998) and genetically (ZUBER & WIDMER 2000). The weak genetic differentiation of the three races observed in the phylogenetic analysis can be interpreted as a relatively recent formation of the host races (ZUBER & WIDMER 2000). Recently, a new subspecies from Crete, *V. album* subsp. *creticum* (growing on *Pinus halepensis* subsp. *brutia*) has been described (BÖHLING et al. 2002).

The taxon *V. album* subsp. *coloratum* - often referred to as *V. coloratum* - grows on deciduous trees and differs from *V. album* subsp. *album* by the more eastern distribution, the yellow or orange berries, and another formation of the mucilaginous threads between the inner and the outer layer (GRAZI & URECH 1981). *Viscum album* populations in Japan have the same chromosome system as in Europe, but in Japan the greatest translocation complexity has been found (BARLOW 1981). Similar to *V. album* in Europe, *V. coloratum* has been divided into different races or subspecies (see comments to Fig. 2). Furthermore WALLDÉN (1961) suggested a north climatic race of *V. a. album*, which can better defends itself against cold temperatures than others grown more southerly.

The second 'good' *Viscum* species in Europe is *Viscum cruciatum*. It grows on deciduous trees in southern Spain, Israel and Morocco. *V. cruciatum* has red berries, and all leafpairs are decussate. DNA sequence data further supported the distinction of this taxon from *V. album* (ZUBER & WIDMER 2000).

Another European mistletoe is *Loranthus europaeus* (Loranthaceae). At first sight it is similar to *V. album*, but the main differences are that *L. europaeus* is summer-green and that the flowers respectively berries are located in stipitate inflorescences (KUIJT 1969; CALDER 1983)

3.13. Continuative literature

A biogeography of the family Viscaceae and of the genus *Viscum* is given in WIENS & BARLOW (1979) and BARLOW (1983, 1987).

Biological flora literature of *V. album* exists:

TUBEUF (1923); WANGERIN (1937); STOPP (1961); LUTHER & BECKER (1986), but is written in German.

For general information about mistletoes see

BARLOW (1987); CALDER & BERNHARDT (1983); KUIJT (1969); WEBER (1993a)

An extensive bibliography of mistletoes can be found at

http://www.rms.nau.edu/mistletoe/

Acknowledgements

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Genetic evidence for host specificity in the hemiparasitic *Viscum album* L. (Viscaceae)

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Abstract

Nuclear ribosomal DNA (nrDNA) ITS sequences and partial sequences of three non-coding chloroplast DNA (cpDNA) introns and spacers were used to assess genetic variation within and among three presumed host races of the hemi-parasite *Viscum album* L. Currently, identification of host races occurs via the host-trees, and morphological differences are minute at best. cpDNA and nrDNA ITS sequences revealed little sequence variation, but the variation found consistently supported the distinction of three host-races. cpDNA and ITS sequences were not incongruent, as assessed by the incongruence length difference test. A combined analysis supported the sister group relationship between mistletoes from deciduous trees and fir.

Keywords: *Abies*, cpDNA, gene flow, host-races, ITS, *Pinus*

Introduction

The European mistletoe, *Viscum album* L. (Viscaceae), is widely distributed across Europe. Male and female plants live as evergreen hemiparasites (*sensu* Weber 1993) on woody plant species from which they obtain water and dissolved inorganic compounds directly from the xylem. The inconspicuous flowers are insect pollinated and ripe pseudoberries are dispersed by a variety of birds, the most important being the Mistle Trush (*Turdus viscivorus*) (Luther & Becker 1987). *V. album* is able to infect a large number of host plants. In fact, a recent compilation of the host range of *V. album* by Barney *et al*. (1998) has shown that 384 taxa of shrubs and trees may become infected under natural conditions. These include a significant number of woody plants that are not native to the geographical range of *V. album* but have been introduced into Europe. This large host range and the ability to readily infect a variety of potential new hosts may suggest that *V. album* is not host-specific. On the other hand, three sub-species are currently recognised, which presumably differ in their host specificity. *V. album* ssp. *abietis* (Wiesb.) Abromeit is restricted to fir (*Abies* spp.), *V. album* spp. *austriacum* (Wiesb.) Vollmann occurs mainly on pine (*Pinus* spp.), and *V. album* ssp. *album* L. grows on a wide variety of deciduous trees (Ball 1993). These sub-species, hereafter called host races, may only be identified via characteristics of the ripe pseudoberries that differ among host races (Grazi & Urech 1981); male plants and females without ripe pseudoberries, however, can not be identified. Therefore, identification of the three host races relies on the identification of their hosts. Evidence supporting the distinction of three host races of *V. album* comes from biochemical investigations. Schaller *et al*. (1998) have shown that host races differ in viscotoxin composition. Crossinfection experiments, which have proven useful to demonstrate host specificity in other mistletoes (Nickrent & Stell 1990)), have not been carried out adequately in *V. album*. Circumstantial evidence suggests that more infections occur on the primary host than on alternate hosts (Luther & Becker 1987), but more thorough experimental studies are needed to verify these results.

Cross-infection experiments to analyse the host-specificity of presumed *V. album* host races are time-consuming. It may take up to three years before successful seedling establishment can be observed, and onset of flowering as a reliable indicator of successful infection may take four more years (Langbehn & Weber 1995). As an alternative to cross-infection experiments, molecular markers may allow us to assess host specificity. If host specificity occurs, and if it reduces gene flow among host races, e.g. due to geographical separation or selection, genetic divergence may occur. Well known examples in which host

specificity may lead to genetic divergence and ultimately speciation are e.g., leaf beetles (Funk 1995; Köpf *et al.* 1998) smut fungi (Shykoff *et al.* 1999) and holoparasitic plants (Cullings *et al.* 1996). To date, population genetic studies on V. *album* and allied species are lacking despite the large economic interest from both foresters and the pharmaceutical industry (Luther & Becker 1987)). In dwarf mistletoes (*Arceuthobium*), allozymes have proven useful to identify and delimit host races (Nickrent & Stell 1990). For phylogenetic analyses among mistletoe species, sequence information from the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA and non-coding sequences of the chloroplast genome have been used successfully (Nickrent *et al.* 1994); (Molvray *et al.* 1999). As the chloroplast genome is usually either predominantly or strictly uniparentally inherited in angiosperms (Harris & Ingram 1991), cpDNA sequences may provide another picture of evolutionary relationships compared to the ITS sequences. ITS and non-coding chloroplast DNA sequences, however, may not only be useful for phylogenetic studies among taxa, but may also uncover substantial intraspecific variation, such as e.g. in black alder *Alnus glutinosa* (King & Ferris 1998), *Coreopsis grandiflora* (Mason-Gamer *et al.* 1995) and *Draba aizoides* (Widmer & Baltisberger 1999) and may thus be ideal markers to unravel the presence of host races in *V. album*.

The aim of this study was to assess whether molecular markers suggest the presence of host races in *V. album*, whether these host races correspond to those currently identified in the literature based on pseudoberry characteristics, host range, and viscotoxin composition, and to assess the phylogenetic relationships among host races.

Materials and Methods

Plant samples

Twenty-eight *V. album* individuals were collected from a wide variety of host shrubs or trees in Switzerland, Germany and Slovenia (Table 1). Twenty *V. album* samples came from deciduous trees, and four each from fir (*Abies* sp.) and pine (*Pinus silvestris*). A sample from one individual of *V. cruciatum* Sieb. was obtained from the Botanical Garden in Basel (Switzerland). Leaf samples were stored in silica gel at room temperature.

Table 1 Source of the Viscum album s.l. isolates used in this study. All samples were collected in 1998

Host race	Host	Origin	cpDNA haplotype
V.a. abietis	*Abies alba*	Switzerland, Gallenkirch	A
V.a. abietis	*Abies alba*	Switzerland, Gallenkirch	A
V.a. abietis	*Abies alba*	Switzerland, Althäusern	A
V.a. abietis	*Abies nordmanniana*	Switzerland, Zürich	B
V.a. austriacum	*Pinus silvestris*	Switzerland, Grengiols	C
V.a. austriacum	*Pinus silvestris*	Switzerland, Brig	D
V.a. austriacum	*Pinus silvestris*	Switzerland, Leuk	C
V.a. austriacum	*Pinus silvestris*	Switzerland, Fürstenaubruck	C
V.a. album	*Acer campestre*	Germany, Nikolausberg	E
V.a. album	*Acer platanoides*	Germany, Göttingen	E
V.a. album	*Acer sp.*	Switzerland, Basel	E
V.a. album	*Betula* sp.	Switzerland, Robenhausen	E
V.a. album	*Crataegus* sp.	Switzerland, Robenhausen	E
V.a. album	*Malus* sp.	Switzerland, Weesen	H
V.a. album	*Malus* sp.	Germany, Göttingen	E
V.a. album	*Malus* sp.	Slovenia, Idrsho	F
V.a. album	*Populus nigra*	Switzerland, Baar	E
V.a. album	*Populus nigra*	Switzerland, Martigny	E
V.a. album	*Populus nigra*	Germany, Hannover	E
V.a. album	*Prunus padus*	Germany, Hannover	E
V.a. album	*Robinia pseudo-acacia*	Germany, Göttingen	E
V.a. album	*Salix alba*	Switzerland, Weesen	G
V.a. album	*Salix alba*	Switzerland, Martigny	E
V.a. album	*Salix caprea*	Switzerland, Robenhausen	E
V.a. album	*Sorbus aria*	Switzerland, Eptingen	E
V.a. album	*Tilia cordata*	Germany, Tübingen	E
V.a. album	*Tilia cordata*	Switzerland, Berzona	E
V.a. album	*Tilia platyphyllos*	Switzerland, Basel	E

DNA extraction, amplification, sequencing, and phylogenetic analyses

Total DNA was extracted from the dried leaf material using the CTAB method of Doyle &
Doyle (1987). Polymerase chain reaction (PCR) was used to amplify ITS and non-coding
cpDNA sequences. Nuclear rDNA ITS was amplified using primers ITS4 and ITS5 of White
et al. (1990). Non-coding cpDNA was amplified using the universal primers c and f of
Taberlet *et al*. (1991) for *trn*L(UAA) - *trn*F(GAA) and primers developed by Demesure *et al*.
(1995) for *trn*H(GUG) - *trn*K(UUU) and *trn*S(UGA) - *trn*fM(CAU). PCR reactions were
carried out separately for each locus in a total reaction volume of 50µl each, containing 1x
reaction buffer (Promega), 1.5mM of $MgCl_2$, 100µM of each dNTP, 0.4µM of each primer,
0.5U of Taq DNA Polymerase (Promega). For the amplification of the *trn*L(UAA) intron and
the *trn*L(UAA) - *trn*F(GAA) intergenic spacer, 40µM of each dNTP and 0.3µM of each
primer were used instead. PCR amplifications were performed on a Perkin Elmer GeneAmp

PCR System 2400 or a MJ Research PTC-100[TM] thermal cycler. PCR products were purified either with the JETquick PCR purification spin kit (Genomed) or the QIAquick PCR purification kit (Qiagen). Cycle-sequencing was done with AmpliTaq DNA Polymerase FS and fluorescent labelled dNTP's according to the supplier's recommendations (Perkin-Elmer 1995). Sequences were analysed on an ABI PRISM[TM]310 Genetic Analyser (Perkin Elmer). Sequences were then corrected and aligned using Sequence Navigator PPC 1.0.2b3 (Applied Biosystems). Sequences are deposited in GenBank (Accession nos. AF180529-AF180559).

Phylogenetic analyses based on the principle of maximum parsimony were carried out using PAUP* 4.0b2 (Swofford 1999). All characters were treated as unordered and weighted equally. One-base pair (bp) indels were treated as binary presence/absence characters. Longer indels were each considered as one independent evolutionary event and were each treated as one polymorphic position. *V. cruciatum*, which occurs in southern Spain, Northern Africa and Israel, was chosen as outgroup. *V. album* and *V. cruciatum* are closely related (Barlow *et al.* 1978), but they are morphologically different (v. Tubeuf 1923).

Sequences from the different cpDNA fragments were combined for phylogenetic analyses. Nuclear rDNA sequences were analysed separately from cpDNA sequences. To test for incongruence between cpDNA and ITS sequences, we used the incongruence length difference (ILD) test (Farris *et al.* 1995) with 100 replicates. Constant characters were excluded (Cunningham 1997).

Results and Discussion

The lengths of the three cpDNA fragments and of the nuclear ITS sequences after alignment, and the types and numbers of mutations are given in Table 2. Insertions and deletions (indels) varying in length between 1 bp and 10 bp (within the ingroup) and with a length of up to 28bp (between in- and outgroup) were found in the cpDNA fragments. Indels are a typical feature of non-coding cpDNA fragments (van Ham *et al.* 1994). Phylogenetic analysis of ITS sequences, using the exhaustive search option, yielded the single most parsimonious tree shown in Fig. 1a (CI = 0.99, RI = 0.67). Only three out of 87 variable characters were phylogenetically informative. However, although sequence variation was low, ITS sequences grouped individual *V. album* isolates according to their presumed host races. No genetic variation was found within the host races, despite the large geographical area sampled.

Table 2 Number of characters of studied DNA fragments after alignment and the numbers and types of mutations

Fragment	Length (characters)	Ingroup		Outgroup	
		No. of substitutions	No. of indels	No. of substitutions	No. of indels
ITS	668	5	-	75	5
*trn*L-*trn*F	913	-	2	24	10
*trn*H-*trn*K	557	4	3	52	11
*trn*S-*trn*fM	361	6	2	12	4

CpDNA sequences were more variable than ITS sequences. Eight cpDNA haplotypes were found. Parsimony analyses of cpDNA sequences resulted in three shortest trees (CI = 0.96, RI = 0.79). Eleven out of 132 variable positions were phylogenetically informative. All trees identified *V. a. abietis* as sister lineage to the other two host races, *V. a. album* and *V. a. austriacum*, respectively. The topology of the bootstrap 50% majority rule consensus tree (Fig. 1b) is identical with one of the shortest trees. CpDNA sequences also supported the distinction of three lineages and are thus largely congruent with results based on ITS sequences.
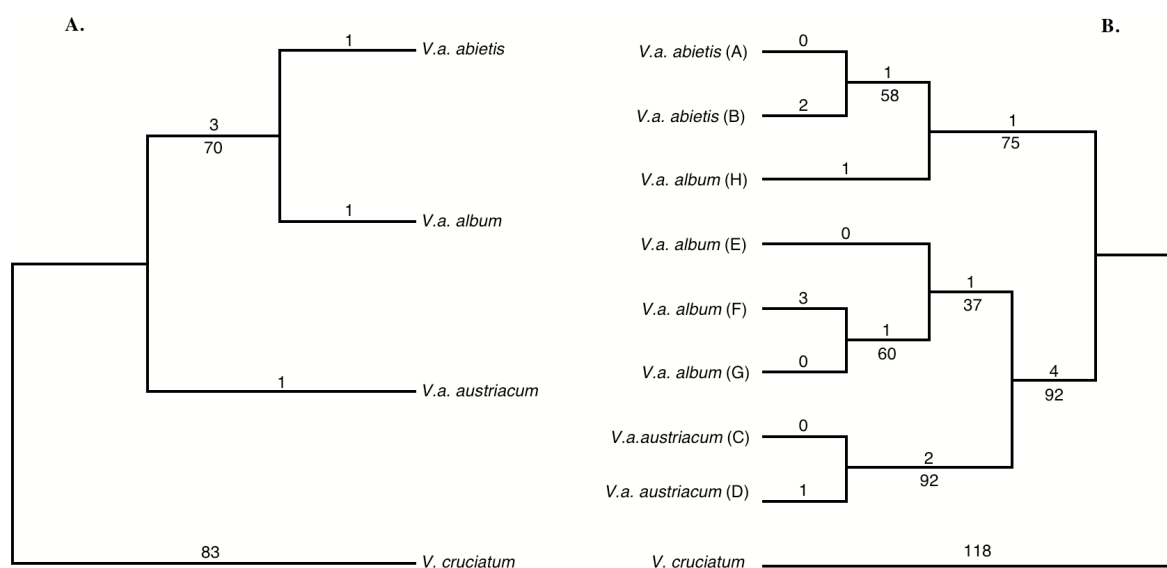
Fig.1 Maximum parsimony trees based on ITS sequences (a) and cpDNA sequences (b). Numbers above branches represent the number of mutations. Numbers below branches are bootstrap values. Letters in parentheses indicate cpDNA haplotypes

Nuclear and cpDNA sequence variation supported the distinction of three host races. We interpret the small genetic distances among host races as evidence for their recent formation. An alternative scenario, where gene flow occurs frequently among the presumed host races,

and genetic distances are therefore small among host races, is not supported by our results because *V. album* isolates from a given host show either identical or very closely related haplotypes.

Interestingly, however, the cpDNA haplotype found in one individual collected from a deciduous tree (Haplotype H) groups with samples collected from fir (Fig. 1b), while its ITS genotype is identical to that found in mistletoes from deciduous trees (Fig. 1a). We suggest that this individual represents a hybrid derivative between the host races from fir and deciduous trees. ITS sequences obtained directly from the PCR product did not show ambiguities at sites polymorphic between host races from fir and deciduous trees, suggesting the absence of ITS additivity. This may either be due to the loss of the fir host race ITS sequence in this hybrid derivative or through under-representation of the fir host race ITS sequences in the PCR product despite its presence in the genome. In either case, however, it suggests that hybridization is possible in natural populations, but further investigations are necessary to determine how frequently hybridization occurs.

Our presumably closely related outgroup species, *V. cruciatum,* which Meyer v. Freyhold (1987) described as a temperature-conditioned modification of *V. album,* turned out to be genetically clearly distinct from *V. album*.

The incongruence length difference test between cpDNA and nrDNA sequences showed that they were not incongruent (P = 0.35). An analysis of combined sequences resulted in two equally parsimonious trees, which both recognise *V. a. austriacum* as sister-lineage to the other two host races, thus supporting the topology already found in the analysis of ITS sequences. These results suggest that *V. a. album* and *V. a. abietis* are more closely related to each other than to *V. a. austriacum* and that hybridization is more likely between *V. a. album* and *V. a. abietis*. Indeed, the only putative hybrid derivative found so far is the result of a cross between *V. a. album* and *V. a. abietis*. However, more data are needed to assess the frequency of hybridization, especially in areas where host-trees grow intermingled and *V. album* host-races are thus not separated geographically

Acknowledgements

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CHAPTER III

Phylogeography and host race differentiation in the European mistletoe (*Viscum album* L.)

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Abstract

Mistletoes are bird dispersed, hemi-parasitic shrubs infecting a large number of woody host plants. Chloroplast fragment length polymorphisms were used to study genetic differentiation among presumed host races, population genetic structure, and to elucidate the postglacial migration history of mistletoe (*Viscum album*) across the entire natural distribution range in Europe. The populations sampled belong to four closely related taxa, three of which are widely distributed and differ in their host trees, whereas a fourth taxon is rare and endemic to the Island of Crete. The molecular analysis of cpDNA variation supported the distinction of these four taxa. We further found evidence for phylogeographic structure in each of the three widely distributed host races. Independent of host race, mistletoe haplotypes from Turkey were distinct and distant from those found elsewhere in Europe, suggesting that highly differentiated populations, and possibly new taxa, exist at the range limit of the species.

Keywords: chloroplast DNA, host race, parasitic angiosperm, *Pinus*, *Abies*

Introduction

The postglacial history of a large number of animal and plant species has been investigated in recent years using cytoplasmic genetic markers (Taberlet *et al.* 1998; Hewitt 1999; Petit *et al.* 2003). Investigations on numerous European tree and shrub species confirmed that chloroplast DNA markers are useful to unravel the main migration routes after the last glaciation (Petit *et al.* 2005b; Gugerli *et al.* 2005). In Europe, the reconstructed recolonization routes of several woody plant species, including *Quercus* sp. (Petit *et al.* 2002a), *Populus nigra* (Cottrell *et al.* 2005), *Fraxinus excelsior* (Heuertz *et al.* 2004), *Abies alba* (Konnert & Bergman 1995; Vendramin *et al.* 1999), or *Hedera* sp. (Grivet & Petit 2002) fit well the classic model of colonization of Europe described by Hewitt (1999). This model postulated major glacial refugia in the peninsulas of Iberia, Italy, and the Balkans. Additional refugia may be found in Turkey and regions further east, such as the Black Sea and the Caspian Sea (Hewitt 1999).

Not all species, however, revealed this classic phylogeographic pattern. For some of the investigated species, only a subset of the potential refugial areas may have served as important refugia. For *Corylus avellana* (Palmé & Vendramin 2002) and *Carpinus betulus* (Grivet & Petit 2003), for example, the Iberian peninsula may not have been an important refugial area, possibly because this area was too arid and the mountain massifs too small for many tree species (Bennett *et al.* 1991). Furthermore, some tree and shrub species may have survived in additional refugia further north, including *Betula pendula* (Palmé *et al.* 2003b), *Pinus sylvestris* (Sinclair *et al.* 1999; Soranzo *et al.* 2000), *Picea abies* (Sperisen *et al.* 1998) or *Tilia cordata* (Fineschi *et al.* 2003).

Viscum album, the European mistletoe, is a dioecious, insect pollinated plant with fleshy fruits that are bird-dispersed. It is a hemi-parasitic shrub that lives on woody plants. As a consequence, its distribution range is directly related to the availability of suitable host trees. *Viscum album* must therefore have been restricted to the refugial areas of its hosts during glacial periods. *V. album* is divided into four taxa that are assigned different taxonomic status, depending on author. Because no general agreement concerning their taxonomic status exists to date, we here use the term host race (Zuber 2004).

The mistletoe is an enigmatic species because of its value as medicinal plant (Luther & Becker 1987), but it also is an important pest species of trees (Janssen 2001; Hawksworth 1983). Information about the phylogeographic history of *V. album*, about genetic differentiation among host races, and about host specificity may therefore be of considerable

applied interest. Furthermore, our knowledge about the phylogeographic history of parasitic and hemiparasitic plant species is limited, and further case studies are needed to improve our understanding of the importance of mutualistic interactions for the recolonization history of species.

To examine postglacial colonization routes, genetic markers have been used together with fossil pollen data (Vendramin *et al.* 2000; Petit *et al.* 2002b; Demesure *et al.* 1996) mainly in wind-pollinated trees or shrubs (Demesure *et al.* 1996). Because *Viscum* is insect pollinated and a sparse pollen producer, its pollen record is of little value for reconstructing the phylogeographic history of the species. Furthermore, it is impossible to distinguish the pollen of the different *V. album* races. Fossil pollen maps only revealed a more northern distribution range of *Viscum* in the mid-Holocene (combined occurrences at 5000, 5500 or 6000 BP) (Huntley & Birks 1983). These authors explained the absence of *Viscum* pollen in western and southern Europe with cooler summers in the west and summer drought in the south at this time. As a consequence of the sparse pollen records, the study of the postglacial history of *Viscum* has to rely on genetic markers. The marker of choice for most phylogeographic studies in angiosperms is chloroplast DNA because it is non recombining and is in the majority of species maternally inherited. The smaller effective population size, compared to nuclear markers reduces the level of genetic diversity in the chloroplast genome and increases genetic differentiation among populations (Petit *et al.* 2005a). This effect is even more pronounced in dioecious species such as mistletoe, because the chloroplast genome has a four times smaller effective population size compared to nuclear genes. An efficient method to assess intraspecific variation is the analysis of cpDNA fragment length polymorphisms (Horning & Cronn 2006).

The aims of the present study were 1) to test whether host races of *V. album* correspond to differentiated gene pools, 2) to compare population genetic structure between host races in relation to host diversity and distribution, and 3) to assess whether the population genetic structure in these host races contains a phylogeographic signal that allows to identify possible glacial refugia and postglacial recolonization routes.

Material and methods

Study organisms

The division of *Viscum album* into host races has originally been proposed based on slight morphological differences (Grazi & Urech 1981) and has later been confirmed by sequencing segments of the nuclear ribosomal DNA (ITS) and non-coding cpDNA (Zuber & Widmer 2000). Three host races differ in their host specificity: *V. album album* grows on a wide variety of deciduous trees, *V. a. abietis* is restricted to fir (*Abies* spp.), and *V. a. austriacum* occurs mainly on pine (*Pinus* spp.). The fourth host race, *V. a. creticum*, is associated with a sole pine host, *Pinus halepensis* ssp. *brutia,* and occurs exclusively on the island of Crete (Zuber & Widmer 2000; Böhling *et al.* 2002).

Sampling

Leaf samples of 82 *V. album* populations comprising of 3-10 individuals were collected, covering the natural range of the species in Europe (Table 1). All individuals were classified on the basis of their host-trees. Based on this criterion, forty-five populations were assigned to *V. a. album*, 19 to *V. a. abietis*, 18 to *V. a. austriacum*, and one to *V. a. creticum*. Leaf material was dried and stored in silica gel.

Table 1 Sampling locations and hosts of *Viscum album*. The longitude and latitude given does not in all cases correspond exactly to the sampling site but to a nearby town.

V.a.abietis hosts	Code	Site	Country	Coordinates	
Abies alba	Sui1	Flüelen	Switzerland	46°54N	8°38E
Abies alba	Sui2	Forch	Switzerland	47°23N	8°33E
Abies alba	Cro1	Skrad	Croatia	45°25N	14°54E
Abies alba	Ger1	Nagold	Germany	48°33N	8°44E
Abies alba	Ger2	Öschelbronn	Germany	48°54N	8°49E
Abies alba	Ger3	Sulz	Germany	48°21N	8°38E
Abies alba	Fra1	St. Germain de Joux	France	46°11N	5°44E
Abies cephalonica	Gre1	Athen/Parnitha	Greece	38°10N	22°41E
Abies alba	Gre2	Parnaß	Greece	38°31N	22°37E
Abies nordmanniana	Aut1	Flysch/Wienerwald	Austria	48°10N	16°10E
Abies alba	Pol1	Brzesko	Poland	49°59N	20°34E
Abies alba	Svk1	Banská Štiavnica	Slovakia	48°27N	18°53E
Abies alba	Svk2	Staré Hory	Slovakia	48°51N	19°07E
Abies alba	Svk3	Zvolen	Slovakia	48°39N	19°02E
Abies alba	Svk4	Bardejov	Slovakia	49°20N	21°17E
Abies alba	Rom	Gura Humorului	Romania	47°31N	25°57E
Abies alba	Esp1	Viella	Spain	42°46N	0°39E
Abies bornmuelleriana	Tur1	Yenice	Turkey	41°08N	32°22E
Abies cilicica	Tur2	Yeniköy	Turkey	37°31N	36°43E

V.a.austriacum hosts	Code	Site	Country	Coordinates	
Pinus sylvestris	Sui9	Flüelen	Switzerland	46°54N	8°38E
Pinus sylvestris	Sui10	Lanquart	Switzerland	46°58N	9°34E
Pinus sylvestris	Sui11	Martigny	Switzerland	46°06N	7°08E
Pinus sylvestris	Cze	Zbraslav	Czech Republic	50°02N	14°23E
Pinus sylvestris	Ger12	Berlin/Dammendorf	Germany	52°08N	14°25E
Pinus pinaster	Esp5	Avila-Las Navas del Marques	Spain	40°35N	4°27W
Pinus sp	Esp6	Cazorla	Spain	37°55N	3°00W
Pinus pinaster	Esp7	Cebreros-Robledo de Chavela	Spain	40°28N	4°20W
Pinus sylvestris	Esp8	Fuente de la Gitana	Spain	41°50N	3°02W
Pinus sp	Fra8	Comps sur Artuby	France	43°43N	6°31E
Pinus nigra	Fra9	Corsica/Col de Bavella	France	41°48N	9°15E
Pinus sylvestris	Aut4	Gutenstein	Austria	47°52N	15°52E
Pinus sylvestris	Pol4	Jesiona	Poland	52°00N	15°59E
Pinus sylvestris	Svk6	Malacky	Slovakia	48°25N	17°08E
Pinus nigra	Tur6	Andirin	Turkey	37°30N	36°20E
Pinus nigra	Tur7	Karabük/Safranbolu	Turkey	41°16N	32°42E
Pinus sylvestris	Tur8	Bolu- Mengen	Turkey	40°56N	32°13E
V.a.creticum host					
Pinus halepensis subsp. *brutia*	Gre3	Crete/Thripti	Greece	35°03N	25°36E

V.a.album hosts	Code	Site	Country	Coordinates	
various	Bul	Nessebar	Bulgaria	42°40N	27°43E
various	Sui3	Flüelen	Switzerland	46°54N	8°38E
Malus sp.	Sui4	Jegenstorf	Switzerland	47°03N	7°31E
various	Sui5	Martigny	Switzerland	46°08N	7°04E
Populus sp.	Sui6	Riveo/Maggia	Switzerland	46°15N	8°42E
various	Sui7	Weesen	Switzerland	47°07N	9°05E
Quercus robur	Sui8	Zumikon	Switzerland	47°19N	8°37E
various	Cro2	Zagreb	Croatia	45°48N	15°58E
various	Ger4	Darmstadt/Erfelden	Germany	49°50N	8°30E
Crataegus sp.	Ger5	Dresden	Germany	51°03N	13°45E
Malus sp.	Ger6	Göttingen	Germany	51°32N	9°57E
various	Ger7	Hannover	Germany	52°23N	9°42 E
Populus sp.	Ger8	Hildesheim /Marienburg	Germany	52°05N	10°07E
various	Ger9	München/Nymphenburg	Germany	48°9N	11°30E
various	Ger10	Potsdam/Belzig	Germany	52°08N	12°35E
Tilia cordata	Ger11	Tübingen	Germany	48°34N	9°07E
Populus nigra s.l.	Esp2	Burgos	Spain	42°21N	3°41W
Populus nigra s.l.	Esp3	Pola de Siero	Spain	43°24N	5°39W
Tilia cordata	Esp4	Poncebos-Sotres	Spain	43°47N	4°15W
various	Fra2	Monieux	France	44°02N	5°21E
Robinia pseudoacacia	Fra3	Luberon/Saune	France	43°44N	5°31E
Populus sp.	Fra4	Paris	France	48°52N	2°20E
Populus nigra s.l.	Fra5	Druillat-Pont d Ain	France	46°03N	5°20E
various	Fra6	Riez	France	43°49N	6°06E
various	Fra7	Sologne	France	47°33N	1°48E
various	Gbr	Oxford	Great Britain	51°46N	1°15W
Castanea sativa	Ita1	Calabrien/Piano Lago	Italy	39°11N	16°10E
Quercus sp.	Ita2	Castrovillari/Cosenza	Italy	39°48N	16°12E
various	Ltu	Kaunas	Lithuania	54°50N	24°02E
Malus sp.	Aut2	Fußach/Bodensee	Austria	47°28N	9°40E
Acer sp.	Aut3	Wien/Schönbrunn	Austria	48°10N	16°18E
Tilia cordata	Pol2	Kornik	Poland	52°15N	17°05E
Populus sp	Pol3	Okalina	Poland	50°49N	21°25E
Malus sp.	Svk4	Sebechleby	Slovakia	48°17N	18°55E
Populus nigra	Svk5	Viničky	Slovakia	48°24N	21°45E
Tilia cordata	Swe	Västerås/Tidö	Sweden	59°36N	16°32E
Amygdalus communis	Tur3	Atabey	Turkey	37°57N	30°38E
Pyrus communis	Tur4	Karabük/Safranbolu	Turkey	41°16N	32°42E
Pyrus eleagnifolia	Tur5	Kastamonu	Turkey	41°22N	33°45E
various	Ukr1	Lviv	Ukraine	49°50N	24°00E
various	Ukr2	Znesinnya Natural Park	Ukraine	49°50N	24°00E
various	Hun1	Esztergom	Hungary	47°46N	18°42E
Populus sp.	Hun2	Balatonszentgyörgy	Hungary	46°42N	17°17E
Populus sp.	Hun3	Nagykanizsa	Hungary	46°23N	17°04E
various	USA	Sebastopol	USA	38°23N	122°50W

DNA extraction, amplification, fragment analysis

Total DNA was extracted from 20mg silica gel-dried leaf material using the adapted CTAB method of Doyle & Doyle (1987). A subset of 24 individuals covering all host races and the geographic area sampled were sequenced with five chloroplast primer pairs to find polymorphisms: *trn*C-*trn*D, *trn*H-*trn*K, *trn*S-*trnfM* (Demesure *et al.* 1995), cf (Taberlet *et al.* 1991) and *ccmp4* (Weising & Gardner 1999). Three fragments, *trn*C-*trn*D, *trn*H-*trn*K and *ccmp4*, revealed overall 40 length polymorphisms (Table 2). To discriminate the detected cpDNA haplotypes based on fragment length polymorphisms, we developed three new primer pairs. Fluorescently 5`labelled (two FAM and one HEX) internal primers were designed to visualise fragments on ABI PRISM^TM 310 or 3130xl Genetic Analysers (Applied Biosystems,

Table 2 Summary of polymorphisms found in three cpDNA fragments of *Viscum album*.

	ccmp4	*trn*C-*trn*D	*trn*H-*trn*K	all
Indels	19	3	16	38
SNP	1	1	6	8
mikrosat.	1	1	-	2

Foster City, CA, USA). Primers are given in Table 3. Polymerase chain reactions (PCR) for the three fragments were multiplexed. The reaction was performed in 10μl volumes consisting of 0.5U *Taq* DNA Polymerase (Eppendorf), 1x reaction buffer, 1.5mM of MgCl$_2$, 200μM of each dNTP, 0.2μM of each of the six primers. PCR amplifications were performed on a

Table 3 Description of the primer pairs used for fragment length amplification.

Fragment	Forward primer labelled with	Internal primer
*trn*C-*trn*D	FAM	5´TTTGTTTTCTACTGCAACTATCCCG 3´
		5´AAAGCAGCCCAAGTGAAACTGAC 3´
*trn*H-*trn*K	FAM	5´CGGGAATTGAACCCGCG 3`
		5´CGTAATGCTCATAACTTTCCTCTAGAC 3´
ccmp4	HEX	5`CCAAAATATTBGGAGGACTC 3`
		5`GTGGTTTGAAATCCAGATGC 3`

Perkin Elmer GeneAmp PCR System 2400 or 9700 and the fragment sizes were analysed using GENOTYPER 2.1. New haplotypes were sequenced with internal unlabelled primers. The sequences have been deposited in GenBank (accession numbers EU651900-EU651992; Table 4).

Gene differentiation across all populations (G_{ST}) was computed using the program HAPLONST according to Pons & Petit (1995). To estimate the partitioning of haplotype variation within and among populations, analyses of molecular variance (AMOVA) were calculated with ARLEQUIN ver. 3.01 (Excoffier *et al.* 2005). According to Pons & Petit (1996), significantly higher values for N_{ST} than for G_{ST} indicate the existence of a phylogeographic structure. To test whether N_{ST} is significantly larger than G_{ST}, the program PERMUT was used with one thousand random permutations. CLUSTALW version 1.7 (Thompson *et al.* 1994) was used to determine sequence alignments. Haplotype networks were constructed using TCS1.21 (Clement *et al.* 2000).

Results

The three sequenced cpDNA fragments (*trn*C-*trn*D, *trn*H-*trn*K and *ccmp4*) revealed overall 48 polymorphisms. Of these, 38 were due to length variants, eight corresponded to single nucleotide polymorphisms (SNPs), and two were microsatellites (Table 2). The combined analysis of the fragments length variants allowed to distinguish 31 different haplotypes (Table 4) among 734 individuals in 82 populations (Table 5). The great majority of cpDNA haplotypes was found only in mistletoes growing on the same host tree group (fir, pine or deciduous trees), indicating that host races are genetically distinct and that mistletoes can in most cases be assigned to host races based on host tree identity. AMOVA indicated that 68% of the variation was distributed among the three host races, whereas 26% of the variation was found among populations within the three host races, and only 6% of the total variation was found within populations (Table 6). Because of the clear differentiation of the host races, the geographic distribution of haplotypes and haplotype networks are illustrated separately for each host race (Fig. 1a,b,c; Fig. 2a,b,c).

Table 4 Characteristics of the haplotypes detected with three combined polymorphic chloroplast DNA fragments.

Haplotype	ccmp4		CD		HK
V.a.abietis					
A1	0 1 1 1 1 1 1 0	10 1 1 1 0 1 1 0 1 0 0	0 0	9 0	0 1 1 0 1 0 1 0 1 0 0 0
A2	0 1 1 1 1 1 1 0	10 1 1 1 0 1 1 0 1 0 0	0 0	8 0	0 1 1 0 1 0 1 0 1 0 0 0
A3	0 1 1 1 1 1 1 0	9 1 1 1 0 1 1 0 1 0 0	0 0	9 0	0 1 1 0 1 0 1 0 1 0 0 0
A4	0 1 1 1 1 1 1 0	10 1 0 1 0 1 1 0 1 0 0	0 0	9 0	0 1 1 0 1 0 1 0 1 0 0 0
A5	0 1 0 0 0 0 0 0	10 1 0 1 0 1 1 0 1 0 0	0 0	8 0	0 1 1 0 1 0 1 0 1 0 0 0
A6	0 1 1 1 1 1 1 0	10 1 1 1 0 1 1 0 1 0 0	0 0	8 0	0 1 1 0 1 0 1 0 1 0 0 0
A7	0 1 0 0 0 0 0 0	10 1 0 1 1 1 0 0 1 0 0	0 0	9 0	0 1 1 0 1 0 1 0 1 0 0 0
A8	0 0 1 0 0 0 0 0	10 0 0 0 0 0 0 1 0 1 0	0 1	8 0	0 1 0 1 0 1 1 1 0 1 1 1
V.a.austriacum					
P1	0 0 0 0 0 0 0 0	11 1 1 0 1 0 1 1 0 1 0	0 0	7 0	0 1 0 1 0 0 0 1 1 0 0 0
P2	0 1 0 0 0 0 0 0	11 1 1 0 1 0 1 1 0 1 1	0 0	7 0	0 0 1 0 0 0 1 0 1 0 0 0
P3	0 1 0 0 0 0 0 0	11 1 1 0 1 0 1 1 0 1 0	0 0	7 0	1 1 0 1 0 0 0 1 1 0 0 0
P4	0 1 0 0 0 0 0 0	11 1 1 0 1 1 1 1 0 1 0	0 0	7 0	0 1 1 0 1 0 0 0 1 1 0 0
P5	0 1 0 0 0 0 0 0	11 1 1 0 1 0 1 1 0 1 0	0 0	8 0	0 1 0 1 0 0 0 1 1 0 0 0
P6	0 0 0 0 0 0 0 0	12 1 1 0 1 0 1 1 0 1 0	0 0	7 0	0 1 0 1 0 0 0 1 1 0 0 0
P7	0 1 0 0 0 0 0 0	11 1 1 0 1 0 1 1 0 1 0	0 0	8 0	0 1 1 0 1 0 0 0 1 1 0 0
P8	0 1 0 0 0 0 0 0	11 1 1 0 1 0 1 1 0 1 0	0 0	8 0	0 1 0 1 0 0 1 0 1 0 0 0
P9	0 1 1 1 1 1 1 1	11 1 1 0 1 0 1 1 1 0 0	0 0	8 0	0 1 1 0 1 0 0 0 1 1 0 0
P10	0 1 1 1 1 1 1 1	11 1 1 0 1 1 0 1 1 0 0	0 0	8 0	0 1 1 0 1 0 0 0 1 1 0 0
V.a.album					
H1	0 1 0 0 0 0 0 0	9 1 0 1 0 1 1 0 1 0 1	0 0	8 0	0 1 0 1 0 0 0 1 1 0 0 0
H2	0 1 0 0 0 0 0 0	9 1 0 1 0 1 1 0 1 0 1	0 0	7 0	0 1 0 1 0 0 0 1 1 0 0 0
H3	0 1 1 0 0 0 0 0	9 1 0 1 0 1 1 0 0 0 1	0 0	8 0	0 1 0 1 0 0 0 1 1 0 0 0
H4	1 1 0 0 0 0 0 0	9 1 0 1 0 1 1 0 1 0 1	0 0	8 0	0 1 0 1 0 0 0 1 1 0 0 0
H5	0 1 1 1 0 0 0 0	10 1 0 1 0 1 1 0 1 0 1	0 0	7 0	0 1 0 1 0 0 0 1 1 0 0 0
H6	0 1 0 0 0 0 0 0	9 1 0 1 0 1 1 0 1 0 1	0 0	8 0	0 1 0 1 0 0 1 0 1 0 1 1
H7	0 1 0 0 0 0 0 0	9 1 0 1 0 1 1 0 1 0 1	0 0	8 0	0 1 0 1 0 0 0 1 1 0 0 0
H8	0 1 1 1 0 0 0 0	9 1 0 1 0 1 1 0 1 0 1	0 0	8 1	0 1 0 1 0 0 0 0 1 0 0 0
H9	0 1 1 0 1 1 0 0	9 1 0 1 0 1 1 0 1 1 1	0 0	8 0	0 1 0 1 0 0 0 1 1 0 0 0
H10	0 1 1 1 1 1 1 1	10 1 0 1 0 1 0 1 1 1 0	0 0	7 0	0 1 0 1 0 0 0 1 1 0 0 0

Table 5 Details on sample size, host race, location, and number of individuals per haplotype detected.

V.a.abietis populations	Code	Sample size	Haplotypes								
			A1	A2	A3	A4	A5	A6	A7	A8	H1
Flüelen (SUI)	Sui1	10	10								
Forch (SUI)	Sui2	10	10								
Skrad (CRO)	Cro1	10	8	1	1						
Nagold (GER)	Ger1	10	10								
Öschelbronn (GER)	Ger2	5	4			1					
Sulz (GER)	Ger3	8	8								
St. G. de Joux (FRA)	Fra1	10	10								
Athen/Parnitha (GRE)	Gre1	7					7				
Parnaß (GRE))	Gre2	5						5			
Flysch (AUT)	Aut1	10	10								
Brzesko (POL)	Pol1	3	3								
Banská Štiavnica (SVK)	Svk1	10	8						2		
Staré Hory (SVK)	Svk2	7	6			1					
Zvolen (SVK)	Svk3	10	10								
Bardějov (SVK)	Svk4	8	8								
Gura Humorului (ROM)	Rom	10	10								
Viella (ESP)	Esp1	10	10								
Yenice (TUR)	Tur1	6								5	1
Yeniköy (TUR)	Tur2	7								7	

			Haplotypes										
V.a.austriacum populations	Code	Sample size	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	A1
Flüelen (SUI)	Sui9	10		10									
Lanquart (SUI)	Sui10	10	10										
Martigny (SUI)	Sui11	10	9		1								
Zbraslav (CZE)	Cze	5	5										
Berlin (GER)	Ger12	10	10										
Avila (ESP)	Esp5	10				10							
Cazorla (ESP)	Esp6	10		10									
Cebreros-R. de Ch. (ESP)	Esp7	10				10							
Fuente de la Gitana (ESP)	Esp8	10	7				3						
Comps sur Artuby (FRA)	Fra8	10			10								
Corsica (FRA)	Fra9	5					5						
Gutenstein (AUT)	Aut4	10	9										1
Jesiona (POL)	Pol4	10	10										
Malacky (SVK)	Svk6	10	9					1					
Andirin (TUR)	Tur6	10							8	2			
Karabük/Safranbolu (TUR)	Tur7	9								9			
Bolu- Mengen (TUR)	Tur8	10								10			
V.a.creticum													
Crete (GRE)	Gre3	10									6	4	

V.a.album populations	Code	Sample size	Haplotypes													A1	P1
			H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	A1	P1
Nessebar (BUL)	Bul	10	10														
Flüelen (SUI)	Sui3	10	7													3	
Jegenstorf (SUI)	Sui4	10	10														
Martigny (SUI)	Sui5	9	3	4													2
Ticino (SUI)	Sui6	7	7														
Weesen (SUI)	Sui7	10	9													1	
Zumikon (SUI)	Sui8	10	10														
Zagreb (CRO)	Cro2	10	4		6												
Darmstadt/Erfelden (GER)	Ger4	9	7			2											
Dresden (GER)	Ger5	10	3				7										
Göttingen (GER)	Ger6	10	10														
Hannover (GER)	Ger7	10	10														
Hildesheim (GER)	Ger8	10	10														
München (GER)	Ger9	10	10														
Potsdam (GER)	Ger10	8	5														3
Tübingen (GER)	Ger11	10	10														
Burgos (ESP)	Esp2	10	10														
Pola de Siero (ESP)	Esp3	10	10														
Poncebos-Sotres (ESP)	Esp4	10	10														
Monieux (FRA)	Fra2	9	9														
Luberon/Saune (FRA)	Fra3	8	8														
Paris (FRA)	Fra4	10	10														
Pont d A in (FRA)	Fra5	10	10														
Riez (FRA)	Fra6	10	4	4				2									
Sologne (FRA)	Fra7	10	8						2								
Oxford (GBR)	Gbr	5	5														
Calabrien/P. Lago (ITA)	Ita1	7	1						3	3							
Castrovillari/Cosenza (ITA)	Ita2	6	2						2								2
Kaunas (LTU)	Ltu	8	7								1						
Fußach (AUT)	Aut2	9	9														
Wien (AUT)	Aut3	10	7		3												
Kornik (POL)	Pol2	10	5		5												
Okalina (POL)	Pol3	7	7														
Sebechleby (SVK)	Svk4	10			10												
Vinicky (SVK)	Svk5	10	8		1				1								
Västerås/Tidö (SWE)	Swe	9	9														
Atabey (TUR)	Tur3	10										10					
Karabük/Safranbolu (TUR)	Tur4	9										1	8				
Kastamonu (TUR)	Tur5	10										8	2				
Lviv (UKR)	Ukr1	9	6		3												
near Lviv (UKR)	Ukr2	8	7											1			
Esztergom (HUN)	Hun1	9	4	1	4												
Balatonszentgyörgy (HUN)	Hun2	7	7														
Nagykanizsa (HUN)	Hun3	10	1		7										2		
Sebastopol (USA)	USA	6	6														

Table 6 Analysis of molecular variance (AMOVA) of *Viscum album* genetic variation for the three host races (*V. a. creticum* in *V. a. austriacum*) and the total sample range. All fixation indices and variance components were significant.

	Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
V.a.abietis	Among populations	18	295.645	1.98386	90.88
	Within populations	137	27.290	0.19920	9.12
$F_{ST} = 0.91$	Total	155	322.936	2.18306	
V.a.austriacum	Among populations	17	405.164	2.52305	93.22
	Within populations	151	27.700	0.18344	6.78
$F_{ST} = 0.93$	Total	168	432.864	2.70649	
V.a.album	Among populations	44	351.335	0.83072	65.47
	Within populations	364	159.502	0.43819	34.53
$F_{ST} = 0.65$	Total	408	510.836	1.26892	
Overall	Among groups	2	1702.901	3.86017	68.42
	Among populations within groups	79	1052.144	1.45289	25.75
	Within populations	652	214.492	0.32898	5.83
$F_{SC} = 0.82$ $F_{ST} = 0.94$ $F_{CT} = 0.68$	Total	733	2969.537	5.64204	

Table 7 Gene diversity parameters estimated according to Pons & Petit (1995) with standard error in brackets.

Host race	*h*-type parameters		
	h_S	h_T	G_{ST}
V.a.abietis	0.092 (0.0367)	0.441 (0.1263)	0.791 (0.0957)
V.a.austriacum	0.109 (0.0419)	0.819 (0.0723)	0.867 (0.0524)
V.a.album	0.223 (0.0401)	0.467 (0.0713)	0.521 (0.0685)
Overall	0.168 (0.0260)	0.798 (0.0324)	0.790 (0.0324)
Host race	*v*-type parameters		
	v_S	v_T	v_S
V.a.abietis	0.054 (0.0348)	0.442 (0.1679)	0.054 (0.0348)
V.a.austriacum	0.080 (0.0332)	0.820 (0.1102)	0.080 (0.0332)
V.a.album	0.178 (0.0387)	0.468 (0.1116)	0.178 (0.0387)
Overall	0.090 (0.0173)	0.799 (0.0528)	0.090 (0.0173)

Viscum on *Abies* spp.

In a total of 156 individuals representing 19 populations, we found 9 haplotypes, including one haplotype common in mistletoe growing on deciduous trees (Table 5, Fig. 1a). Haplotype A1 is the most widespread type in central and western Europe. 11 populations were fixed for type A1. Haplotypes A2 and A3 were each found in a single individual from one population in Croatia. Haplotype A4 was found in two individuals, one from Germany (Ger2), one from Slovakia (Svk2). Haplotype A7 was found twice in a Slovakian population (Svk1). In southeastern Europe, two populations from Greece were monomorphic for haplotypes A5 and A6, whereas haplotype A8 was restricted to Turkey. The network shows that haplotype A8 from Turkey is highly differentiated from all others. It could not be connect to the network because of its high genetic divergence from other haplotypes (Fig. 2a). One sample collected on fir in Turkey carried haplotype H1, the most common haplotype found elsewhere in mistletoes growing on deciduous trees.

Gene differentiation across all *V. a. abietis* populations was high, with $G_{ST} = 0.791$ and $N_{ST} = 0.879$ (Table 7). The permutation test indicated that N_{ST} was significantly higher than G_{ST} ($P < 0.05$) indicating the presence of a phylogeographic structure for *V. a. abietis* haplotypes. The AMOVA further indicated that only 9% of variation was found within populations, whereas 91% of the variation was found among populations (Table 6).

Viscum on *Pinus* spp.

In a total of 169 individuals representing 18 populations we found 11 haplotypes, including haplotype A1 that was otherwise common in mistletoes growing on fir (Table 5, Fig. 1b). The most frequent haplotype P1 was found in central and northern Europe and southwards to northern Spain. Some haplotypes were restricted to southern Europe. In the southwest, haplotype P4 was found exclusively on the Iberian peninsula, whereas in the southeast haplotypes P8 and P7 were restricted to Turkey. Other southern haplotypes were more widely distributed. Haplotype P2 was found in two monomorphic populations, one from southern Spain (Esp6) and one from Switzerland (Sui9). Haplotype P5 is distributed in Spain and Corsica. Haplotype P3 from a monomorphic population in southern France was also found in one individual in Switzerland (Sui11). Haplotype P6 was rare and found only in one individual in Slovakia. In Austria, one individual with the main *V. a. abietis* haplotype A1 was found. The population from Crete, which belongs to *V. a. creticum*, showed two distinct haplotypes, P9 and P10, that were not found anywhere else. The network also shows *V. a.*
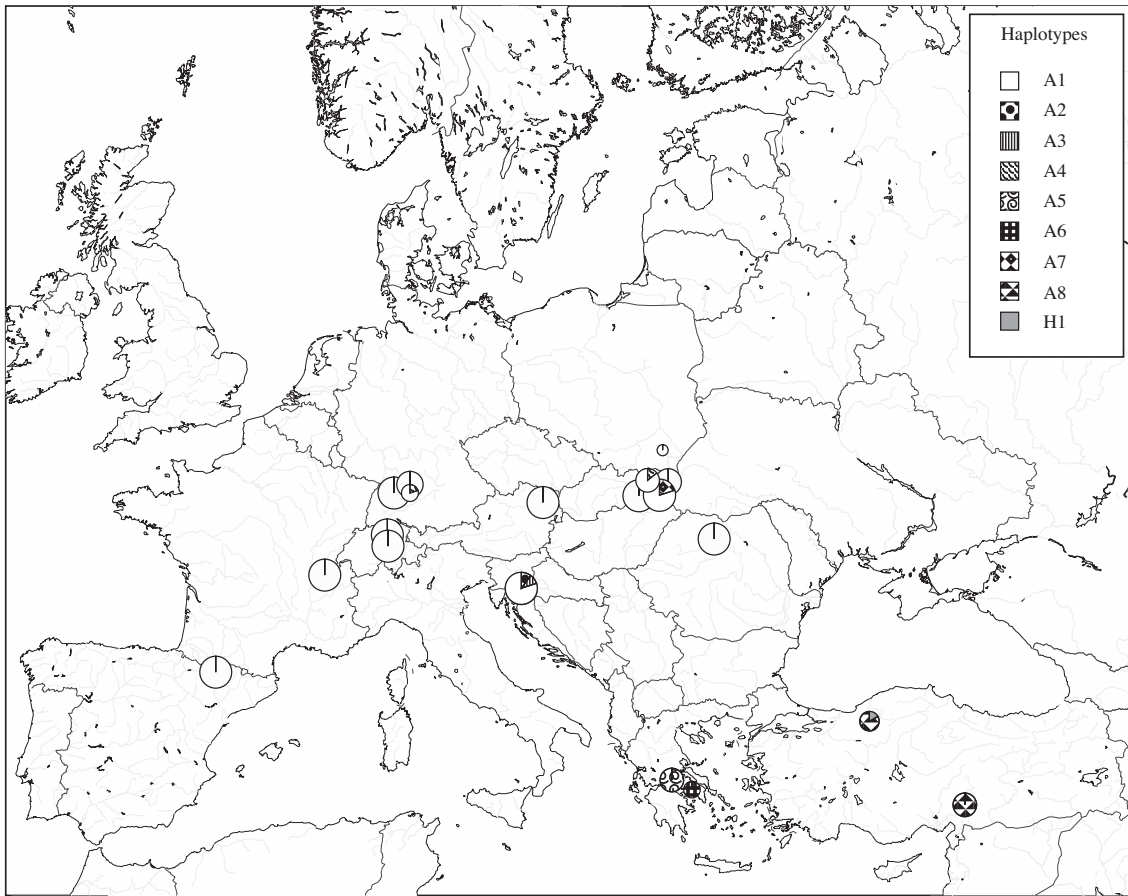
Fig. 1a Geographical distribution of *V. a. abietis* chloroplast haplotypes The size of the circles reflect, but are not totally proportional to the number of analysed samples per population (n = 3-10).
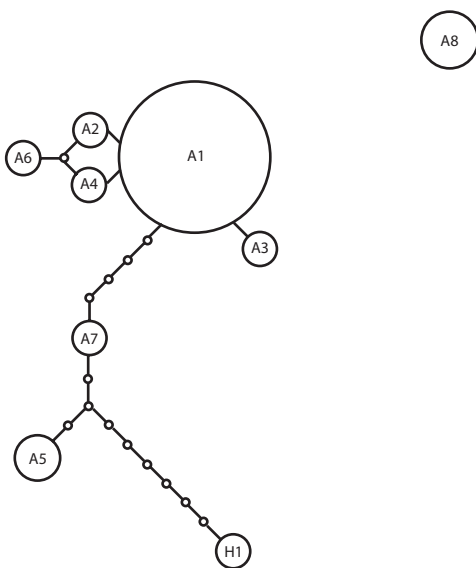

Fig. 2a Haplotype network based on the fragment length data: *V. a. abietis*. The size of the circles reflect, but are not totally proportional to the frequency of the haplotypes.
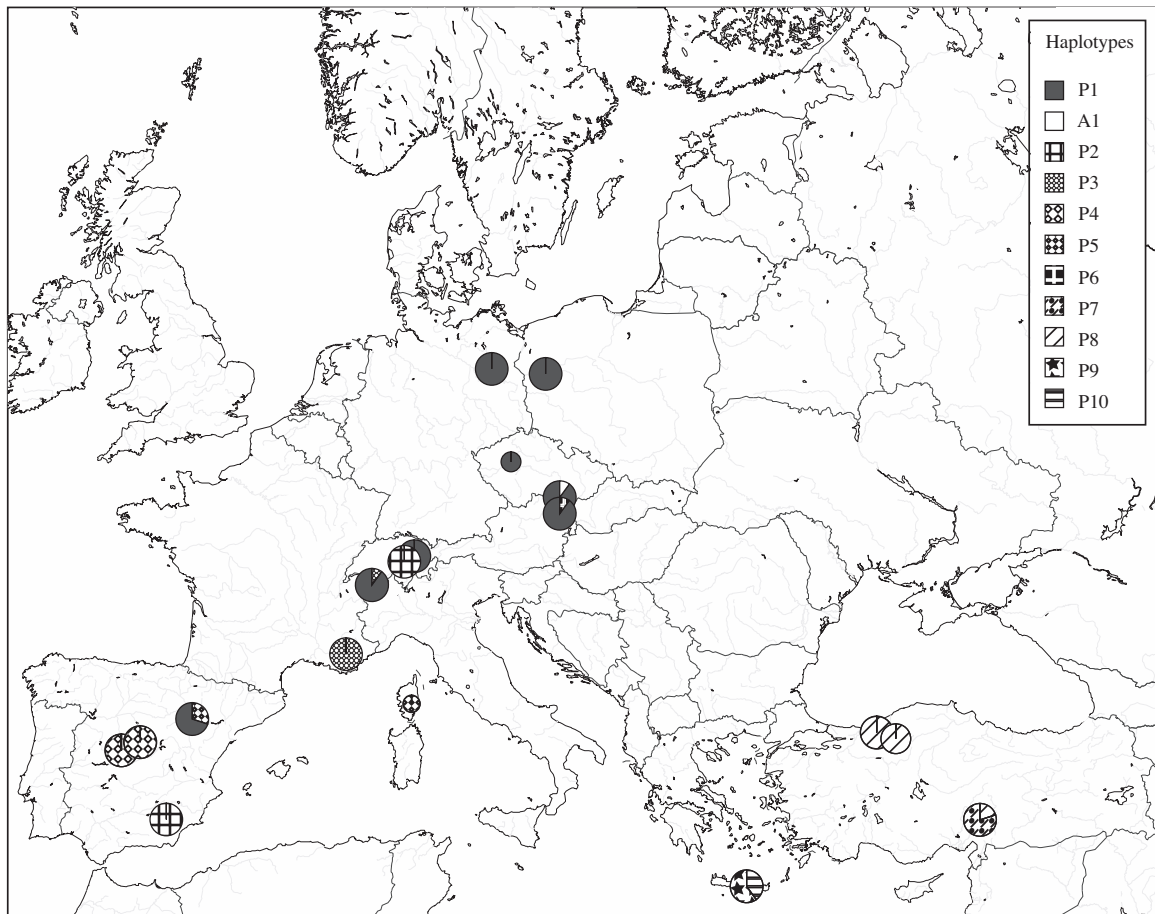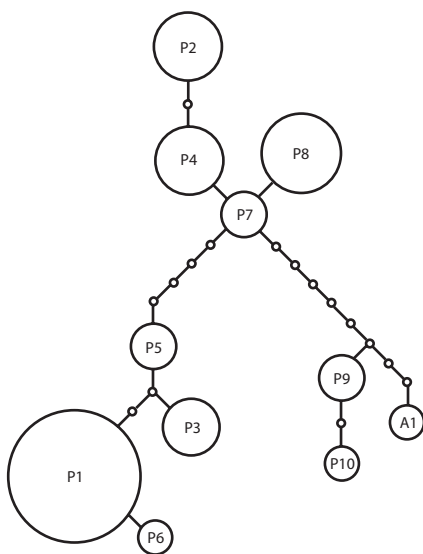
Fig. 1b Geographical distribution of *V. a. austriacum* chloroplast haplotypes The size of the circles reflect, but are not totally proportional to the number of analysed samples per population (n = 3-10).

Fig. 2b Haplotype network based on the fragment length data: *V. a. austriacum*. The size of the circles reflect, but are not totally proportional to the frequency of the haplotypes.

creticum separated from other *Viscum* haplotypes from pine and grouped instead together with *Viscum* haplotype A1, common in mistletoes from fir (Fig. 2b).

The G_{ST} value for *V. a. austriacum* was the highest found among the three widely distributed host races, with $G_{ST} = 0.867$ and $N_{ST} = 0.903$ (Table 7). The permutation test indicated that N_{ST} was significantly higher than G_{ST} ($P < 0.05$) indicating the presence of a phylogeographic structure for *V. a. austriacum* haplotypes. As in *V. a. abietis*, the variation in *V. a. austriacum* was high among populations (93%), and much lower within populations (7%) (Table 6).

Viscum on deciduous trees

In a total of 409 individuals representing 45 populations we found 15 haplotypes, including haplotype A1, common in mistletoes from fir, and haplotype P1, common in mistletoes from pines (Table 5, Fig. 1c). Haplotype H1 was the most widespread one, occurring across the entire surveyed part of the distribution range, with the notable exception of the southeast (Turkey). In one population from Slovakia (Svk4), H1 was absent. This population was monomorphic for haplotype H3, a less frequent haplotype occurring in the eastern part of Europe (Ukraine, Poland, Austria, Slovakia, Hungary, Croatia). Haplotypes H7 and H2 were the most widely distributed of the rare haplotypes. H7 was found in France, Italy and Slovakia, whereas H2 was found in France, Switzerland and Hungary. Haplotypes H10 and H11 were restricted to Turkey. The network shows that these Turkish haplotypes are separated from the other haplotypes common in *Viscum* from deciduous trees (Fig. 2c). The remaining haplotypes (H4, H5, H6, H8, H9, H12, H13) were rare and found in one single population each. While most of these types were found just in one or two (H8 in three) individuals per population, haplotype H5 (Ger5) was slightly more common and was found in seven individuals. Two populations from Switzerland contained in addition to haplotype H1 also haplotype A1 from *V. a. abietis*, and some individuals from Germany (Ger10), Italy (Ita2) and Switzerland (Sui5) carried haplotype P1 from *V. a. austriacum*. A population from North America, where *Viscum a. album* has been introduced, carried the most common haplotype H1.

Gene differentiation across the *V. a. album* populations ($G_{ST} = 0.521$, and $N_{ST} = 0.620$) was lower than among mistletoe populations growing on conifers (Table 7). Again, N_{ST} was significantly higher than G_{ST} ($P < 0.05$), indicating the presence of a phylogeographic
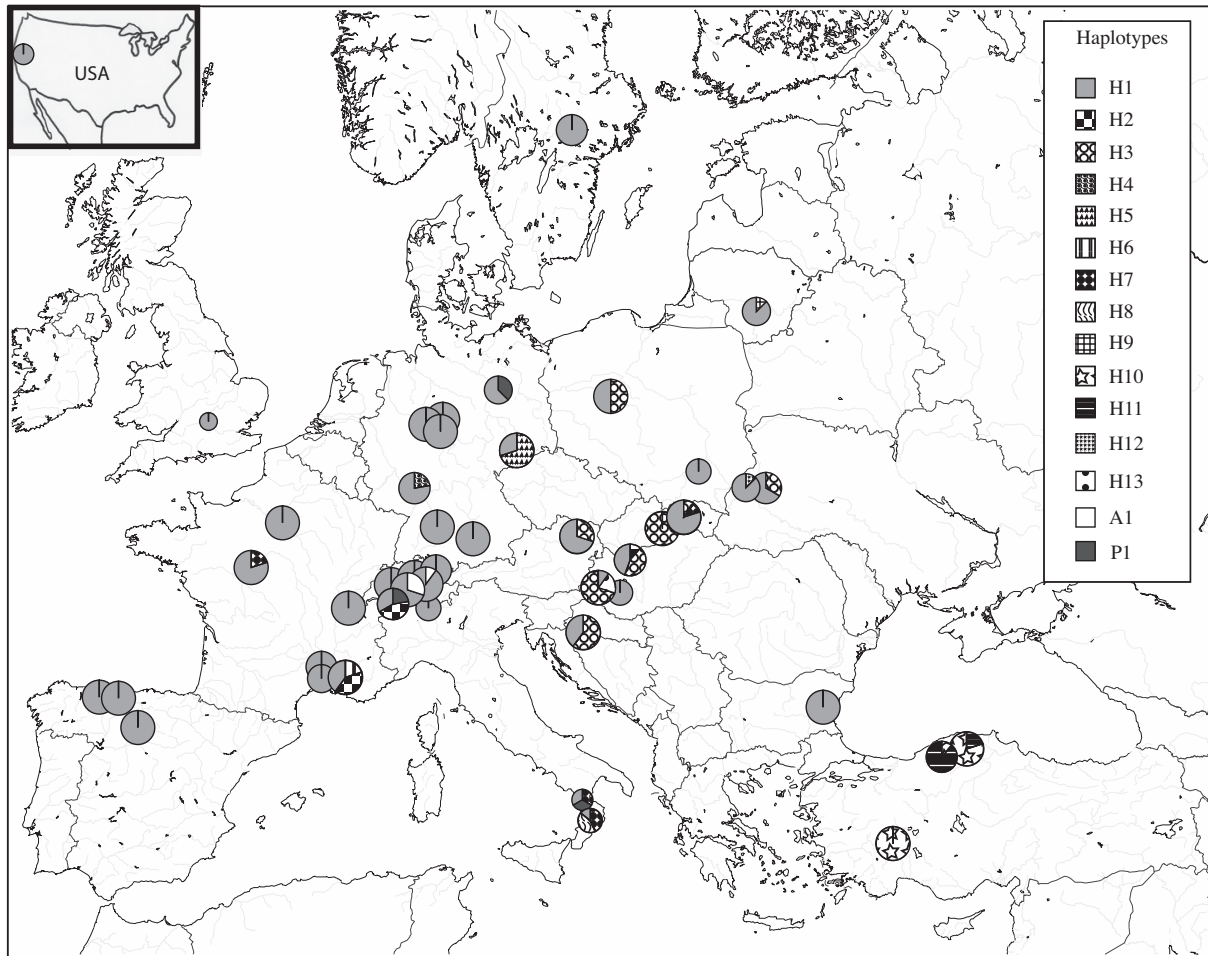
Fig. 1c Geographical distribution of *V. a. album* chloroplast haplotypes The size of the circles reflect, but are not totally proportional to the number of analysed samples per population (n = 3-10).
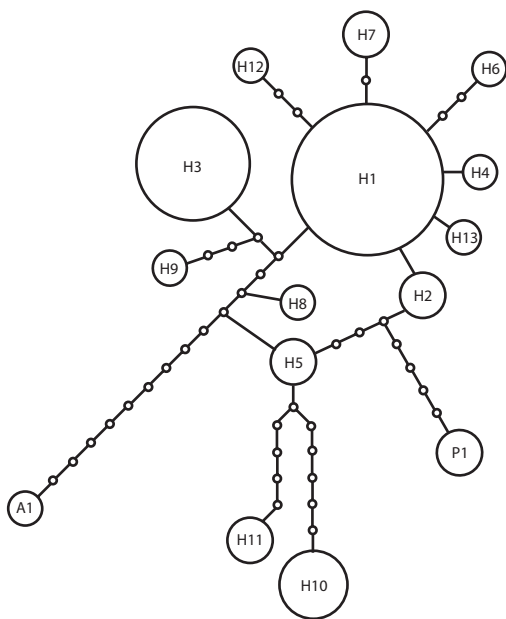

Fig. 2c Haplotype network based on the fragment length data: *V. a. album.* The size of the circles reflect, but are not totally proportional to the frequency of the haplotypes.

structure for *V. a. album* haplotypes. Variation among populations was less pronounced than in conifer mistletoes, with 65% of the variation occurring among populations, and 35% within populations (Table 6). Overall, variation within mistletoe populations from conifers was substantially lower (6-7%) than in *Viscum* populations growing on deciduous trees (35%).

Discussion

We found that the European mistletoe can be divided into four genetically well differentiated taxa that correspond to three widely distributed host races and one geographically highly restricted fourth host race that have previously been described as subspecies (Zuber & Widmer 2000). In addition to these known host races, other, probably new ones, may be found in Turkey, as indicated by the presence of highly divergent haplotypes in this area. The most common host race, growing on a wide range of deciduous trees, also harboured the most genetic variation and revealed the lowest genetic differentiation among populations. Chloroplast DNA variation within each of the three widely distributed host races revealed a phylogeographic signal.

The strong genetic differentiation among the mistletoe host races observed in the present study corroborates an earlier study based on much smaller sample sizes that suggested that host races are genetically well differentiated from each other (Zuber & Widmer 2000). Our analysis may underestimate host race differentiation, because it includes individuals that carry haplotypes otherwise found commonly in plants of other host races. These individuals may either be hybrids between host races, or pure individuals growing on the "wrong" host tree. To distinguish between these two alternatives it would be necessary to screen nuclear markers.

Speciation via host race formation is a typical process in parasitic plants. Examples include the desert mistletoe, *Phoradendron californicum,* and the dwarf mistletoe, *Arceuthobium americanum,* in North America (Glazner *et al.* 1988; Jerome & Ford 2002). Similar to *Viscum*, *Arceuthobium* could be divided into genetic host races despite the lack of obvious morphological differences. The type and strength of reproductive isolation among the *Viscum* host races is presently unknown. Prezygotic reproductive isolation could arise as a consequence of differences in the ability to colonize particular host trees. Preferential pollen transfer among individuals growing on the same or neighbouring trees may then further reduce chances for gene flow between host races (Glazner *et al.* 1988). The few observed occurrences of mistletoe on "wrong" host trees suggest that these individuals originated from

"seeds" that were transferred from plants growing on other host trees and were able to establish on the new host. These host switches were typically observed where the different hosts grow in close proximity. The rarity of such cases, however, may indicate that the ability to colonize non-host trees is indeed reduced. Postzygotic barriers may occur when hybrids cannot develop or suffer from reduced fitness in the parental habitats. Hybridization among host races is possible but rare and hybrids have been reported only between *V. a. album* and *V. a. abietis* (Piotrowski *et al.* 2003; Zuber & Widmer 2000).

Viscum produces fleshy berries that are eaten and dispersed by birds. The most important birds for mistletoe dispersal are mistle thrush (*Turdus viscivorus*), fieldfare (*Turdus pilaris*), blackcap (*Sylvia atricapilla*), and waxwing (*Bombycilla garrulus*) (Zuber 2004). These are partly migratory birds, which may act as long distance dispersers of *Viscum* (waxwing only in evasion-years) on their migration back north. The birds are known, depending on their breeding site in Northern Europe, to take a migration route to their wintering area either in SW - SSW direction to Spain or in SSE - SE direction to Italy, Turkey and the Caucasus (Huttunen 2004; Bauer 2005). The migration routes, breeding and wintering areas fits the distribution area of mistletoes. The birds mainly promote the local spread of mistletoes when they consume berries, with an estimated maximum spread of 20km (Frochot & Sallé 1980). Long distance dispersal of seeds by birds, however, cannot occur due to seed consumption, because retention times of seeds in the digestive tracts are short. Instead, seeds are most likely dispersed over greater distances by birds when seeds remain attached to the plumage or bill as a consequence of their sticky coat, and are later stripped off (Wangerin 1937).

In general, the level of population subdivision estimated from cpDNA analysis tends to be low in plants with animal-ingested propagules (Petit *et al.* 2003). Exceptions, however, are known and include *Frangula alnus* (Hampe *et al.* 2003) and *Hedera* sp. (Grivet & Petit 2002). These species have fleshy fruits that are dispersed by birds, such as *Viscum*. *Viscum* host races were also found to have strong population differentiation as estimated by high G_{ST} values. One possible explanation for this high value in *Viscum* is the threefold dependence on host trees, seed vectors (i.e. birds) and pollinators, and the small effective population size of cpDNA in a dioecious species. The dwarf mistletoe *Arceuthobium* has a comparatively low G_{ST} (0.04-0.361), but is wind and insect pollinated and seed dispersal is achieved through a hydrostatically controlled explosive discharge mechanisms (Jerome & Ford 2002). The high population differentiation in *Viscum* could be further caused by the scattered distribution of host trees. Generally, parasites have been observed to have a high degree of differentiation if the distribution range of the hosts is fragmented (Burban *et al.* 1999).

Because of the strong differentiation of the host races in *Viscum album,* the phylogeography of each of the three widely distributed host races is discussed individually. The rare *V. a. creticum* is included in the discussion of *V. a. austriacum* with which it shares the host tree genus.

Viscum on *Abies* spp.

All mistletoes with the widely distributed haplotype A1 were collected from *Abies alba*, with the exception of one population that was collected from *A. nordmanniana*. For *Abies alba*, five glacial refugia have been reported: the Pyrenees, central and/or eastern France, central Italy, Calabria and the Balkan peninsula. Recolonisation into central Europe has occurred only from three refugia, not from the Pyrenees and Calabria (Konnert & Bergman 1995; Vendramin *et al.* 1999). For *Viscum a. abietis*, the haplotype distribution indicated no refugium in the southwest. Because of the parasitic life form, *Viscum* is depending on host availability and distribution, therefore possible refugia for *V. a. abietis* haplotype A1 are the Pyrenees and southeastern France following the firs north and eastwards. The rare haplotypes were found in central to eastern Europe, matching the southeast migration routes of the migratory birds, with one haplotype occuring slightly more to the west (A4). All monomorphic populations carrying a haplotype other than A1 were situated in southeastern Europe. Haplotypes A5 and A6 in Greece and A8 in Turkey were restricted to the these areas.

An analysis of cpDNA variation among *Abies* species occurring in central an eastern Europe revealed little differentiation among species (Parducci & Szmidt 1999) and we found no strong indication that mistletoes growing on different *Abies* species are genetically differentiated. Instead, genetic differentiation reflects primarily geographic differentiation, as indicated by the two Turkish populations. These were collected from different *Abies* species (*A. bornmuelleriana, A. cilicica*) but revealed the same haplotype (A8). This haplotype, however, was highly distinct from all other *Viscum* haplotypes found in this study and could not be connected to the *V. a. abietis* haplotype network (Fig 2a). This leads us to propose that these populations represent a new taxon within *V. a. abietis* that occurs at the geographic range margin. This is similar to the situation in *V. a. austriacum*, where a distinct race, *V. a. creticum*, occurs at the range margin.

Populations of *V. a. abietis* revealed little intrapopulation variation and were highly differentiated (G_{ST} = 0.791), whereas populations of the main host, *Abies alba*, are substantially less differentiated (G_{ST} = 0.133) (Vendramin *et al.* 1999), as is often observed for hosts and their parasites (Burban *et al.* 1999; Jerome & Ford 2002; Price 1980). The

scattered distribution of fir may have contributed to the strong differentiation among the parasite populations.

Viscum on *Pinus* spp.

In contrast to mistletoes from fir, mistletoes from pine revealed the highest cpDNA diversity in southwestern Europe and a decrease of variation northwards. Iberia is a probable refugium for this taxon. The haplotype distribution in southwestern and central Europe fits the southwestern migration route of birds. According to this scenario, haplotypes P2 could have spread from Spain northwards to Switzerland and type P3 from southwestern France to Switzerland. Of the haplotypes found in the Iberian peninsula, haplotype P5 has an interesting distribution. It occurs not only in Spain but was also found in Corsica. This haplotype is more closely related to haplotypes from central Europe than to other haplotypes found in Spain, which suggests that this haplotype migrated to Spain via Corsica (Fig. 2b). According to Afzal-Rafii & Dodd (2007), the host species of *V. a. austriacum* in Corsica, *Pinus nigra*, was present on Corsica during the last glacial period. It is conceivable that Corsica has therefore also served as a refugium for *V. a. austriacum*. The other pine host, *Pinus pinaster,* had a glacial refugium in Iberia (Burban & Petit 2003; Bucci *et al.* 2007), whereas *Pinus sylvestris* shows three evolutionary units: one in Spain, one in north-central Europe and a northern Fennoscandia one, each of which had a different glacial refugium (Sinclair *et al.* 1999; Soranzo *et al.* 2000). Because of the low haplotype diversity found in northern European mistletoes growing on *P. sylvestris*, only Spain appears to be a possible refugium. As in mistletoes from fir, we found no indication that mistletoes growing on different *Pinus* species are genetically differentiated, therefore the glacial refugia for *V. a. austriacum* seem to be, according to the available hosts: Iberia with *P. pinaster, P. sylvestris* and *P. nigra*; Corsica and the Balkan with *P. nigra*. As in *Viscum* from fir, populations from Turkey showed two distinct haplotypes. Interestingly, these are more closely related to haplotypes from Spain than to haplotypes from central Europe (Fig. 2b).

Populations of *V. a. austriacum* have a highly scattered distribution, reflecting the distribution of its host species. Populations display little variation but high differentiation, as in *V. a. abietis*.

V. a. creticum was found to have two haplotyes that are distinct from other cpDNA haplotypes found in *Viscum* growing on pine. These two haplotypes grouped with haplotype A1 from fir, thus supporting the presumed morphologic and genetic proximity to mistletoes from fir (Böhling *et al.* 2002) rather than pine (Fig. 2b).

Viscum on deciduous trees

Mistletoes growing on deciduous trees were not differentiated according to host trees and represent a single taxon with broad host range. Populations of *V. a. album* have higher genetic variation and lower genetic differentiation than mistletoe populations on conifers. This difference in population genetic structure may be a consequence of the broad host range and thus of the more continuous distribution and larger number of suitable host trees. While mistletoes growing on conifer hosts are restricted to hosts belonging to the same species or genus, mistletoes of deciduous trees have a host range that includes not only trees from different species, but also from different genera and even families.

Chloroplast DNA variation was found in southern Europe, mainly in southeastern France, Italy and Turkey, but not in Spain. Another region with relatively high diversity was eastern Europe (Slovakia, Hungary). Deciduous forest were restricted during the ice ages to southern Iberia, Calabria, Greece, the southern Balkan, northern Turkey, the Caucasus and the Caspian Sea (Hewitt 1996). These refugia have been confirmed for several host tree species, including *Populus nigra* (Cottrell *et al.* 2005), *Tilia cordata* (Fineschi *et al.* 2003), *Corylus avellana* (Palmé & Vendramin 2002), *Alnus glutinosa* (King & Ferris 1998), *Fraxinus excelsior* (Heuertz *et al.* 2004) and *Quercus* (Petit *et al.* 2002a; Petit *et al.* 2002b).

The mistletoe haplotype H1 was the most widely distributed and common one, being found in all populations (including North America), except one in Slovakia and three populations in Turkey. The distribution of haplotype diversity suggests refugia in Italy, southeastern France or eastern Europe, rather than in Spain or on the Balkans. A second frequent type, H3, has a clear eastern distribution. For some deciduous host trees, refugia have also been proposed to occur in eastern Europe: in western Russia for *Tilia cordata* (Fineschi *et al.* 2003), in the Russian Arctic for the cold tolerant tree *Betula pendula* (Palmé *et al.* 2003b), or in Hungary for *Salix* sp. (Palmé *et al.* 2003a). These tree refugia are a possible explanation for the observed haplotype diversity of *Viscum* occurring on deciduous trees in eastern Europe.

As in the other *Viscum* host races, plants from Turkey carried distinct haplotypes. Interestingly, however, haplotype H5 provides a link between the distinct Turkish haplotypes and those found in central Europe (Fig 2c).

Turkey

Although we found strong genetic support for the three main host races, it is noticeable that all *Viscum* individuals from Turkey differed genetically from the European ones. While it is difficult to delimit *Viscum* host races based on morphological differences, the molecular

results strongly suggest that distinct evolutionary units occur in Turkey, especially on *Abies*, but presumably also on other host trees. The haplotypes found in *V. a. album* do not belong to *Viscum coloratum*, which grows exclusively on deciduous trees and has a more eastern distribution than *V. album* up to Korea and Japan (unpublished result). Clearly, mistletoes from Turkey need to be studied in more detail, not only morphologically, but also genetically and biochemically. This aspect may be of applied interest, because *Viscum* host races are known to differ substantially biochemically, and the distinct lineages in Turkey may do so as well. Consequently, they may provide a valuable natural resource for biochemical compounds that are of medical interest.

Conclusions

Chloroplast DNA variation in the European mistletoe can be divided into four groups that correspond to the previously described host races. In addition, two major geographic groups can be distinguished, a southeastern group (Turkey and Greece) and the rest of Europe. In the southeast of Europe chloroplast haplotypes did not substantially spread northwards or westwards. Populations occurring at the southeastern range margin, i.e. in Turkey, are genetically differentiated from more central European populations, and deserve further study to test whether these range marginal populations have diverged from range central ones.

Possible glacial refugia and postglacial immigration routes of *Viscum album* host races are only roughly comparable with those of their host trees, because each mistletoe host race is able to parasitize several tree species and may have switched among closely related hosts during glacial survival and subsequent recolonization.

The high degree of population differentation found in *Viscum* supports the finding that plants with bird dispersed seeds has not necessarily low levels of cpDNA differentation (Petit *et al.* 2003). In mistletoes, high population differentiation is probably a consequence of the parasitic life form and the scattered distribution of hosts. All host races have high G_{ST} value, but *V. a. album* with the widest host range and the most continuous distribution of hosts has the lowest G_{ST} value.

Acknowledgements

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Synthesis

Genetic differentiation of Viscum album

While it is difficult to delimit *Viscum* host races based on morphological differences, the molecular results strongly suggest the distinction of three host races: *Viscum album album* from deciduous trees, *V. a. abietis* from fir and *V. a. austriacum* from pine. The host races correspond to the subspecies previously proposed in the literature. We interpret the small genetic distances among these host races as evidence for their recent formation. *V. album* may undergo first steps of speciation via host race formation. An alternative scenario, where gene flow occurs frequently among the presumed host races and genetic differentiation remains weak, is not supported by our results because *V. album* isolates from a given host show either identical or most closely related chloroplast haplotypes. Our results further suggest that *V. a. album* from deciduous trees and *V. a. abietis* from fir are more closely related to each other than to *V. a. austriacum*. Hybrids are between host races are rare and are more likely to occur between *V. a. album* and *V. a. abietis*. Indeed, the presumed hybrids found so far are the result of crosses between *V. a. album* and *V. a. abietis*.

The type and strength of reproductive isolation among the *Viscum* host races is presently unknown. Prezygotic reproductive isolation could arise as a consequence of differences in the ability to colonize particular host trees. Preferential pollen transfer among individuals growing on the same or neighbouring trees may then further reduce chances for gene flow between host races. The few observed occurrences of mistletoes on "wrong" host trees (as determined by their cpDNA haplotypes) suggest that these individuals originated from "seeds" that were transferred from plants growing on other host trees and were able to establish on the new host. These host switches were typically observed where the different hosts grew in close proximity. The rarity of such cases, however, may indicate that the ability to colonize non-host trees is reduced.

Postzygotic barriers may occur when hybrids cannot develop or suffer from reduced fitness in the parental habitats. The failure of hybrid development in mistletoes might be explained by the lack of suitable habitats for their establishment. Hybrids combine the genetic characteristics of two species, and may be best adapted to intermediate habitats. The habitat of mistletoes is defined by their host trees, and intermediate habitats are therefore unlikely to occur, which may limit the occurrence of hybrids.

Phylogeographic structure of Viscum album

The high degree of population differentiation found in *Viscum* is probably a consequence of the threefold dependence on host trees, birds and pollinators, and the small effective population size of cpDNA in a dioecious species. The scattered distribution of hosts may further add to the observed high population differentiation.

Chloroplast DNA variation within each of the three widely distributed host races revealed a phylogeographic signal. Possible glacial refugia and postglacial immigration routes of *V. album* host races were only roughly comparable with those of their host trees, because each mistletoe host race is able to parasitize several tree species and may have switched among closely related hosts during glacial survival and subsequent recolonization. However, for *Viscum* from fir (*V. a. abietis*), possible refugia are the Pyrenees and southeastern France, following the firs north and eastwards. Mistletoes from pine (*V. a. austriacum*) have a probable refugium in Iberia and possibly also one in Corsica. The distribution of haplotype diversity found in mistletoes from deciduous trees (*V. a. album*) suggests southern refugia in Italy and southeastern France, with an additional refugium in eastern Europe.

Viscum album creticum and Viscum from Turkey

Viscum album populations from Crete grow exclusively on pine hosts and occur at the distribution range margin. These populations were found to belong to a distinct race: *V. a. creticum* (Appendix).

The situation in Turkey may be similar to the one investigated in Crete. All haplotypes in each host race found in Turkey (and in *V. a. abietis* from Greece) were distinct and restricted to this area. The distribution and high sequence divergence of these haplotypes suggests that they represent new races. Additional results show that these haplotypes do not belong to *Viscum coloratum*, which grows exclusively on deciduous trees and has a more eastern distribution than *V. album*.

Further research options

This thesis has answered several questions with respect to the evolution of *V. album*, but has also raised new questions. Two of these I consider to be particularly interesting:

- Examination of individuals that carry cpDNA haplotypes otherwise found commonly in individuals from other host races. These individuals may either be hybrids between host races, or pure individuals growing on the "wrong" host tree. To distinguish between these two alternatives, it is necessary to identify and screen nuclear markers.

- Mistletoes from Turkey need to be studied in more detail, not only morphologically, but also genetically and biochemically. This aspect may be of applied interest, because *Viscum* host races are known to differ substantially biochemically, and the distinct lineages in Turkey may do so as well. Consequently, they may provide a valuable natural resource for biochemical compounds that are of medical interest.

Notes on the Cretan mistletoe, *Viscum album* subsp. *creticum* subsp. nova (Loranthaceae/Viscaceae)

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Abstract

Viscum album subsp. *creticum,* known from two mountain areas of E. Crete, is described as new to science. It combines features of the two presently accepted conifer mistletoes, resembling the fir parasite, subsp. *abietis*, in gross morphology but genetically (by nrDNA ITS sequence data) close to the pine parasite, subsp. *austriacum,* while differing from either in hypocotyl shape and nucleotide sequences of cpDNA. Its host is *Pinus halepensis* subsp. *brutia,* a taxon not normally affected by mistletoes. It is thus likely that the new subspecies has its own, particular host tree. Hypotheses of the Cretan mistletoe's history and endemic status are discussed on the background of documented size fluctuations of Cretan pine forests and their propensity to burning.

Introduction

Taxonomic background

The genus *Viscum* L. (mistletoes) comprises ca. 100 species, with a center of diversity in the Old World tropics and subtropics (Barlow, 1983). Two species occur in Europe: *V. cruciatum* Boiss., with disjunct Mediterranean occurrences in southern Spain and Portugal, Morocco, and Palestine, parasitic on trees of *Olea europaea* L. and several other dicotyledonous genera of various families; and the widespread, mainly palaeotemperate *V. album* L. (map in Meusel et al., 1965).

Viscum album is a variable species and has been further subdivided. Tubeuf (1923) recognised three host-specific varieties or "races" in European material, but also found that leaf and fruit morphology partly depend on nutritional conditions – which is why some authors (e.g., Hess et al., 1967) did not formally recognize infraspecific taxa. Several others (e.g. Ehrendorfer, 1967; Bergmeier, 1994; Lindacher, 1995), on the contrary, split *V. album* into three species. Others still (e.g. Schwarz, 1949, Rothmaler, 1990) accepted two species, one of them (*V. laxum* Boiss. & Reut., the conifer mistletoe) with two subspecies. A large majority of modern authors (e.g., Miller, 1982; Pignatti, 1982; Ball, 1993; Snogerup and Snogerup in Phitos et al., 1997; Wisskirchen and Haeupler, 1998; Karlsson, 2000) recognized (sometimes with doubt: Greuter et al., 1989) one species with three European subspecies. Further subspecies have been described on morphological grounds from E. Asia (subsp. *coloratum* Kom.: see Grazi and Urech, 1982) and the Sino-Himalayan region (subsp. *meridianum* (Danser) D. G. Long: Grierson & Long, 1982), but the variation of *V. album* in Asia is not yet properly understood. The formerly described European subspecies are:

- *Viscum album* subsp. *album* (*V. album* var. *mali* Tubeuf), parasitic on a wide range of woody dicots;
- *Viscum album* subsp. *abietis* (Wiesb.) Abrom. (*V. austriacum* var. *abietis* Wiesb., *V. album* var. *abietis* (Wiesb.) Tubeuf, *V. austriacum* subsp. *abietis* (Wiesb.) O. Schwarz), on *Abies* species; and
- *Viscum album* subsp. *austriacum* (Wiesb.) Vollm. (*V. austriacum* Wiesb., *V. laxum* Boiss. & Reut., *V. album* var. *pini* Tubeuf), on *Pinus* and more rarely *Larix* and *Picea.*

The traditional distinction between the subspecies is largely based on leaf and internode dimensions, but these are strongly influenced by environmental and nutritional factors and

their reliability has been justifiably challenged. Indeed, when material from a broad geographical range is considered, differences in vegetative characters are likely to break down. Yet, they retain their usefulness as taxonomic indicators, especially when no female, fruiting individuals are available. Berry color is a notoriously unreliable feature.

Better criteria, as worked out by Tubeuf (1923) and Grazi and Urech (1982), are those based on the fruit (presence of viscine threads between the two mesocarp layers; structures adhering to the endocarp), seed (shape; number of embryos), and embryo (shape of the hypocotyl).

As all these characters can only be observed on fruiting plants, molecular data might be the answer. Zuber and Widmer (2000) studied the genetic variability of the three European subspecies of *Viscum album* by sequencing segments of non-coding chloroplast DNA and nuclear ribosomal DNA. They found but slight differences, but their pattern was consistent with the recognition of the present taxa. Each had its own, unique nucleotide sequence of the internal transcribed spacer (ITS) region of nrDNA, but they differed only by 2-5 mutation steps among themselves. The cpDNA sequences were slightly more varied: their analysis yielded 8 different haplotypes, characterizing subsp. *abietis* (type A and B), subsp. *austriacum* (types C and D) and subsp. *album* (types E-H). The trees resulting from a cladistic analysis cannot be expected to be very robust. The ITS cladogram shows subsp. *album* and subsp. *abietis* in a sister-group relationship, both together being sister to subsp. *austriacum*. The cpDNA tree differs in twinning up subsp. *album* and subsp. *austriacum* to form the sister group of subsp. *abietis*, except for one sample of subsp. *album* (type H) whose anomalous position is explained by possible hybridization.

Viscum in Greece and Turkey

The distribution of the *Viscum album* subspecies, even within Europe, is still imperfectly known (Jalas and Suominen, 1976), but subsp. *album* appears to be the more northerly taxon (the only one to reach Britain, northern France and Scandinavia). There is an enormous bulk of literature on host preferences (see Barney et al., 1998), which may be less specific than is generally acknowledged, as the reliability of the data sometimes suffers from uncertainty of identification of the parasite. Gäumann (1946) claimed that there is a limited overlap in hosts between the three taxa, with some species of *Salix* and North American (!) *Acer* being infected by both subsp. *album* and one each of the two conifer parasites – but unfortunately the source of his data is not mentioned.

All three European subspecies are known to occur in Greece (see maps in Phitos et al., 1997) as well as Turkey (Miller, 1982, Ergun et al., 1994), but with a very different pattern. *V. album* subsp. *album* is very rare in Greece, with only two isolated records close to the northern border; whereas it is the prevalent taxon in Turkey, growing throughout the country. *V. album* subsp. *abietis* is widespread on the Greek mainland, wherever *Abies* grows, except in the Rodopi Mts. (also avoiding the fir-wooded islands, Thasos and Kefallinia); it is less frequent in Turkey, and naturally absent from central and western Anatolia where no *Abies* exists. *V. album* subsp. *austriacum* in Greece is totally confined within the range of *Pinus nigra* J. F. Arnold, and patchy even there, steering clear of the stands of the Mediterranean pine species, *P. halepensis* Mill. and *P. pinea* L.; in Turkey, it is recorded as a parasite of *P. nigra* and *P. sylvestris* L., with one remarkable exception, from Adana Province in the south, where *P. halepensis* (almost certainly subsp. *brutia*) is given as the host. Barney et al. (1998) do not mention *P. halepensis* subsp. *brutia* among the known mistletoe hosts. Those floras that give details of the host species of mistletoes (many just mention the genus) do not as a rule list *P. halepensis,* the exceptions being two Spanish floras (Bolós and Vigo, 1990; Catalán and Aparicio, 1997). From specialized literature (López-Sáez & Sanz de Bremond, 1992; López-Sáez, 1993) it would appear that an unspecified mistletoe taxon parasitizing *P. halepensis* is found in, and probably limited to, eastern Spain (provinces of Barcelona, Lérida, Navarra, Tarragona, Teruel, and Zaragoza; with one record in Burgos, in planted stands).

Mistletoe in Crete

If one compares the known Mediterranean ranges of distribution of *Pinus nigra* (including its subspecies) and *Viscum album* subsp. *austriacum* (Greuter et al. 1984, 1989), one notes good general congruence. There are a few areas with black pine from where the parasite is unrecorded, such as Cyprus, but only a single one where the reverse situation obtains: Crete.

The mistletoe populations in Crete are the southernmost of Europe. Their host tree is *Pinus halepensis* subsp. *brutia* (Ten.) Holmboe. Subspecific placement of these plants was and is controversial: Barclay (1986), Jahn and Schönfelder (1995), and Snogerup and Snogerup (in Phitos et al., 1997) assigned them to *Viscum album* subsp. *abietis,* based mainly on leaf shape and general aspect; whereas others, following the concept of host-specific races, listed them under subsp. *austriacum* (Rechinger, 1944; Jalas and Suominen, 1976; Greuter et al., 1989; Turland et al., 1993). The peculiarity of the Cretan plants was noted by Greuter (1977), Jahn and Schönfelder (1995), and Snogerup and Snogerup (in Phitos et al., 1997). The latter

authors foreshadow the possibility that they may constitute a yet undescribed, fourth European subspecies, approaching subsp. *abietis* in habit but differing in host preference.

Materials and Methods

Measurements were performed on air-dried, freshly collected material representative of the two populations. The leaf sample consisted of 30 mature leaves, 10 each from *Böhling 10656* (Lasithi Mts), *Böhling 5532,* and *Böhling 7213 and Raus* (Mt Afendis Kavousi). For internode measurements, the five youngest fully developed distal internodes of each of nine branches were considered: 4 branches of *Böhling 10656,* 3 of *Böhling 5532,* and 2 of *Böhling 7213 and Raus.* Seed morphology was studied on the three same gatherings as well as the *Jahn* and *Broussalis* specimens. Hypocotyl shape was examined on seeds longisected by hand.

DNA sequence analyses of Cretan *Viscum* material were performed (by D.Z.) using air-dried, recent herbarium material from two individual plants representing the two known populations *(Böhling 7213* and *10656.1)*. The sequences examined (nuclear ribosomal DNA ITS sequences and partial sequences of three non-coding chloroplast DNA introns and spacers) were the same as those previously studied by Zuber and Widmer (2000) for the three known European subspecies, and the procedures of DNA extraction, PCR amplification, choice of primers, purification and sequence analysis were exactly the same as are described in that paper. The additional sequences have again been deposited in GenBank (accession nos. AF419257-AF419260).

The sequence data were added to the previous set and a new phylogenetic analysis was performed in the same way, with identical settings and conventions, using the PAUP™ version 4.0b6 software package.

Results

After thorough study of the material and a balanced consideration of the available information, we feel justified in describing the Cretan populations of *Viscum album* as a new subspecies, being physiologically, phytogeographically, genetically, and to some extent morphologically distinct from the other European representatives of the species.

Viscum album subsp. ***creticum*** N. Böhling, Greuter, Raus, B. Snogerup, Snogerup & Zuber, **subsp. nova** – Holotype: Greece, Crete, Nomos Lasithi, Eparchia Ierapetra, between

Males and Anatoli, 35°03'N, 25°36'E, alt. 650 m, parasitic on *Pinus halepensis* subsp. *brutia*, 8.i.2000, *Böhling 10656* (B; isotypes: UPA, herb. Böhling) (Fig. 1,2).

A *Visci albi* subspecie *austriaca* differt habitu erecto et foliis obovato-oblongis latitudine (2.1-)2.2-3.4(-3.8)-plo longioribus indistincte venosis; a *V. albi* subspecie *abietis* foliis et baccis subminoribus distinguitur; ab ambabus insuper hypocotylo doliiformi atque hospite (nam in *Pino halepensi* subsp. *brutia* parasitica) discrepat. A *Visci albi* subspecie *alba* itidem hospite, praeterea stratis mesocarpii inter se liberis nec filamentis viscosis junctis et seminibus compresso-ellipsoideis reticulo filamentoso obtectis longius distat.

Specific traits of vegetative morphology distinguishing the new subspecies from *Viscum album* subsp. *austriacum* include its upright growth and peculiar dense, hemispherical to globose habit (Fig. 2), the short and stout stem internodes, and the short, relatively broad leaves (Fig. 1; Table 1). The first, basal internodes are about twice as long as those produced later, and sometimes the stems are trifurcate at the lower nodes, which according to Snogerup and Snogerup (in Phitos et al., 1997) would be a feature of subsp. *austriacum* (but is also commonly found in subsp. *abietis,* e.g. on Mt. Parnon: *Böhling 11356-11358*). *V. album* subsp. *abietis* is similar to our subspecies in leaf shape, but on average the leaves are larger, although the values overlap. Fruit and seed characters, viz., the absence of viscine threads between the mesocarp layers, the loosely reticulate fibrous pattern covering the endocarp, and the compressed-ellipsoid shape of the monoembryonic seeds, are reliable criteria for separating the conifer mistletoes from subsp. *album*, and the Cretan specimens predictably conform with the former. The size (6-8 mm across) of the Cretan plants' whitish berries is smaller than the average dimensions for subsp. *austriacum* and subsp. *abietis,* but the values overlap. With respect to hypocotyl shape, Grazi and Urech's (1982) new criterion for separating the two conifer mistletoes, the Cretan plants match neither, even though their barrel-shaped hypocotyl is more akin to the cylindrical one of subsp. *austriacum* than to the centrally slimmed, "waisted" outline observed in subsp. *abietis* (Fig. 3). These differences, however, have yet to be confirmed statistically.

Fig.1 *Viscum album* subsp. *creticum,* live branch of the plant from which the holotype gathering, *Böhling 10656,* was prepared (photo: Böhling).

Fig.2 *Viscum album* subsp. *creticum,* habit and habitat of the plant from which the holotype gathering *(Böhling 10656)* was prepared (photo: Böhling).

Table 1 *Viscum album* subsp. *creticum*: Leaf and internode dimensions and length : width ratios

	minimum	maximum	normal range[*]	mean	n
leaf length (mm)	13	27	15.5–25	21	30
leaf width (mm)	4.5	10.0	6–9.5	7.8	30
leaf ratio	2.1	3.8	2.2–3.4	2.72	30
internode length (mm)	6	28	10-23	15.6	45
internode width (mm)	0.9	3	1-2	1.6	45
internode ratio	3.5	21.5	6.3-12.9	10.1	45

[*] Disregarding the 10 % lowest and 10 % highest measurements.

Fig.3 Seed longisections of *Viscum album* subsp. *abietis* (left: Peloponnisos, Mt Parnon, *Böhling 11381,* B), subsp. *creticum* (centre: holotype specimen), and subsp. *austriacum* (right: Turkey, 42 km SE of Ankara, *Walter & Walter 1070,* B), showing the embryo. The hypocotyl, pointing upward, is centrally slimmed in the former, barrel-shaped in the second, and cylindrical in the last. Scale unit = 1 mm (photo J. Bansemer).

Distribution and habitat

According to Raulin (1869) the discovery of the Cretan mistletoe would be due to Franz Wilhelm Sieber, who visited Crete in 1817. This, however, is a doubtful attribution. *Viscum* does not appear in any of the printed lists of Cretan plants offered for sale by Sieber, and we have not been able so far to locate a corresponding specimen. The first to collect the Cretan mistletoe was probably Theodor von Heldreich who, according to his manuscript inventory kept in the archives of the Conservatoire botanique, Geneva, collected it in April 1846 "Parasiticum in *Pino laricione* [an error for *P. halepensis* subsp. *brutia*] in m. Psychro" [i.e., on Mt. Afendis Kavousi].

The Cretan mistletoe is found in two areas of eastern Crete, both located in the Ierapetra district, Lasithi province (see map in Phitos et al., 1997): in the pine forests extending around the village of Males, on the SE slopes of Mt. Dikti, from the Selakano valley to near Anatoli, at altitudes of 650-1100(-1500) m (3 records); and in the Thripti valley above Monastiraki, west of Mt. Afendis Kavousi, at 500-950 m (10 records).

Both areas are mountain-locked valleys, protected from strong winds from almost any direction but particularly from the northerly gales frequent in the Cretan mountains in summer time. The altitudinal range coincides with the belt of maximum cloud formation, where average air humidity is highest. The mountain relief provides shelter from factors such as exsiccation by climatic aridity and frost that are likely to adversely affect the seedlings, which need one year to penetrate the host and tap the water supply of its xylem (Seybold in Sebald et al., 1992).

A census of the demography and age structure of the Cretan populations is a desideratum for the future. One plant, with an overall spread of 70 cm and basal internodes up to 13 mm thick, was 22 years old judging by the number of stem internodes. The remote position of the mistletoes on their host trees suggests that they are harvested by local people, perhaps to be used as fodder for goats or as a source of bird-lime, if not for medicinal, sacred or magic purposes (Kabbadas, 1957-1964: 892-893).

Specimens seen

Lasithi Mts: "entre Anatoli et Males, sur *Pinus brutia*", xii.1975, *Broussalis* (herb. Greuter; fruiting); "Mavro Dasos NW Selakano, auf *Pinus brutia*", 1080 m**,** 35°05'N. 25°32'E, 25.vi.1993, *Jahn* (herb. Jahn, fruiting); "between Males and Anatoli, parasitic on *Pinus halepensis* subsp. *brutia*", 35°03'N, 25°36'E, alt. 650 m, 8.i.2000, *Böhling 10656* (B, herb. Böhling; fruiting).

Mt Afendis Kavousi: "in m. Psychro, parasiticum in *Pino laricione*", iv.1846, *Heldreich* (G-BOIS); "Western slope of Afendis Kavousi, ca. 2 km E of Monastiraki, on *Pinus brutia*", 800 m, 29.v.1976, *Landström 3137* (LD); "SW of the village Thripti, sandy stony ground with phrygana and *Pinus brutia*", 900-950 m, 18.vi.1977, *Stamatiadou 19992* (ATH); "Ortseingang von Thripti, Straßenrand, auf *Pinus brutia*", 800 m, 20.xii.1984, *Kypriotakis 1025* (B); "SW Thripti, Abstieg zur Monastiraki-Schlucht, auf *Pinus brutia* im Kiefernwald", 750 m, 35°05'N, 25°51'E, 12.iv.1992, *Jahn 5* (herb. Jahn, flowering and fruiting); "1 km SV Thripti, växte på *Pinus brutia*", 35°04.96'N, 25°51.61'E, 22.v.1993, *Svensson 1621* (LD); "1,5 km SW Thripti, lockerer Kiefernwald, Phyllit-Quarzit", 35°04'46''N, 25°50'39''E, 500 m, 17.iv.1996, *Diewald* (REG), "near the chapel Ag. Anna SW Thripti, *Pinus brutia* stand on dolomitic rock and in NW exposition", 35°05'N, 25°51'E, 660-670 m, 6.v.1997, *Böhling 5532* (B, herb. Böhling; fruiting and flowering); id. (also observed at 720 m), 24.iii.1998, *Böhling 7213 and Raus* (B, herb. Böhling; fruiting and flowering).

DNA sequence analysis

In both Cretan plants examined, nrDNA ITS sequence proved to be identical with that known for *Viscum album* subsp. *austriacum* (Zuber and Widmer 2000). This means that the Cretan mistletoe does not belong to subsp. *abietis* as had often been assumed.

Parsimony analysis of cpDNA sequences resulted in the four shortest trees (CI = 0.96, RI = 0.79). The topology of the bootstrap 50 % majority-rule consensus tree is shown in Fig. 4. The cpDNA sequences of *Viscum album* subsp. *creticum* proved to be a new, additional haplotype (K). Unfortunately, the cladistic analysis of the cpDNA sequence data did not yield a clear picture. Obviously the additional data set from Crete introduced a significant amount of homoplasy into the system. The tree, which was fully resolved before, saw many of its nodes break down to form a two-level polytomic structure. Whereas the two haplotypes (C and D) of *V. album* subsp. *austriacum* still group together, they now branch off a polytomy together with the three "normal" haplotypes (E-G) of subsp. *album*. The *album-austriacum* clade as a whole, again, sits in a polytomy from which the two haplotypes (A and B) of subsp. *abietis,* the anomalous, suspected hybrid one (H) of subsp. *album* and the new one (K) of subsp. *creticum* depart.
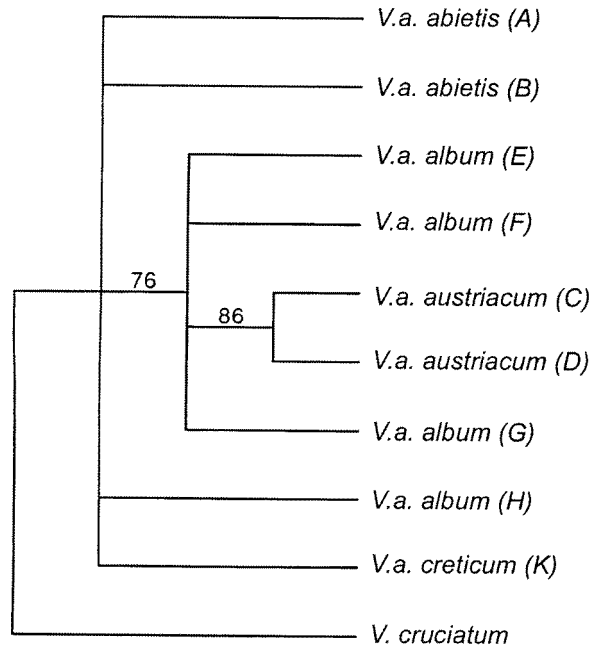
Fig.4 Strict consensus tree based on Viscum album cpDNA haplotypes, using V. cruciatum as outgroup taxon. The numbers above the branches represent bootstrap values.

For what it is worth, this tree lends support to the autonomous status of the new subspecies and underlines its ambiguous relationship with the two other conifer parasite taxa. *Viscum album* subsp. *creticum* branches off the common tree at the same basal level as subsp. *abietis* but by its nrDNA ITS profile matches subsp. *austriacum* instead.

Discussion

So far, *Viscum album* subsp. *creticum* must be regarded as a Cretan endemic. If indeed it is, it may either have evolved anew in its present location, in insular isolation; or it may be the last remnant of a formerly more widespread taxon. Supposing it is not endemic, the question then arises: Where else should we look for it?

Viscum album subsp. *creticum* is exceptional, if not unique, in parasitising *Pinus halepensis* subsp. *brutia*. Few other records of *Viscum album* growing on Aleppo pine exist, and they need to be verified as they might perhaps refer to subsp. *creticum*. We already mentioned one from S. Anatolia [*Balls 1301* from Adana: Bürücek], and both Miller (1982) and Erun et al. (1994) cite many additional mistletoe gatherings from the same general area for which the host tree is not known. A second area which to look is eastern Spain, the only region where *Viscum* is known to grow on *P. halepensis* subsp. *halepensis*.

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Irrespective of the question of endemism, it is tempting to speculate on the mode of survival of the Cretan mistletoe in relation to the past and present condition of its host. It is a proven fact that *Pinus halepensis* subsp. *brutia,* Crete's single wild pine species, is a member of the old autochthonous flora: Pine pollen, plentiful in the single polliniferous core, was found to reach back to pre-settlement (pre-Neolithic) time, from a location near Ayia Galini in South Central Crete (Bottema, 1980). However, while native status is undisputed, past presence of pine woods in areas where they stock today may not been taken for granted. The extension of pine woods on the island has varied greatly through historical times. Rackham and Moody (1996) demonstrated that it has never before been as large as it is now. Their map (p. 62) shows old pine woods or stands on the southern slopes of all major mountain massifs, most of which, especially in the east, have spread considerably in recent years, conquering large new areas. Rackham (1972) gives details for the southern foothills of the Lasithi Mts., where pine, he believes, arrived around 1850 and gradually spread ever since, having been rare if at all present in the Early Bronze Age, and absent in the 16th century. Higher up in the Selakano Valley, presently the centre of extensive pine woods, Tournefort in 1700 found a woodland of *Quercus ilex* L., *Quercus coccifera* L. and *Acer sempervirens* L.

Size fluctuation of host stands and consequent population bottlenecks may be one factor accounting for the present rarity and patchy occurrence of Cretan mistletoe. Another factor of obvious importance is forest fires. To quote Rackham and Moody (1996), "Pine is the most ferociously combustible Cretan plant", and they go on with their own, plausible hypothesis: "Cretan pine is not flammable by misfortune: these features are adaptations to promoting fire. It is the business of this [pine] ... to ignite from time to time and burn up its less fire-adapted competitors" - and why not, may we add, its parasites.

Forest fires are not a human invention, but their number and frequency have dramatically increased through the presence and action of Man. The published record is patchy, but includes the burning of 5000 acres of pinewoods in the Selakano valley on 23 June 1969 (Zaharês, 1977) and of several square kilometres of pine forest above Monastiraki in 1987, in a conflagration that Rackham & Moody (1996) compare with a small atomic bomb in terms of energy release. These two places happen to coincide with the known occurrences of the Cretan mistletoe. But anyway, all Mediterranean pine stands are bound to burn down sooner or later.

Fire is likely a major problem for mistletoes, and may be the cause for their general rarity and patchy occurrence on Mediterranean pine trees. Sometimes a fire is not strong enough to kill the trees, and in that case the parasite might survive within the host and re-sprout along

with it, but we know nothing positive of potential fire resistance of mistletoe in general or of the Cretan taxon in particular. Survival as seed in the guts of dispersing birds, and migration from one unburned patch of wood to the next, are another likely answer. Whereas no direct observations of dispersal by birds have been made in Crete, one of the notorious dispersers, the mistle thrush *(Turdus viscivorus),* is known to occur on the island (Peterson et al., 1983). Birds play an essential role in mistletoe dispersal, but "the normal dispersal range of mistletoes is probably small ... and distance dispersal may depend on unusual events" (Barlow, 1983).

Whether endemic or not, old relict or newly evolved, *Viscum album* subsp. *creticum* is an interesting plant full of riddles for us to solve. This paper is but a first step destined to ignite its further study.

Acknowledgements

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