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Abstract

The *Helianthemum oelandicum* complex shows considerable morphological variation across its geographic distribution in Europe and western Asia. By combining four primer pairs and four restriction enzymes I identified nine cpDNA haplotypes with distinct geographical distributions. Two haplotypes were most frequent over most of the range; one in western Europe and one in eastern Europe. Moreover, differences in haplotype composition were much more strongly pronounced in the west-east direction than in south-north direction. There was no correlation between chloroplast haplotype and morphological variation. Parallel evolution in the regional populations produced similar morphologies without necessarily being closely related. The complex on Öland is represented by two endemic and allopatric varieties, differing in flowering phenology and indumentum. Variety *oelandicum* has a wide distribution, while var. *canescens* is restricted to small areas in the southernmost Öland. Only two, allopatrically distributed, cpDNA haplotypes are detected in the present study. The border between the distributional areas of these two haplotypes in southern Öland is distinct and cuts across extensive, more or less continuous populations of var. *oelandicum*. This border coincides with marked differences in the frequency of hairy and glabrous plants. The phylogenetic analysis of the ITS and cpDNA revealed poorly resolved trees in *Helianthemum oelandicum*; taxa were polyphyletic.

The *H. nummularium* complex is a morphologically variable species that has been subdivided into several subspecies, primarily based on indumentum characters. I investigated five of these subspecies for variation in chloroplast DNA and leaf and petal shape in Europe. The highest haplotype diversity was found in the Alps and the surrounding lowland areas, whereas marginal areas such as northern Europe and southeastern Balkans had low diversity. Most of the common haplotypes were shared between subspecies and showed geographic structures across the species range. Leaf and petal shape descriptors could not differentiate between subspecies. It is concluded that the poor correspondence between chloroplast haplotype distribution and subspecies circumscription is due to multiple origins of morphologically similar morphs (grouped into taxonomic subspecies) in different parts of the distribution range of the complex.

Key words: *Helianthemum oelandicum*, *H. nummularium*, cpDNA, morphometry, Pleistocene, genetic polymorphism, parallel evolution, introgression, postglacial migration.

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Eman Soubani

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II Soubani E, Hedrén M and Widén B. Colonization, establishment and introgressive hybridization between two post-glacial immigration lineages of *Helianthemum oelandicum* (Cistaceae) on the Baltic island, Öland, Sweden: evidence from chloroplast DNA haplotype and indumentum data. *Submitted*

III Soubani E, Hedrén M and Widén B. Phylogeography of the European rock rose *Helianthemum nummularium* (Cistaceae); incongruent patterns of differentiation in chloroplast DNA and morphology. *Manuscript*

IV Soubani E, Hedrén M and Widén B. Postglacial history of the rock rose *Helianthemum nummularium* in Scandinavia: combined cpDNA haplotype and indumentum suggests two postglacial immigration lineages. *Manuscript*

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Paper I: Participation in field collection; responsible for DNA extraction of most of the material and data collection; main responsible for data analysis; shared responsibility for manuscript preparation.

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Paper III: Main responsible for DNA extraction, data collection and analysis and shared responsibility for manuscript preparation.

Paper IV: Shared responsibility for data collection and analysis and manuscript preparation.

Paper V: Main responsible for data collection and analysis and manuscript preparation.

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INTRODUCTION

Patterns and levels of genetic diversity in plants, allows for detection and estimation of past and present evolutionary processes shaping the present genetic structure. Historical events (i.e. the Pleistocene glaciations), the species ecological requirements (i.e. selection pressure) and life history traits are responsible for shaping the genetic variation in species. Also, interactions between different evolutionary processes such as genetic drift and gene flow account for the present genetic structure of different species

Below the species level, the circumscription of taxonomic units or morphs is based on morphological characters supported by ecological factors of the species. However, taxa circumscription does not necessarily agree with the distribution of genetically defined lineages; taxa may not represent different genetic lineages.

In less well-studied groups such as species complexes, incongruence between the traditional taxonomy based on morphological characters and genetic data is a common problem. And the phylogeographic or phylogenetic analyses building on the present taxonomy may result in circular reasoning and fallacious conclusions. Therefore, taxa belonging to taxonomically complex groups should not be studied independently, on the contrary the whole group should be studied and treated as one species and that taxonomic subdivision must be discussed along with phylogeography and phylogeny.

Systematics

Systematics is defined as the "*scientific study of the kinds and diversity of organisms, and of any and all relationships between them*" (Simpson 1961). The field is continually incorporated with other fields of biology (such as ecology, palynology, genetics and phytpgeography) to describe the variation pattern in species in order to develop a significant system of classification. Modern approach to systematics aims to reconstruct the species evolutionary history with the help of various molecular techniques. It takes into considerations not only the basic traditional taxonomy of the species but in addition the latest evolutionary concepts or models and modes of data analysis. Most of the systematic studies usually investigate the relationships above the species level such as between genera and families without necessarily facing major conflicts or problems. However, it has often been problematic when investigating systematics below the species level especially within species complexes due to the complexity or insufficient amount of variation depicted (Singh 2004). Species complexes with unresolved taxa circumscription are found in some plant species such as in the genus *Helianthemum* (Cistaceae).

Phylogeography

Phylogeography is a means by which intraspecific genetic lineages are interpreted in the light of their geographic distributions. It has successfully interpreted the historical and the evolutionary events governing the distribution of plants at temporal and spatial scales.

The Quaternary cold periods in Europe starting 2.4 Myr ago had a dramatic influence on the distribution and differentiation of many plant species (Huntley & Briks 1983). The repeated contraction and expansion of species' ranges are considered one of the main causes of modification and reorganization of the genome structure within species (Hewitt 1996). The intraspecific differentiation was especially pronounced during the Pleistocene, and many extant lineages may have differentiated during the two most recent glacial periods (Comes & Kadereit 2003). During the last ice age (which ended about 15000 yr BP), glaciers covered large parts of northern Europe, including the whole of Scandinavia and northern Britain (Björck 1995, Svendsen *et al.* 2004). Two of the major mountain ranges in southern and central Europe, the Pyrenees and Alps, were almost entirely covered by ice caps, while the more eastern Carpathians and Balkans were less extensively glaciated (Charlesworth 1957, Messerli 1967). Between the main ice sheet in the north and the southern mountain ranges was a plain of permafrost covered by tundra and cold steppe (Hewitt 1999). Populations that inhabited the southern parts of Europe and Asia Minor, including the Iberian Peninsula, Italy, Turkey and the Balkans survived where the climate was more favourable and recolonized northern areas as glaciers withdrew (Hewitt 1996, Taberlet *et al.* 1998). Parallel to this latitudinal migration, are fluctuations in the altitudinal distribution of mountain and alpine species in southern and central European mountain ranges (e.g. Kropf *et al.* 2003, Ronikier *et al.* 2008). Some trees and shrubs could also have survived in pockets of favourable conditions in the southern parts of the steppe-tundra in eastern, central and south-western Europe (Willis *et al.* 2000, Carcaillet & Vernet 2001). Moreover, pollen from the steppe-tundra element was deposited close to the southern margins of the main ice cap during the last glacial maximum (Lang 1994). Species belonging to open habitats, e.g. *Helianthemum* were deposited in central Britain during the late glacial period, probably at the end of the Allerød Interstadial (12000 yr BP) and also in southern Scandinavia during the same period (Iversen 1944, Berglund 1966, Smith *et al.* 2005). Many temperate-adapted species may thus have survived the long, unfavourable Quaternary cold periods north of the southern European Peninsulas (Stewart & Lister 2001, Provan & Bennett 2008).

Evolution

The biological concept of evolution is "*change in the properties of populations of organisms, or groups of such populations, over the course of generations*" (Futuyma 1986). The modern research of evolutionary processes is based on the nature, origin and amount of genetic variation and the processes that govern this variation. The causes of evolution can be mutation, gene flow, genetic drift (i.e. bottleneck and founder effects) and adaptation to specific environments.

An interesting aspect of evolution is "parallel evolution or parallelism" and is defined as the independent evolution of similar phenotypic traits across geographically separated groups (i.e. populations or closely related species) that inhabit similar environmental conditions favoured by natural selection (Futuyma 1986). An

important outcome of this parallelism is the independent evolution of morphologically similar taxa across the species range followed by an increase in reproductive isolation between these taxa as explained by the "isolation of distance" theory. Ultimately, parallel evolution of similar but reproductively isolated taxa can play an essential and productive role in the early stages of speciation (Futuyma 1986, Schluter et al 2004).

Natural hybridization and introgression

Natural hybridization and introgression are common and play an essential role in evolution and speciation of different plants species. They produce new combinations of genetic material from divergent evolutionary lineages and have therefore strong influences on shaping the genetic structure and thus the evolutionary history of species (Arnold 1992, 1997).

A common consequence of hybridization and introgression is the formation of contact (between genetic lineages) or hybrid (between taxa) zones, induced by historical events e.g. the Pleistocene glaciations. When populations of a species representing different refugial areas, meet in the deglaciated areas, secondary contacts are established between the migrant populations. The hybrid or contact zones are characterized by high genetic diversity with clinal transition between different morphs and genetic lineages (Endler 1977, Hewitt 2000, Hewitt 2001).

AIMS OF THE THESIS

I have studied two species complexes belonging to the genus *Helianthemum* and family Cistaceae. Both *Helianthemum oelandicum* and *Helianthemum nummularium* are poorly taxonomically resolved and no consensus exists regarding the delimitations of taxa in any of the complexes. Molecular evidence has been urgently required to understand the evolutionary history of the two species, test the subspecific delimitations of species based on traditional morphological characters.

This thesis sought to investigate the genetic variation of the two species complexes by means of molecular markers supported by morphological characters to:

1. Study large-scales of chloroplast DNA variation in *Helianthemum oelandicum* (**Paper I**) and *H. nummularium* (**Paper III**) in Europe; to deduce the postglacial history of the two species; postglacial lineages and potential glacial refugia.
2. Study regional-scales of genetic differentiation in *H. oelandicum* (**Paper II**) and *H. nummularium* (**Paper IV**); to infer historical events and contemporary evolutionary processes responsible for shaping the observed pattern of variation.
3. Examine the utility of ITS and chloroplast DNA markers for inferring intraspecific phylogeny of *H. oelandicum* **(Paper V**).

STUDY SPECIES

The family Cistaceae comprises eight genera (*Cistus*, *Tuberaria*, *Croanthemum*, *Hudsonia*, *Halimium*, *Lechea*, *Fumana* and *Helianthemum*) and about 180 species that are widely distributed in the warm temperate regions of North America, North Africa and Western Eurasia (Arrington & Kubitzki 2003). The species of interest in this study is *Helianthemum* in Europe. Thirty-one *Helianthemum* species have been recorded in Europe with a centre of diversity in the Mediterranean region (see Fig. 1) (Flora Europaea, Tutin *et al.* 1968). Only two species have expanded northward into the temperate regions of northern Europe; *Helianthemum oelandicum* and *H. nummularium.* Both species are widespread in Europe and display a broad morphological and ecological variation across their geographical ranges. They are considered polymorphic species and the recognized subspecies are poorly defined.

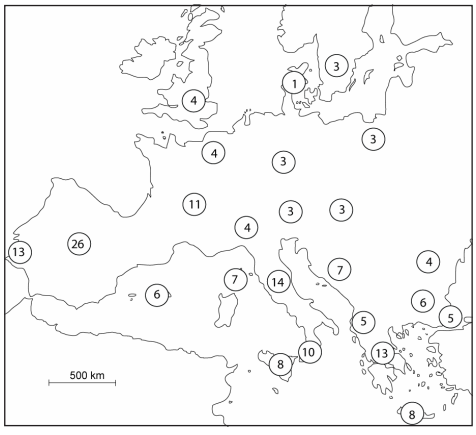

Figure 1. Distributional map and frequency of 31 *Helianthemum* species in Europe obtained from Flora Europaea (Tutin *et al.* 1968).

Helianthemum oelandicum (L.) Willd

The rock rose *Helianthemum oelandicum* (L.) Willd. (Cistaceae) is a diploid (2n = 22), self-incompatible, insect and wind pollinated perennial dwarf shrub. The species is restricted to calcareous soils and limestones in open lowland to alpine grasslands, often with exposed outcrops (Janchen 1907, Proctor 1956). The species is distributed mainly in central and southern Europe, and it also extends into Turkey and the Caucasus. Marginal populations occur in the British Isles and in Scandinavia, where the complex is an important component in the alvar grasslands of the Baltic island of Öland (Sterner 1936a&b). Isolated populations are also found in the Pinega area in northern Russia and in the Atlas mountain range in North Africa (Proctor 1956).

H. oelandicum comprises a variety of morphs that show complex variation in both indumentum and flowering phenology and has therefore been treated as a species complex (Janchen 1907, Sterner 1936a&b, Widén 1980, 2010). Three main types of trichomes are found in different combinations in the *H. oelandicum* species complex (Widén 1988): stellate hairs, bristles (or simple hairs) and glandular hairs. Indumentum may vary both within and between populations and shows considerable geographical variation even on the local scale (Widen 1988, 2010). Two flowering phenologies are recognized in the species complex, the concentrated flowering (CF) where the inflorescences are developed at the apex of the previous year's growth and the protracted flowering (PF) with inflorescences developed laterally on the current year's growth

as well as at the apex of the previous year's growth. Flowering phenologies have been studied thoroughly on Öland, where CF plants flower early in the season (early June), while PF plants flower throughout the season with one flowering peak in early June from inflorescences developed on the previous year's growth and another peak in July-August with inflorescences developed on the current year's growth (Widén 1980, 2010).

The taxonomy of the species complex in Europe has been treated in different ways. Janchen (1907) recognized five species: *H. canum* (L.) Baumg., *H. oelandicum* (L.) Willd., *H. italicum* (L.) Pers., *H. rupifragum* Kerner and *H. alpestre* (Jacq.) DC. Proctor in Flora Europaea (Tutin *et al.* 1968) divided the complex into two species based on the presence/absence of dense felt of stellate hairs on the abaxial surface of the leaves: *H. canum* with seven subspecies and *H. oelandicum* with five subspecies. Widén (2010) has revised the species-complex on Öland and recognized one subspecies *H. oelandicum* subsp. *oelandicum* with two varieties; var. *oelandicum* (plants with a CF phenology and without stellate hairs) and var. *canescens* (plants with a PF phenology and variable with respect to stellate hairs on the abaxial surface of the leaf).

In the present study, the taxonomic subdivision of the European part of the complex is treated as following. The complex is represented by one species, *H. oelandicum* with five subspecies. Plants from Öland were regarded as the subspecies *oelandicum*. Following Widén (2010) var. *oelandicum* (with the CF phenology) and var. *canescens* (with the PF phenology) are distinguished from each other based on the flowering phenology. The subspecies *alpestre* refers to an alpine taxon, usually with large flowers, from higher altitudes in the Alps, Pyrenees, Tatra Mountains, Carpathians and other high mountains in south-eastern Europe. The subspecies *rupifragum* is restricted to lower and intermediate altitudes in south-eastern Europe. The subspecies *italicum* (relatively small flowers) habituates low altitudes in the western and central Mediterranean areas. All morphs from continental Europe and the British Isles with a dense felt of stellate hairs on the lower side of the leaves were provisionally lumped together into the subsp. *incanum*, despite marked regional differences in the growth form, leaf and petal size, and indumentum (B. Widén unpublished). In several Mediterranean mountain areas morphs with and without stellate hairs on the lower side of the leaf may occur sympatrically (B. Widén & E. Soubani, personal observations). A sequence of two or three subspecies often occurs in distinct zones in many mountain ranges, usually in the order *italicum/rupifragum, incanum* and *alpestre* from low altitudes to the alpine zone, but also with *italicum/rupifragum* at intermediate altitudes. When tested, progeny from individual plants in mixed populations could be segregated based on the presence/absence of stellate hairs, showing hybridization and gene flow between morphs (B. Widén unpublished). Artificially produced hybrids between taxa in the species complex indicate weak crossing barriers between morphs in many but not all combinations (Widén 1986 and unpublished).

Two other closely related species were included as reference material: *H. hymettium* (Greece) and *H. marifolium* (Spain) (cf. Guzmán & Vargas 2009). They belong to the same section (*Plectolobum*) as *H. oelandicum*.

Helianthemum nummularium (L.) Mill

The rock rose *Helianthemum nummularium* (L.) Mill is a species complex that consists of evergreen, diploid (2n = 20), insect-pollinated and out-breeding dwarf shrubs that produce multiple racemes of yellow or pink flowers in early summer. It favours dry open grasslands, rocky places and meadows. The species complex has a wide distribution in Europe but disjunct populations can be found in Turkey, the Caucasus and northern Iran (Widén 2010). The morphological variation in *H. nummularium* is complex and especially the indumentum characters that have been considered to be taxonomically important (cf. Janchen 1909). According to Flora Europaea (Tutin *et al.* 1968), the *H. nummularium* complex consists of eight subspecies of which five display yellow flowers; *H. nummularium* (L.) Mill. subsp. *glabrum* (WDJ Koch) Wilczek, subsp. *grandiflorum* (Scop.) Schinz & Thell, subsp. *nummularium* (L.) Mill., subsp. *obscurum* (Čelak.) Holub and subsp. *tomentosum* (Scop.) Schinz & Thell. The subspecies *nummularium* and subsp. *tomentosum* are very similar in morphology and both have a dense cover of stellate hairs, at least on the lower side of the leaf. The subsp. *tomentosum* has larger leaves and petals and is considered to be an alpine taxon (Janchen 1909), whereas subsp. *nummularium* has a more diffuse distribution; occupying lowlands to alpine altitudes. The subspecies *obscurum,* subsp. *grandiflorum* and subsp. *glabrum* are all characterized by the absence of a dense cover of stellate hairs on the lower side of the leaf. The subspecies *glabrum* has glabrous (hairless) leaves, whereas subsp. *obscurum* and subsp. *grandiflorum* are characterized by the presence of long bristles on the leaves. The subspecies *grandiflorum* has considerably large petals, and the sepals are glabrous between the ribs as opposed to subsp. *obscurum*.

According to Tutin *et al.* (1968), Meusel (1978) and Hultén & Fries (1986), the complex has a wide distribution in almost all of Europe except for the extreme north. The subspecies *nummularium* is widespread in Europe. The subspecies *obscurum* is distributed in central Europe and parts of eastern and southern Europe and extends northwards into Sweden and Denmark. The complex shows the greatest diversity in mountain areas of southern and central Europe, where several subspecies are sympatric, such as subsp. *tomentosum,* subsp. *glabrum* subsp. *grandiflorum,* subsp. *obscurum* and subsp. *nummularium.* In the present study, the taxonomic designation of all plants was determined from the dried (cultivated) reference specimens based on the morphological characters described in Flora Europaea (Tutin *et al.* 1968). The five subspecies were arranged into two groups based on the absence/presence of a felt of stellate hairs on the lower side of the leaf. Group (1) included subsp. *glabrum*, subp. *obscurum* and subsp. *grandiflorum* which have no felt of stellate hairs on the lower side of the leaf. These three subspecies were distinguished from each other based on the previously described morphological characters. Group (2) included subsp. *nummularium* and subsp. *tomentosum* which have a felt of stellate hairs. However, I was not able to distinguish between subsp. *nummularium* and subsp. *tomentosum* based on the size of leaf and petal (as they overlapped broadly) or any other character and therefore the two subspecies were joined into one; subsp. *nummularium*.

METHODS

Three molecular and two morphometric methods have been carried out to investigate the levels and patterns of genetic and morphological variation of *Helianthemum oelandicum* and *H. nummularium.*

PCR-RFLP

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has been broadly applied for investigating genetic polymorphism at intraspecific level in both plant and animal species, where inferences about the species evolutionary history can be made. I have used the PCR-RFLP to search for chloroplast DNA variation in *H. oelandicum* (Paper I, II) and *H. nummularium* (Paper III, IV).

Chloroplast DNA microsatellites

Microsatellites or simple sequence repeats (SSRs) are hypervariable loci found in nuclear and organelle DNA. In the chloroplast genome microsatellites consist of tandemly repeated mononucleotides, generally <15 base pairs in length. They are neutral and have been extensively applied in a wide range of studies to investigate levels and patterns of chloroplast variation in wild plant populations of different species. I have designed three specific cpDNA microsatellites in three regions: *trnL-trnF*, the *trnL intron* (Taberlet *et al.* 1991) and *trnS-trnG* (Hamilton 1999) to look for genetic variation in *H. nummulariun* and infer the postglacial history of the species in Papers III and IV.

ITS and cpDNA sequencing

The advent of DNA sequencing has allowed the detection of greater genetic polymorphism and higher phylogenetic resolution compared to the previously described methods. The comparison between phylogenies of nuclear (e.g. ITS: internal transcribed spacer) and cytoplasmic (e.g. cpDNA: chloroplast DNA) genomes has become essential in many studies, especially when reticulate evolution and hybridization/introgression is expected in the studied species. A pilot study was performed to examine the utility of cpDNA and ITS markers for phylogenetic inference at intraspecific level in the *H. oelandicum* complex. I have sequenced the ITS and four noncoding cpDNA regions: *trnL-trnF,* the *trnL intron* (Taberlet *et al.* 1991), *trnS-trnT* (Demesure *et al.* 1995) and *trnT-psbD* (Widén *et al.* submitted) of 42 accessions belonging to *H. oelandicum* and three others belonging to two closely related species, *H. hymettium* and *H. marifolium,* and one outgroup, *H. nummularium* in Paper V.

Morphometry

Morphometry is a traditional and valuable method used to study the morphological variation of species in e.g. shape (i.e. leaf) and density (i.e. hairs) of characters. Since morphometric analyses produce quantitative description of characters, it allows for a more accurate estimation of variation and therefore rigorous comparisons. I have used an automated image acquisition program (ARBO, White *et al.* 1988) and statistical methods to describe the shape of leaf and petal in 96 popu-

lations of *H. nummularium* in Europe (Paper III) and the shape of leaf in 70 populations of *H. nummularium* in Scandinavia (Paper IV).

The hairiness in *H. oelandicum* (Paper II) was categorized accordingly: (i) presence/absence (1/0) of bristles (BR) on leaf and (ii) presence/absence (1/0) of a dense cover of stellate hairs (ST) on the lower side of the leaves.

In the Scandinavian *H. nummularium* (Paper IV) the variation pattern in hairiness based on the density of stellate hairs on the lower side of the leaf was scored accordingly, 0: no stellate hairs, 1: stellate hairs mainly on the mid-rib, 2: cover of large, sparse stellate hairs on the leaf surface and 3: a dense grey cover of stellate hairs all over the leaf surface. Individual samples can fall between these four categories and are thus given any of these scores 0, 0.5, 1, 1.5, 2, 2.5 or 3. Plants with hair index of 0–2 were assigned to subsp. *obscurum* and plants with hair index of 2.5–3 were assigned to subsp. *nummularium*.

RESULTS AND DISCUSSION

Despite the fact that *H. oelandicum* and *H. nummularium* belong to the same genus within the family Cistaceae, and share many life history traits (i.e. perennial and outbreeding), they differ in the distributional patterns; *H. oelandicum* is relatively confined to areas with open calcareous bedrocks, which has an island-like distribution whereas *H. nummularium* has a more diffused and widespread distribution. Furthermore, the two species do not share similar postglacial migration histories. They differ in the timing, rates and direction of their migrations after the last ice age. Apparently the two species reacted individually during the Pleistocene period by tracing its particular set of environmental and ecological requirements (cf. Huntley 1991).

Phylogeography of *H. oelandicum*

A total of 91 populations belonging to *H. oelandicum* and distributed across the species range in Europe were analysed (Fig. 2). Seven polymorphic primer-enzyme combinations identified a total of nine chloroplast DNA haplotypes in the *H. oelandicum* complex plus *H. marifolium* and one haplotype (H10) in the closely related species *H. hymettium*.

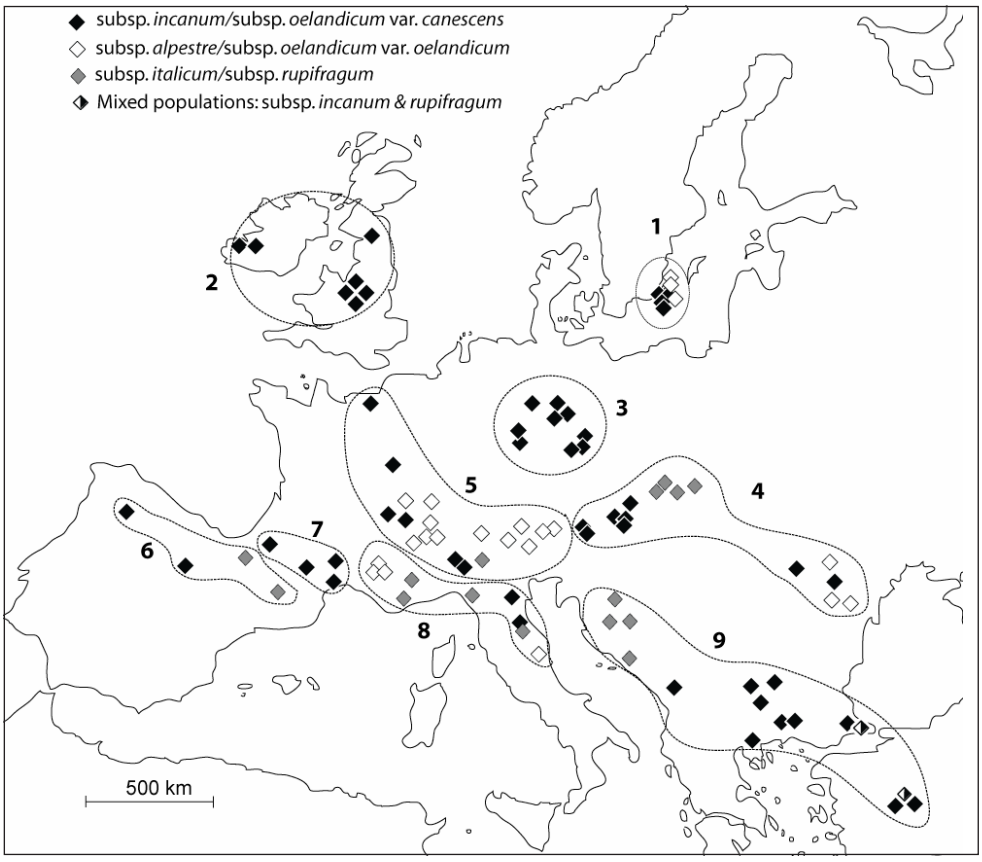

Figure 2. Location of the 91 populations of the *Helianthemum oelandicum* complex studied. Taxonomic affiliation is indicated by different symbols. The populations were grouped into nine regions for comparison of genetic diversity (see Paper I for details).

Postglacial history

The geographic distribution of cpDNA haplotypes was highly structured. The number of haplotypes is greatest in the south-western part of the geographical area covered by the complex (Spain and the Alps surroundings), with a pronounced decrease in the numbers of haplotypes in the northern and eastern parts of the distribution range (see Fig. 3a).

The two most frequent haplotypes - H1, characterizing a western, central and northern lineage and H2, characterizing an eastern, central and northern lineage - meet in central Europe north of the Alps and on the Baltic island of Öland. H1 may have persisted in one or several potential refugia in areas north of the Pyrenees and west/north-west of the Alps and expanded to Scandinavia and western Europe during or after the last ice age. The H2-lineage may have persisted in the periglacial zone and/or re-colonized the eastern part of central and northern Europe from potential refugia in the Balkans (Fig. 3a). The H3-lineage is confined to the regions

surrounding the Alps and middle Italy. The presence of potential glacial refugia on calcareous bedrock along the border of the Alps has also been suggested for several mountain plants (Schönswetter *et al.* 2005). The northern boundary between H3 and the two most frequent haplotypes (H1 and H2) may suggest an earlier arrival of the two latter haplotypes, which may have blocked a further northward expansion of H3 (Taberlet *et al.* 1998). A contact zone between adjacent populations of H1 and H3 is evident in the south-western part of the Alps. Another contact zone between the H2- and H3-lineages in the eastern part of the Alps is suggested. H2 has also been detected in two populations in Southern Alps and one in central Italy (Fig. 3a, 3b).

Most haplotypes were found in the Italian peninsula south of the Alps (the three major haplotypes and two rare ones) Fig. 3a). H8 was found in two populations in northern-central Italy; in an area that Schönswetter *et al.* (2005) identified as a southern-alpine peripheral refugium for mountain plants. H9, which differs from H3 by two polymorphic sites, is found in a single population in central Italy. Haplotype H7 was only found in one population in southern France.

The two related and distinct haplotypes H4 and H5 (Fig. 3a, 3c) have been found only in Spain. They possibly survived several glaciations in the Iberian Peninsula, but did not expand to the rest of Europe after the last ice age. H6 is quite distinct (Fig. 3a, 3c), and has only been found in a deglaciated area along the coast of the Adriatic Sea. It may have been present in this region throughout the Pleistocene and may represent an old evolutionary lineage that had been early separated from the *H. oelandicum* lineages. Adding to that, the plants with H6 have some unique morphological traits not found in the complex (B. Widén and E. Soubani, personal observations).

Evolution

The three most common cpDNA haplotypes (H1, H2 and H3) comprised the whole range of morphological variation in the complex. For instance, plants with H1 are assigned to subsp. *incanum*, *alpestre*, *italicum*, *oelandicum* var. *oelandicum* and *oelandicum* var. *canescens*. Among H2 plants we find subsp. *incanum*, *alpestre*, *rupifragum* and *oelandicum* var. *oelandicum*, while H3 comprises subsp. *incanum*, *alpestre* and *italicum*. The remaining cpDNA haplotypes sampled from more than one population were either restricted to one or two taxa.

The traditionally recognized taxa in the *H. oelandicum* complex are based on few morphological characters (stellate hairs, flowering phenology and to some extent leaf and petal size), but also on altitudinal distribution in Europe. All taxa show considerable morphological variation in the growth form, shape of leaf, petal, capsule, inflorescence and seed and in the distribution of indumentum on different parts of the plant (Janchen 1907, Sterner 1936 a&b, Proctor 1957, Davies 1975, Widén 1980, 1988, 2010). All these traits are stable in cultivation (B. Widén personal observation), only the proportion of inflorescences borne on the current year's growth shows phenotypic plasticity (cf. Widén 1980).

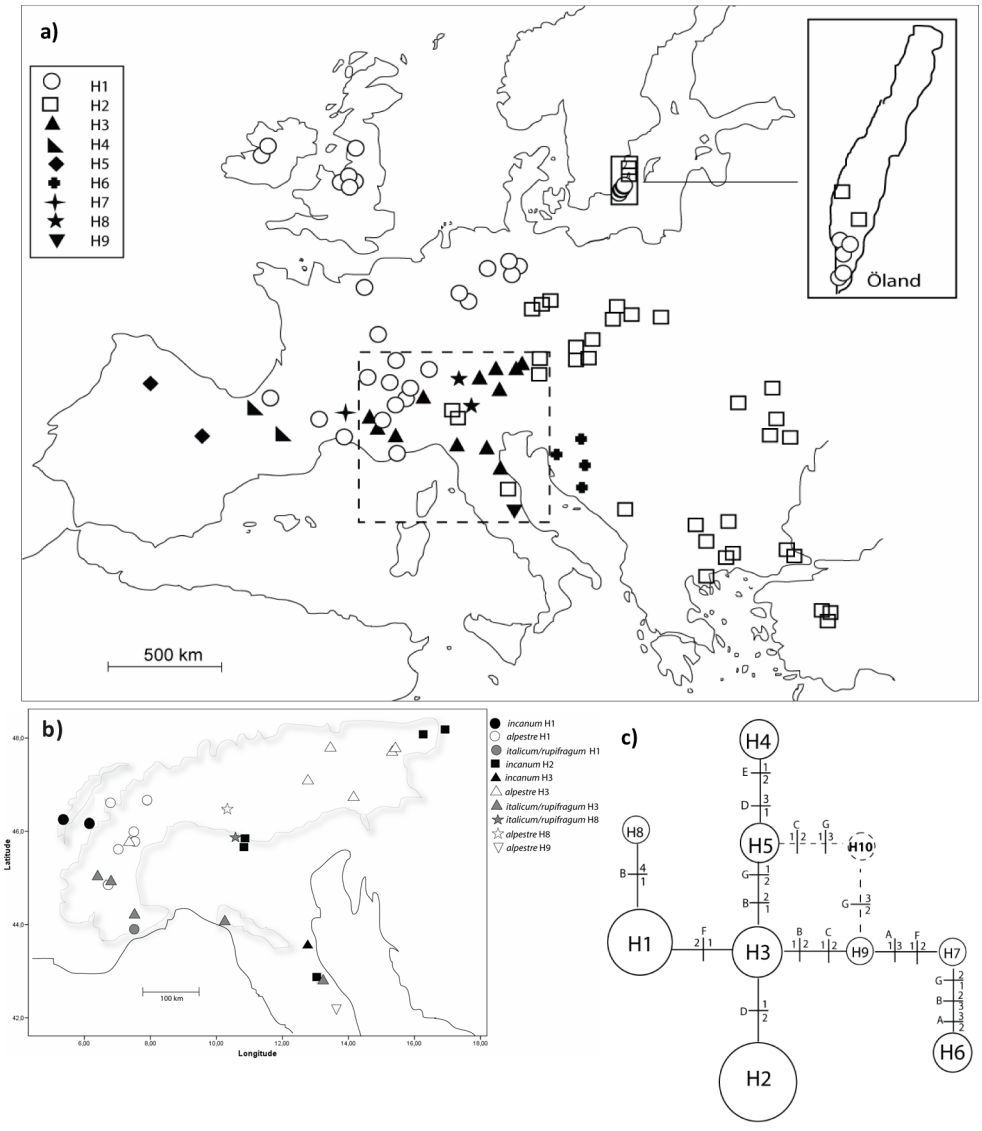

Figure 3. a) Distribution of the nine cpDNA haplotypes identified in the *H. oelandicum* complex in Europe and Turkey. The taxonomic affiliation of haplotypes in the Alps and northern Italy (framed by dotted line) is given as Fig. 3b. b) Map of the Alps and northern Italy showing the locations of the sampled populations of the *H. oelandicum* complex. The shape of the symbols denotes the haplotypes and shading denotes the taxonomic affiliation. c) Minimum spanning network summarizing the relationship between the nine cpDNA haplotypes detected in the *H. oelandicum* complex. H10 was only present in *H. hymettium*. The size of the circles is proportional to haplotype frequency. The superimposed lines correspond to mutation sites with character state inferences based on the results given in Paper I (Table 2).

Based on the morphological variation of the complex, there are various indications of hybridization and introgression between taxa. On Öland, where detailed studies have been performed, a narrow hybrid zone between the distribution of *oelandicum* and *canescens* has been established (Widén 1980, 1988 and unpublished, Soubani *et al.* submitted). When seeds from individual plants in a polymorphic Turkish population of subsp. *incanum* were sown in cultivation, the progeny segregated into two categories; with and without stellate hairs on the lower side of the leaves (B. Widén & E. Soubani, personal observation).

There is a remarkable parallel distribution of taxa in the mountain ranges of the European Alpine system. The different taxa are often distributed in altitudinal zones where populations may be allopatric or parapatric, but sometimes also sympatric. It is also important to point out the similarity between the parallel distribution of taxa in the European Alpine system and the parapatric distribution of the two varieties of subsp. *oelandicum* on Öland (Widén 1980, 1988, 2010, Soubani *et al.* submitted). The varieties *oelandicum* and *canescens* cover the variation in indumentum and flowering phenology traditionally used to delimit taxa in the whole complex.

Postglacial history of H. oelandicum on Öland

Helianthemum oelandicum subsp. *oelandicum* on the Baltic island of Öland is represented by two endemic varieties, which differ in their flowering phenology (var. *oelandicum* has a CF phenology, and var. *canescens* has a PF phenology) and indumentum (bristles and stellate hairs). Variety *oelandicum* varies from glabrous to hairy plants with bristles on leaves, sepals and inflorescences. Variety *canescens* varies from plants with bristles to plants with both bristles and a dense cover of stellate hairs on the lower side of the leaves. The presence of a felt of dense stellate hairs on the lower side of the leaf, was almost restricted to var. *canescens* and only sporadically occurring in var. *oelandicum* (Widén 1988).

The two varieties are allopatrically distributed; var. *canescens* is restricted to the southernmost part of Öland and var. *oelandicum* has a wide distribution on the Great Alvar and on isolated alvars in middle and north of the island (Fig. 4). However, in the southernmost part of the Great Alvar, where the two varieties meet, a narrow hybrid zone has been established (Widén 1980, 1988 and unpublished). The habitat of var. *canescens*, at least in areas where the two varieties meet, tends to be drier and better drained than the habitat of var. *oelandicum* (cf. Sterner 1936a, Widén 1988), giving rise to an ecological separation between the two varieties.

The two haplotypes H1 and H2, already identified (see above) are mainly allopatric with a narrow, U-shaped southeast-northwest/southwest-northeast contact zone in the southern part of the Great Alvar, extending from the village Segerstad in the east to the village Smedby in the west (Fig. 5). H1 is mainly distributed in the middle/southern part of the Great Alvar, in a disjunct area in the northern part of the Great Alvar (Resmo area), and in two isolated alvars in the southernmost part of Öland. H2 has a more continuous distribution extending from the middle part of the Great Alvar to the northern part of the island, except

for the small area (Resmo) which is fixed for H1 in the northern part of the Great Alvar.

In the southern part of the Great Alvar, across the contact zone between H1 and H2, a clear relationship between the distribution of haplotypes and variation in indumentum was observed (Fig. 5, 6). Hairy plants with H1 dominated the southern and western parts of the contact zone while glabrous plants with H2 dominated the northern and eastern parts of the zone.

The most plausible scenario for the postglacial immigration of *H. oelandicum* into Öland is two lineages; var. *canescens* (chloroplast haplotype H1) and var. *oelandicum* (chloroplast haplotype H2), migrating along the same route but at different times. An early stage of unidirectional gene flow from var. *oelandicum* to var. *canescens*, produced three morphological/-cytoplasm lineages; var. *canescens* (with haplotype H1), var. *oelandicum* (with haplotype H1) and var. *oelandicum* (with haplotype H2). The present geographical pattern of diversity in indumentum and flowering phenology may reflect both migration history, a long period of restricted distribution and natural selection in different habitats on Öland.

Figure 4. The distribution of the two varieties of *H. oelandicum* on Öland according to Sterner (1936b) and Widén (1980, 1988). The distribution of the var. *canescens* is restricted to the "black shaded" southernmost part of the Great Alvar and small alvars. Whereas, var. *oelandicum* is common in the remaining parts of Öland (grey shaded) both on the Great Alvar and in small alvars north of the Great Alvar. The two varieties are spatially separated by a narrow hybrid zone in southern Öland.

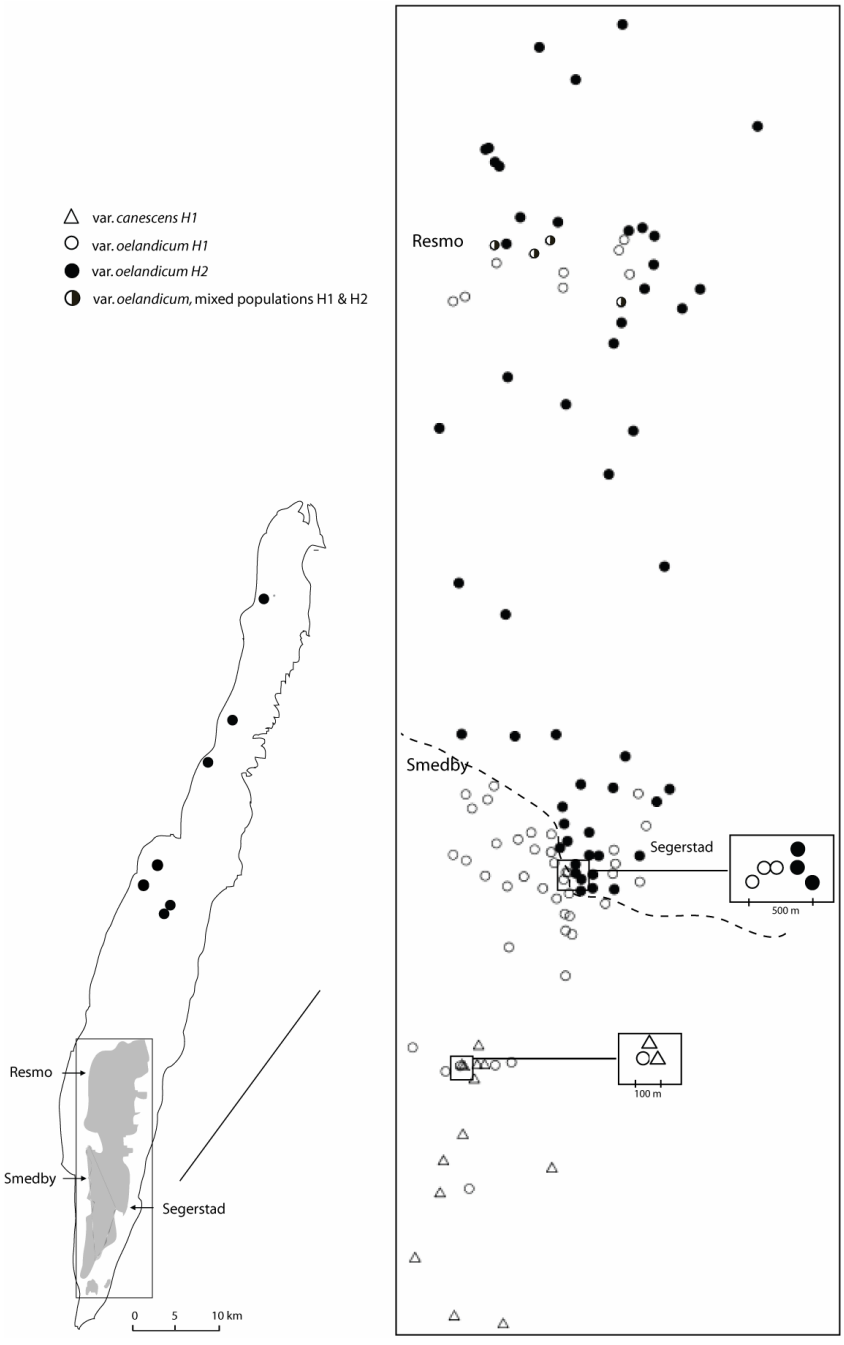

Figure 5. The distribution of the two cpDNA haplotypes on Öland combined with taxa specification (var. *oelandicum* and var. *canescens*). The symbols represent the populations analyzed in this study. The rectangle-marked area represents the Great Alvar and two alvars south of the Great Alvar. The dashed segment shows the genetic boundary detected with Monmonier's Maximum Difference algorithm in southern Öland.

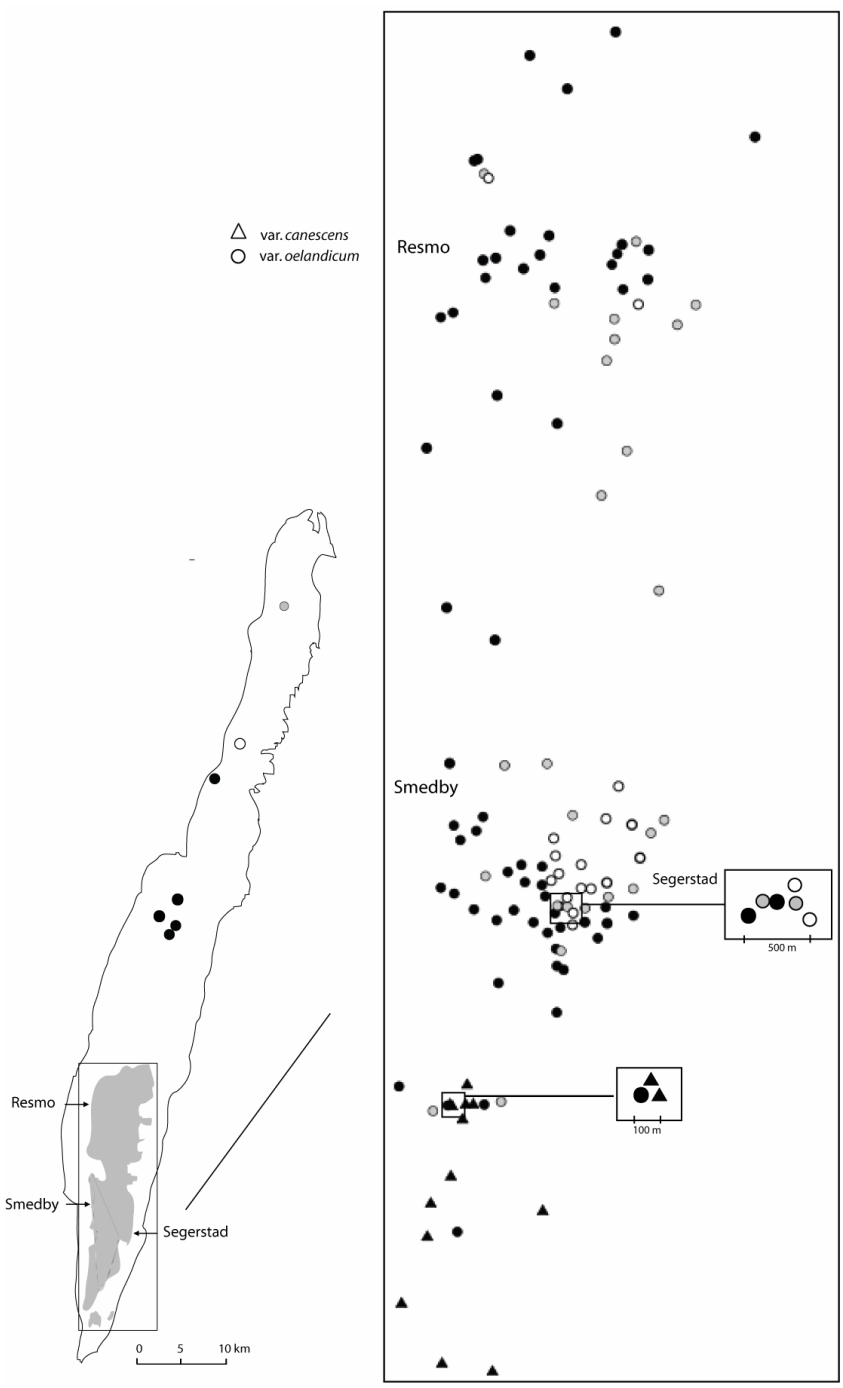

Figure 6. The distribution of taxa combined with bristles specification. Filled symbols denote presence of bristles in 4-5 individuals of the total 5 plants sampled per population, shaded symbols denote presence of bristles in 2-3 individuals/population and empty symbols indicate presence of 0-1 individuals/population with bristles.

Phylogeny of *H. oelandicum*

A total of 44 accessions belonging to the *H. oelandicum* complex (19 subsp. *incanum*, four subsp. *italicum*, four subsp. *rupifragu*m, ten subsp. *oelandicum* var *oelandicum*, three subsp. *oelandicum* var. *canescens* and two subsp. *alpestre*) and two closely related taxa: *H. hymettium* and *H. marifolium* were sequenced and further analyzed. Three potential out-groups were chosen to the species complex, *H. scopulicolum*, *H. squamatum* and *H. nummularium*.

Both ITS (Fig. 7) and the four combined cpDNA regions (Fig. 8) provided a weak phylogenetic substructure within *H. oelandicum* complex. Despite the marked morphological and ecological variation within *H. oelandicum,* these markers have performed poorly in resolving the relationships between species and also between cpDNA haplotypes. The observed polyphyly in *H. oelandicum* may reflects features of allelic histories in the ITS and cpDNA that provide significant insights into the species history. Nevertheless, the observed polyphyly may reflect a weak phylogenetic signal due to inadequate information obtained from the gene trees.

Sequence divergence and potentially informative characters varied slightly between the ITS and combined cpDNA regions. There are some conflicts between the ITS and cpDNA phylogenetic trees but the high number of collapsed branches and the polyphyletic grouping of the six taxa and the two closely related species is predominant in both markers.

Phylogeography of *H. nummularium*

A total of 115 populations belonging to *H. nummularium* and distributed across the species range in Europe were analysed (Fig. 9). Three chloroplast DNA microsatellites were amplified by means of species specific primers and 18 combined haplotypes were identified. The hierarchical partitioning of moment invariants of leaf and petal shape demonstrated that most of the differentiation was found between populations within subspecies. Neither the cpDNA microsatellites nor the morphometric analysis showed any differentiation pattern corresponding to taxonomic subdivision of the *H. nummularium* complex.

Postglacial history

The cpDNA microsatellites detected high haplotype diversity and demonstrated that there is a complex pattern of genetic differentiation of *H. nummularium* in Europe (see Fig. 10, 11). The Alps and the surrounding lowland areas constituted the centre of diversity with a total of 11 haplotypes. Peripheral areas, on the other hand, (e.g. northern Europe and south-eastern Balkans), displayed less chloroplast haplotype diversity. The presence of high genetic diversity in the Alps can either be explained by primary diversity (e.g. in surrounding refugia), and/or by secondary admixture via dispersal between different genetic lineages of taxa with refugia located elsewhere (Petit *et al.* 2003, Taberlet *et al.* 1998). The diversity pattern in *H. nummularium* indicates a west-Mediterranean origin of the species, a pattern typical for many members of the Cistaceae (Guzmán & Vargas 2009, Widén *et al.* submitted).

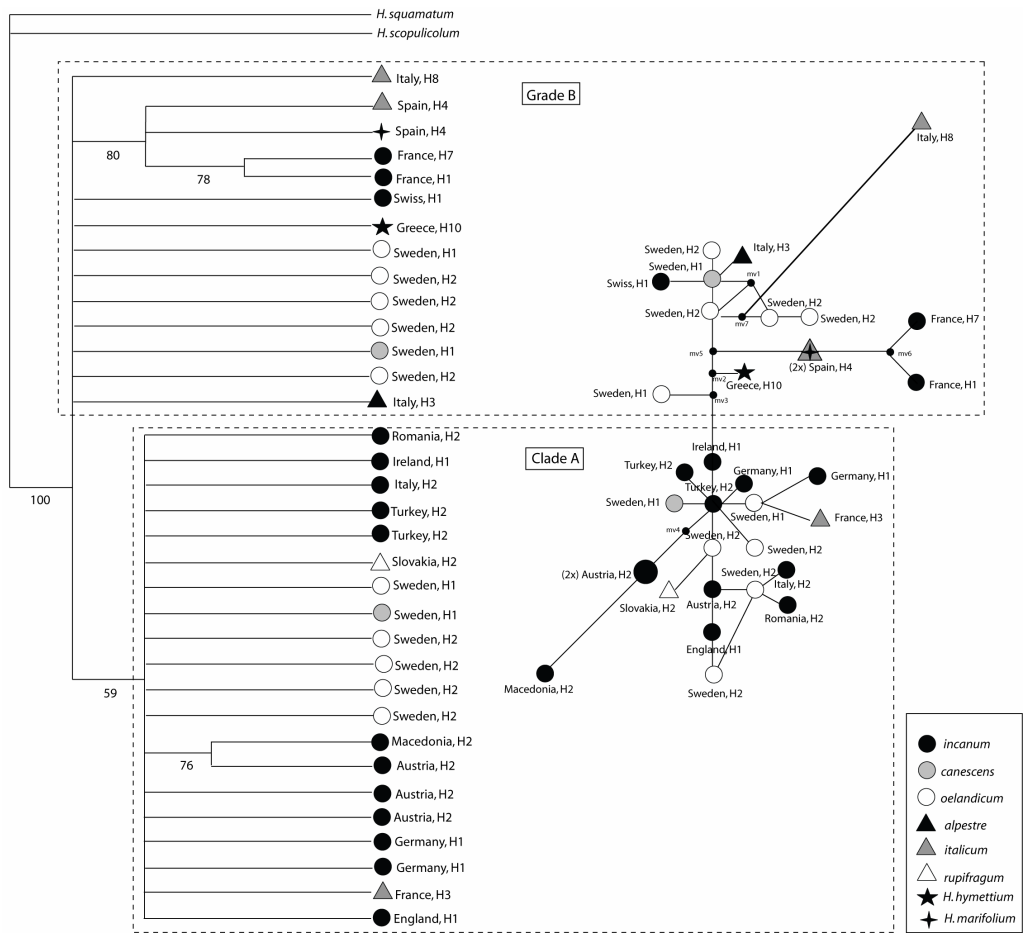

Figure 7. Left: The 50% majority–rule consensus tree of 511 equally parsimonious trees of the *H. oelandicum* complex, *H. hymettium* and *H. marifolium* based on ITS sequences (total length 134 steps; CI = 0.649, RI = 0.675). The tree is rooted with two outgroups, *H. squamatum* and *H. scopulicolum.* Right: The median-joining network based on ITS data. Distances correspond roughly to the number of differences between the accessions. Seven-median joining vectors (mv) are included in the network denoted by small black dots.

Most of the cpDNA haplotypes displayed overlapping distributions in Europe (Fig.10b-d, 11). Still, six of the most frequent haplotypes showed more or less distinct geographical distributions. A total of ten rare haplotypes were found, and seven of these were restricted to the Mediterranean regions. The two closely related haplotypes H3 and H6 have a restricted occurrence and are well established in Scandinavia and Estonia; suggesting the presence of a potential refugium in eastern Europe (e.g. Russia, not covered in this study) in which these haplotypes may have survived (cf. Van Rossum & Prentice 2004). Haplotypes H12 and H14 are closely related and are mainly found in the Alps and surrounding areas (Fig. 11). H12 is concentrated mainly in western Alps, whereas H14 has a wider distribu-

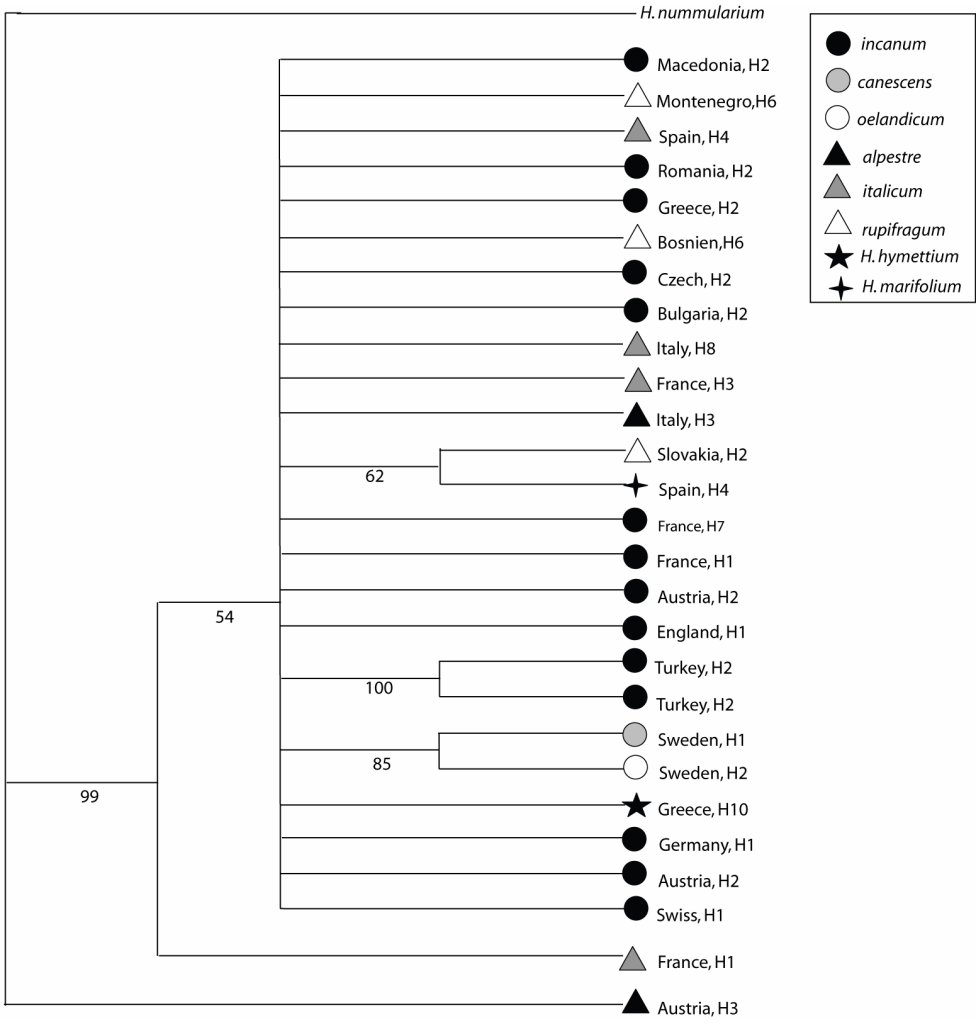

Figure 8. The 50% majority–rule consensus tree of 1342 equally parsimonious trees of *H. oelandicum*, *H. hymettium* and *H. marifolium* based on combined cpDNA regions (total length 1510 steps, CI = 0.547, RI = 0.200). The tree is rooted with one outgroup, *H. nummularium.*

tion in areas surrounding the Alps. The distribution of H12 and H14 suggests the presence of potential glacial refugia in the Alps and the surrounding area. Surprisingly, H12 was also found in one plant individual on Åland, Finland and this may suggest two scenarios: (i) a long distance dispersal; subspecies *obscurum* was first recorded in southern Finland in 1951 (out of its natural range in Europe) and persisted to the late 1950's. It probably arrived accidentally to southern Finland during the Second World War (Widén 2010) and/or (ii) perhaps more likely, occurrence of an independent mutation from the widespread haplotype H5.

Haplotypes H5, H4, H2 and H1 were the most frequent haplotypes and widely distributed across the species´ range (Fig. 10b-d). They are genetically closely rela-

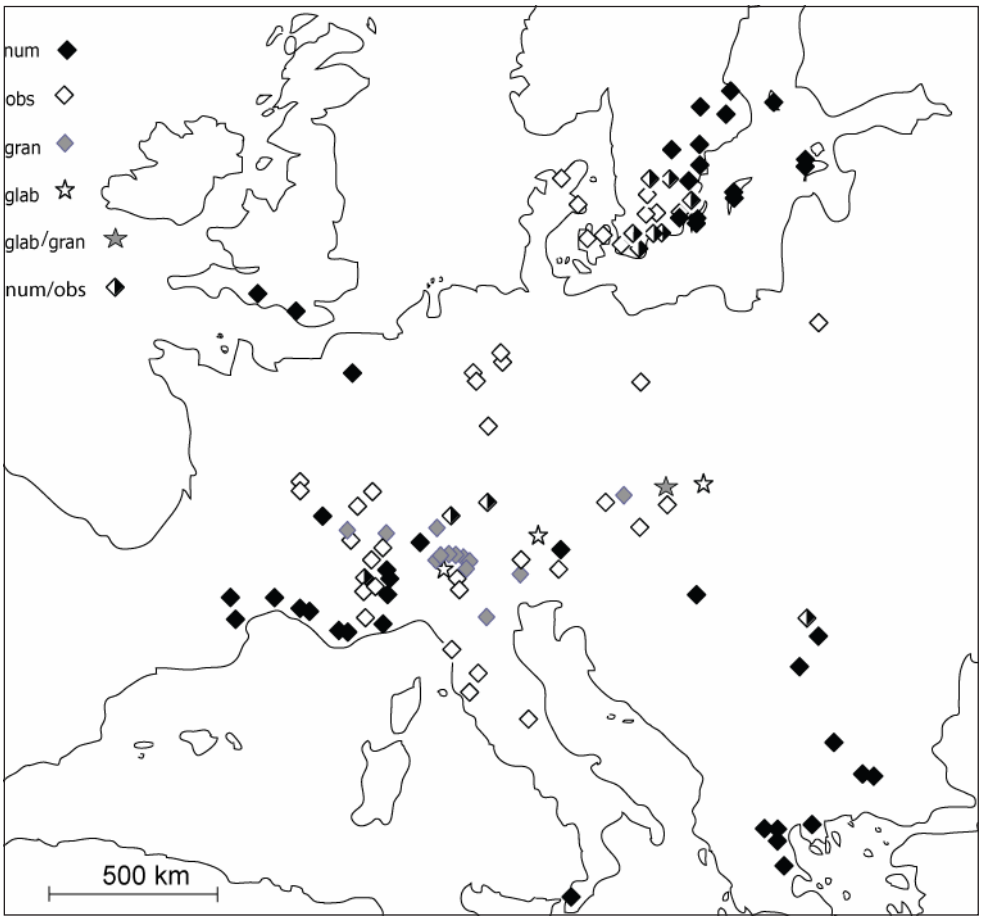

Figure 9. The distribution of 115 sampled populations of the *H. nummularium* complex in Europe. Each symbol represents a population with taxa specification according to Flora Europaea (Tutin *et al.* 1968), num: *nummularium,* obs: *obscurum*, gran: *grandiflorum*, glab: *glabrum*.

ted and their distributions overlap considerably in southern, central and northern Europe. At least three main refugia located in southern Europe (e.g. north and south of the Alps) and south-eastern Europe (e.g. the Balkans) should have contributed to the northward expansion of *H. nummularium* after the last ice age. These refugia must have contained the haplotypes H1, H2, H4 and H5, which subsequently met and admixtured in several contact zones during range expansion into ice-free territories. It is also possible that H1, H2, H4 and H5 may have survived in refugia located in the ice-free tundra areas close to the periglacial zone; south of the main ice sheet. H5 and H2 were absent from western continental Europe and the British Isles, but present in eastern Europe and Scandinavia, which

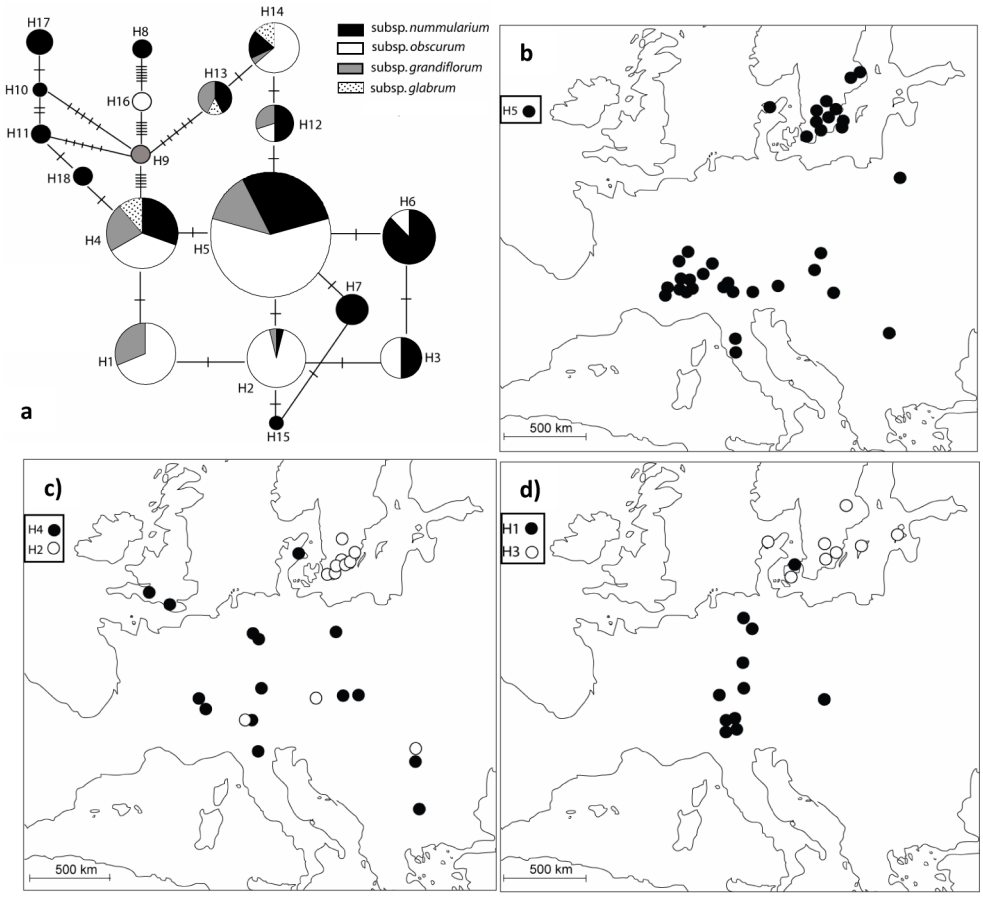

Figure 10. a) Minimum spanning network of the 18 cpDNA haplotypes detected in *H. nummularium*. The size of each pie diagram is proportional to the number of individuals carrying the particular haplotype and the sectors report the haplotype relative frequency in different subspecies. The frequency of hash lines on the connection lengths is proportional to the squared R_{ST} size difference between a haplotype pair. b-d) Distribution of the most common cpDNA haplotypes of *H. nummularium* in Europe (see each panel for details).

support an eastern immigration lineage to Scandinavia. In contrast, the absence of H1 and H4 from Sweden (even though both are present in Denmark), supports a separate, south-western immigration route into western Scandinavia.

Most of the rare haplotypes may represent ancient and relict refugia that remained isolated in the Mediterranean region as well in the mountainous regions in southern Europe. For instance, three haplotypes were restricted to the Pyrenees and did apparently not contribute to the northward expansion (see Fig. 11).

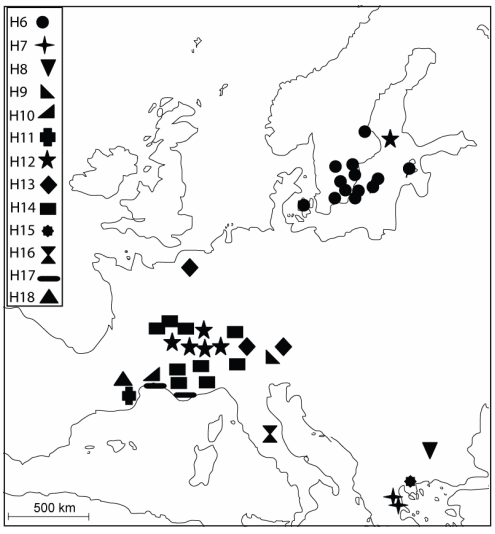

Figure 11. A distributional map of 13 haplotypes, H6-H18 of the *H. nummularium* complex in Europe.

Evolution

No correspondence was found between taxonomy and cpDNA haplotype distribution or relationships (MSN, Fig. 10a). Accordingly, the subspecies do not represent different genetic lineages. All the five subspecies share several haplotypes with one another. The sharing of haplotypes among taxa may be due to homoplasy, introgression or incomplete lineage sorting from a polymorphic ancestor (Petit *et al.* 2002, Palmé *et al.* 2004, Heuertz *et al.* 2006). However, homoplasy in *H. nummularium* is unlikely to be the major process shaping the present genetic variation because there is a geographic structure in the distribution of the most frequent cpDNA haplotypes (cf. Vogel *et al.* 2003). Assuming that haplotype divergence preceded "subspeciation", incomplete lineage sorting from a polymorphic ancestor may explain the sharing of cpDNA haplotypes among taxa. Haplotypes H1-H6 and H12-H14 are the most common ones, and probably represent ancient haplotypes that are likely to have been present in the common ancestor of *H. nummularium*. Some of the frequent haplotypes differ in geographic distribution in the different subspecies, which is another indication of incomplete lineage sorting. The tendency for populations of different subspecies to share the same haplotype in a given region may be explained by local hybridization/introgression. Also, around 23% of the total number of populations were polymorphic for haplotypes, and some populations contained a mixture of subspecies (e.g. in Sweden) which may further indicate past and/or present introgression.

The examined four subspecies within the *H. nummularium* complex had wide zones of overlap in the canonical variates analysis (CVA) obtained from leaf and petal shape descriptors (see Fig. 12). Accordingly, the indumentum characters presently emphasized in the subdivision of *H. nummularium* into subspecies are poorly correlated to the overall morphology. Obviously, the indumentums and leaf and petal shape variation pattern are more functionally and/or developmentally related than taxonomically (Rieseberg & Soltis 1991).

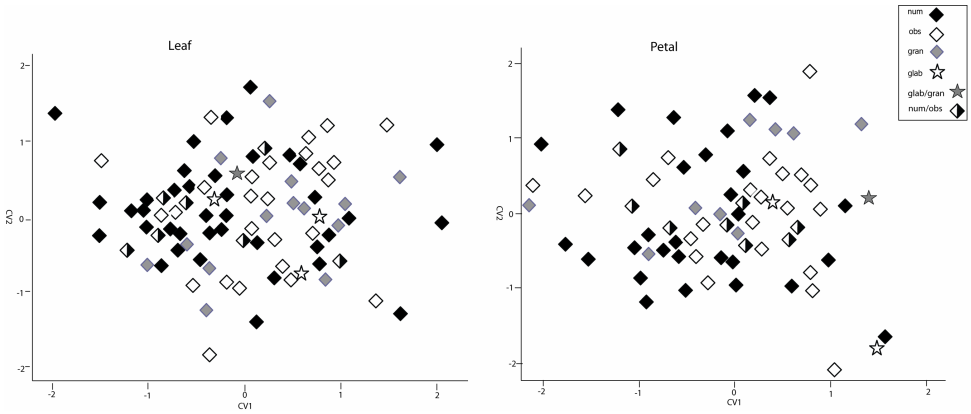

Figure 12. Canonical variates analysis (CVA) of morphometric shape analysis data (moment invariants) in the European *H. nummularium* using population as a grouping variable. Left panel: CVA based on leaf shape descriptors including 96 populations and 586 plants (n = 1172). The first and second CVs accounted for 39.6% and 23.6%, respectively, of the total variance. Right panel: CVA based on petal shape descriptors including 77 populations and 309 plants (n = 927). The first and second CVs accounted for 38.7% and 25.8%, respectively, of the total variance. Each symbol represents a population centroid with taxon specification, num: *nummularium,* obs: *obscurum*, gran: *grandiflorum*, glab: *glabrum*.

Postglacial history of the Scandinavian H. nummularium

Helianthemum nummularium has two subspecies in southern Scandinavia that differ in presence/absence of a dense felt of stellate hairs on the abaxial surface of the leaf. The subspecies *obscurum* (without a dense felt) occurs in southwest whereas subsp. *nummularium* (with a dense felt of stellate hairs) occurs in southeast Scandinavia. The distributions of the two subspecies overlap in a wide contact zone, where natural populations consist of a mixture of the two subspecies in various proportions (Fig. 13). There is no significant difference in the leaf shape between the two subspecies although considerable differentiation in indumentum is observed across the contact zone. Twenty-seven populations displayed six chloroplast DNA haplotypes in Scandinavia. The distribution of four of these haplotypes (H2, H3, H6 and H5) supports an eastern postglacial immigration route into Scandinavia whereas the distribution of two remaining haplotypes (H1 and H4) supports a south-western immigration route into western Scandinavia (see Fig. 10b-d, 11). In a wide contact zone between the two lineages in south-east Scandinavia, hybridization and introgression have produced a range of intermediate populations with high variability in indumentum density.

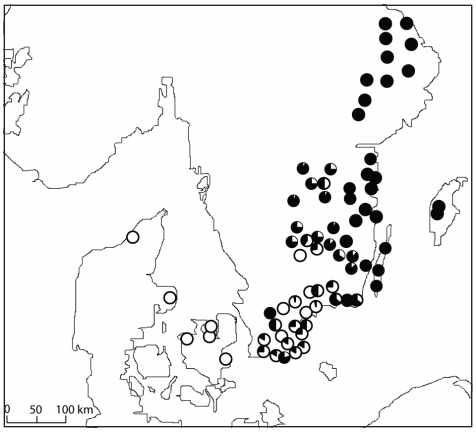

Figure 13. The pie diagrams describe the frequency of the subsp. *nummularium* and subsp. *obscurum* in the sampled populations. The white sectors refer to subsp. *obscurum* and the black sectors to subsp. *nummularium.*

CONCLUSIONS

The distribution of cpDNA haplotype lineages of the *H. oelandicum* complex shows a distinct geographic pattern with three widespread, but allopatric lineages. The morphologically homogenous cpDNA lineages H4, H5 and H6 may represent older evolutionary lineages that never managed to re-colonize areas beyond their glacial or interglacial refugia. If the European Alpine System is considered, it reveals a pronounced parallel differentiation in morphology from lowland to high alpine altitudes that have evolved in three of the most frequent haplotype lineages. The distribution of haplotypes may be due to old dispersal and divergence patterns in the complex, whereas the morphological groups recognized as traditional taxa have arisen independently in many mountain areas as adaptation to specific environmental conditions, e.g. related to altitude. Our results also support periglacial refugia for the complex north of the alpine glacial shields and probably also in central-east Europe. The genetic differentiation in *H. oelandicum* depends primarily on vicariance patterns in a west – eastern direction established during Pleistocene, and secondarily on postglacial migration patterns in south – north direction.

The contact zone between the distributional areas of the two haplotypes of *H. oelandicum* in southern Öland is distinct and cuts across extensive, more or less continuous populations of var. *oelandicum*. This border coincides with marked differences in the frequency of hairy and glabrous plants. The *oelandicum* variety, with the chloroplast haplotype H1, has probably arisen by unidirectional flow of nuclear genes governing morphological recognition of var. *oelandicum* into populations of var. *canescens*.

The current systematics of *H. oelandicum* is based on morphological characters that do not reflect the evolution of the species as all the subspecies appear polyphyletic. The high number of unresolved internal nodes, and the level of incongruence between the ITS and cpDNA trees do not support the subdivision of *H. oelandicum* into clear subspecies. Instead, the species is probably of recent origin and therefore the genetic differentiation has not yet reached the level that gives a clear phylogenetic signal.

The geographical distribution of some rare cpDNA haplotypes in *H. nummularium* in the Mediterranean area may indicate the existence of Pleistocene refugia with a long history in this region, whereas the distribution of common and closely related haplotypes could be used to infer putative migration routes and periglacial

refugia during the last glacial cycles. The complex morphological variation patterns in *H. nummularium* are not correlated with the distribution of haplotypes and do not represent any phylogenetic groups in the species. Instead, the morphological diversity in the species appears to have been shaped by historical processes, e.g. Pleistocene expansion-contraction cycles, contemporary processes, e.g. hybridization and local introgression and adaptation to different habitats. Thus, the poor correspondence between chloroplast haplotype distribution and subspecies circumscription is due to multiple origins of morphologically similar morphs (grouped into taxonomic subspecies) in different parts of the distribution range of the complex.

The distribution of morphological variation and cpDNA haplotypes in *H. nummularium* in Scandinavia shows a stronger geographic structure compared to that of the continental Europe. Two postglacial immigration lineages, one from west and one from east recolonized the deglaciated areas in Scandinavia. These two lineages met in a wide contact zone in south-east Scandinavia, where hybridization/ introgression have created a range of intermediate populations with high variability in indumentum density.

The two species complexes display similar trends regarding incongruence between cpDNA haplotypes and morphology. The most plausible explanation for this poor correspondence is multiple origins of morphologically similar morphs within separate cpDNA lineages in different parts of the distribution range of the complex. However, they differ by the fact that *H. oelandicum* has a distinct geographic structuring of cpDNA haplotypes while *H. nummularium* has a more complicated structuring. In Scandinavia, evidence of postglacial hybridization/ introgression between taxa is well documented in the two species complexes in opposition to the continental Europe where past and/or present hybridization/ introgression is less evident.

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SWEDISH SUMMARY

(SVENSK SAMMANFATTNING)

Istiden har påverkat utbredning och variation hos många växter i Europa, framförallt arter i tempererade områden (dvs. norra Europa). Under den sista istiden (som slutade för mindre än 15000 år sedan) täcktes stora delar av norra Europa, bl.a. hela Skandinavien och norra England, av ismassor. Bergskedjor som finns i södra Europa, t.ex. Karpaterna, Pyrenéerna och Alperna, var täckta med glaciärer. Mellan inlandsisen i norr och bergskedjor i söder fanns stora öppna områden med permafrost. Många växter som finns i norra Europa idag överlevde under istiden i södra Europa; på Iberiska halvön, i Italien, Turkiet och Balkan. Efter istiden vandrade de flesta växter norrut och koloniserade isfria områden. Pollenfynd i torvmossar berättar när och var vissa växter koloniserade olika områden och var de övervintrade under istiden. Detta gäller främst trädarter som producerar massor av pollen. Många växter har låg pollenproduktion och deras migration efter istiden har inte kunnat studeras genom pollenanalyser. Många frågor som gäller utbredningsmönster och migrationshistoria måste därför studeras med alternativa metoder t.ex. molekylära metoder.

Den komplexa *Helianthemum oelandicum* visar betydande morfologisk variation över stora delar av sitt utbredningsområde i Europa och västra Asien. Genom att kombinera tre primerpar och restriktionsenzymer har jag identifierat nio kloroplast(cpDNA)haplotyper med helt åtskilda geografiska utbredningar. Två haplotyper dominerar i större delen av området, en i Västeuropa och en i Östeuropa,. Den västliga haplotypen tyder på att *H. oelandicum* övervintrade den senaste istiden i områden med permafrost väst eller nordväst om den alpina nedisningen. Den tredje vanligaste haplotype är begränsad till Alperna och norra delarna av Italien, och i denna region finns också tre sällsynta haplotyper. Två unika haplotyper har hittats på den Iberiska halvön och en längs den nordöstra kusten av Adriatiska havet. Den genetiska mångfalden är sammantaget mycket högre i södra Europa än i norra Europa. Skillnaden i haplotypsammansättning är dessutom mycket mer uttalad i öst-västlig riktning än i syd-nordlig riktning. Det fanns inget samband mellan kloroplasthaplotyp och morfologiska variation och de tre vanligaste haplotyperna omfattas de flesta morfologiska karaktärer som används för den taxonomiska indelningen av komplexet. Jag tolkar detta så att komplexet har sitt ursprung i västra Medelhavsområdet och har överlevt i södra Europas bergsområden under flera glaciala cykler. Parallell evolution i regionala populationer producerade liknande morfer utan att nödvändigtvis vara nära besläktade, dvs. nyligen dela en gemensam förfader.

Helianthemum oelandicum på Öland representeras av två endemiska och allopatriska (dvs med skilda utbredningar) varieteter, som skiljer sig åt i blomningsfenologi och hårighet. Varietet *oelandicum* har en stor utbredning, medan var. *canescens* är begränsad till små områden på sydligaste Öland. Endast två, allopatriska cpDNA-haplotyper har identifierats på Öland; den sydvästliga postglaciala invandraren H1 med en idag huvudsakligen sydlig utbredning på Öland och den sydöstliga invandringslinjen H2 med en

nordlig utbredning på Öland. Gränserna mellan de två haplotypernas utbredningsområden är distinkta och en skär rak över, mer eller mindre kontinuerliga populationer av var. *oelandicum*. Den sydliga gränsen mellan haplotyperna sammanfaller med markanta skillnader i frekvensen av håriga och kala plantor. Jag drar slutsatsen att gruppen av var. *oelandicum* med kloroplast haplotype H1 har uppstått genom riktat genflöde av de nukleära gener ("pollen swamping") som reglerar de morfologiska karaktärer som kännetecknar var. *oelandicum* in i populationer av var. *canescens*.

Sekvensdata från nukleärt DNA (ITS) och fyra kloroplastregioner har använts för att undersöka förhållandet mellan de sex underarterna i komplexet. Analysen av ITS och cpDNA visade dålig upplösning i de fylogenetiska träden; taxa tycks vara polyfyletiska. Bristen på genetiska skillnader mellan grupperna och det faktum att det saknas en korrelation mellan molekylära markörer och traditionella morfologiska karaktärer stöder behandling av *H. oelandicum* som en artkomplex.

Helianthemum nummularium är en morfologiskt komplex art som har delats upp i flera underarter, av vilket fem har undersökts i denna studie. Jag har utvecklat tre uppsättningar av primers för att studera mikrosatellitevariationen i cpDNA. Den geografiska fördelningen av arton kloroplasthaplotyper antyder ett centrum för diversiteten i Alperna och omgivande områden samt lägre diversitet i marginalområden (Nordeuropa och sydöstra Balkan). De flesta cpDNA-haplotyper delas av flera underarter och de flesta vanliga haplotyperna visar en geografisk struktur. Två haplotyper är begränsad till Nordeuropa och två finns främst i Alperna. Två morfologiska egenskaper, form hos kronblad och blad, visade inte någon betydande skillnad mellan underartena.

Helianthemum nummularium har två underarter i södra Skandinavien som skiljer sig åt i närvaro/frånvaro av en tät filt av sjärnhår på bladens undersida. Underart *obscurum* (utan en tät filt) påträffas i sydväst medan subsp. *nummularium* (med en tät filt av sjärnhår) finns i sydöstra Skandinavien. Utbredningen av de två underarterna överlappar i en bred kontakt zon, där naturliga populationer består av en blandning av de två underarterna i olika proportioner. Det finns inga skillnader i bladformen mellan de två underarterna även om en viss differentiering kan observeras över själva kontaktzonen. Totalt undersöktes 27 populationer som visade sex cpDNA-haplotyper i Norden. Den geografiska fördelningen av fyra av dessa haplotyper stöder en östlig postglacial invandring medan utbredningen av två andra haplotyper stöder en västlig postglacial migration.

Sammanfattningsvis kan jag konstatera att de båda artkomplexen *H. oelandicum* och *H. nummularium* visar samma tendens till svagt samband mellan morfologiska karaktärer och förekomst av cpDNA-haplotyper. Medan *H. oelandicum* har en mycket distinkt geografisk struktur i utbredningen av cpDNA-haplotyper i Europa, är den geografiska strukturen mindre tydlig hos *H. nummularium*. Hybridisering och introgression mellan migrationslinjer kan spåras hos bägge arterna i det tidigare nedisade Skandinavien, men är mindre påtagliga i övriga delar av Europa.

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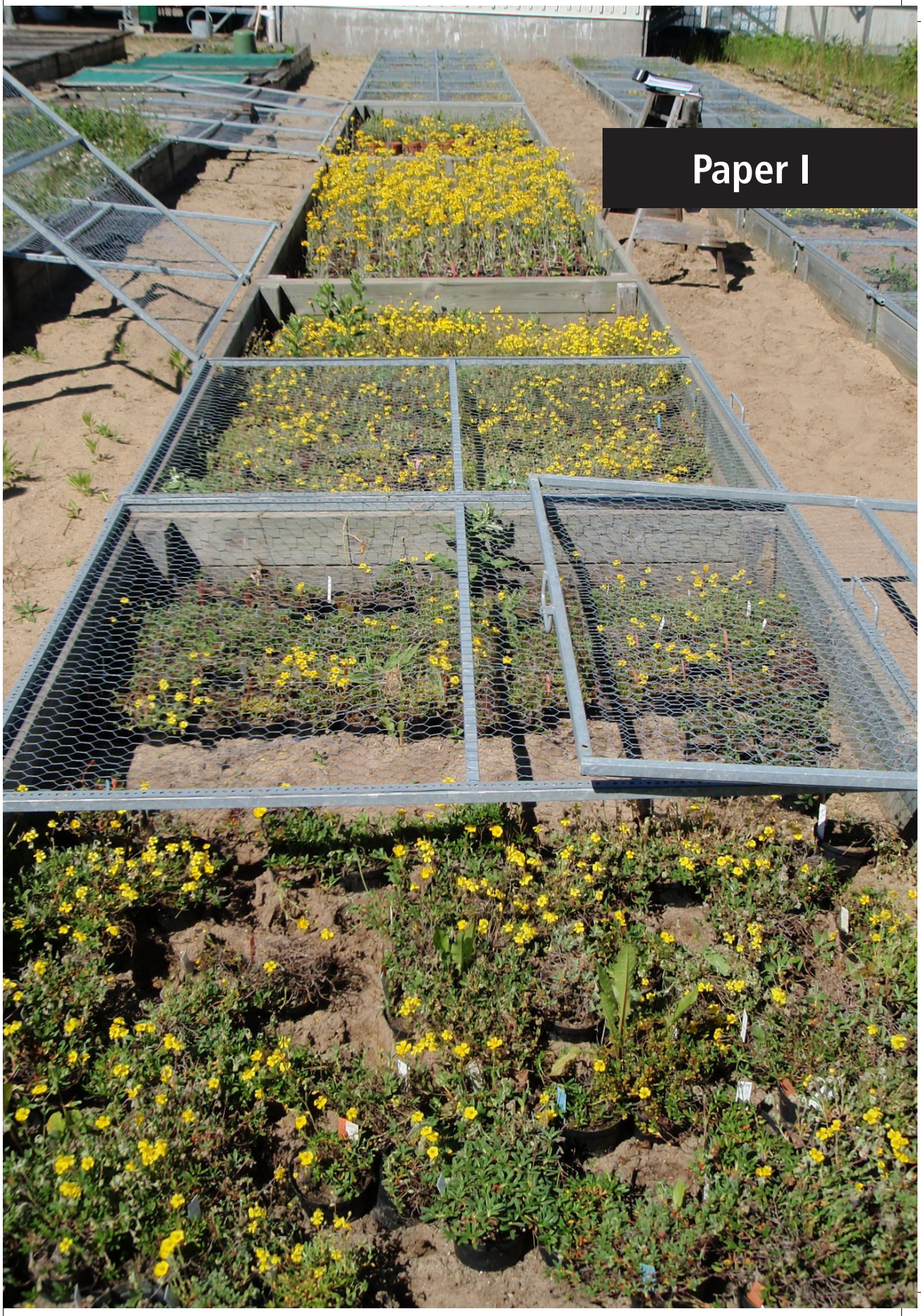
Paper I

Phylogeography of the European rock rose *Helianthemum oelandicum* complex (Cistaceae) – clear geographic structuring of chloroplast DNA haplotypes and multiple allopatric origins of morphospecies

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Keywords: Cistaceae, *Helianthemum*, Quaternary, chloroplast DNA, PCR-RFLP, species complex, vicariance, parallel evolution.

Abstract

The *Helianthemum oelandicum* complex shows considerable morphological variation across its geographic distribution in Europe and Western Asia. By combining four primer pairs and four restriction enzymes we identified nine cpDNA haplotypes with distinct geographical distributions. Two haplotypes were most frequent over most of the range; one in western Europe and one in eastern Europe. The third most common haplotype was confined to the Alps and northern parts of Italy, and this region also had three rare haplotypes. Two unique haplotypes were found in the Iberian Peninsula and one along the northeast coast of the Adriatic Sea. Overall, the genetic diversity was much higher in southern Europe than in northern Europe. Moreover, differences in haplotype composition were much more strongly pronounced in west-east direction than in south-north direction. There was no correlation between chloroplast haplotype and morphological variation as the three most common haplotypes covered most of the morphological variation used for taxonomic subdivision of the complex. We interpret these patterns such that the complex has a west-Mediterranean origin and has survived and existed in southern European mountain systems during several glacial cycles. Parallel evolution in the regional populations produced similar morphologies without necessarily being closely related.

Introduction

Taxa below the species level are usually supported by morphological characters, but their delimitation does not necessarily coincide with the distribution of genetically circumscribed lineages (e.g. Schönswetter *et al.* 2009). Such incongruence between neutral molecular markers and taxonomy (i.e. morphological and ecological characters) in plants has been reported in several studies (e.g. Holderegger *et al.* 2002, Comes & Kadereit 2003, Kropf *et al.* 2003, Ronikier *et al.* 2008b). This type of incongruence may be explained by intraspecific hybridization/introgression or by incomplete lineage sorting (Wendel & Doyle 1998). Parallel evolution, where taxa have arisen independently and repeatedly as adaptation to specific environ-

mental conditions, may also explain the lack of congruence (cf. Comes & Kadereit 2003). Parallel evolution is defined as the independent evolution of similar traits across geographically separated groups (i.e. populations or closely related species) that inhabit similar environments (Futuyma 1986). The phenotypic traits occasionally have a simple genetic basis (i.e. Mendelian) and parallel development of these traits is more frequent than previously thought (Levin 2001, Woods *et al.* 2005). An important outcome of parallel evolution is the increasing degree in reproductive isolation between populations as explained by the "isolation by distance" theory. Ultimately, parallel evolution of similar but reproductively isolated populations can play an essential and productive role in the early stages of speciation (Futuyma 1986, Schluter *et al.* 2004).

In less well-studied groups it could be suspected that the traditional taxonomy based on morphological characters is not well supported by any genetic data and that phylogeographic analyses based on existing taxonomy may result in circular reasoning and flawed conclusions. An example of this is the tetraploid marsh orchids in the *Dactylorhiza traunsteineri* group, to which disjunct populations from Britain, the Balkans, the Alps and Scandinavia have been assigned (Delforge 2001). Detailed analysis has shown that taxa included in this group are more closely related to various other members of the tetraploid marsh orchids than to each other, and that the European marsh orchids as a whole are better subdivided according to geographic area (Nordström & Hedrén 2009). It can be concluded that phylogeography of species belonging to taxonomically complex groups should not be studied in isolation, but that the whole group should be analysed in its entirety and that taxonomic subdivision must be discussed together with phylogeography (cf. Schönswetter *et al.* 2009).

A growing number of molecular studies of intraspecific variation of plant species in the European Alpine system, i.e. the Pyrenees, Alps, Apennines, Tara Mountains, Carpathians and Balkans, have identified multiple lineages connected with different mountain ranges indicating long-term isolation between ranges (e.g. Kropf *et al.* 2003, 2008, Mráz *et al.* 2007, Ronikier *et al.* 2008a) or between putative refugia within ranges (e.g. Schönswetter *et al.* 2002). Comes & Kadereit (2003) discussed the phylogeography of a group of European high mountain taxa with Mediterranean or Asian affinities. They found little evidence of long-distance dispersal between mountain ranges (but see e.g. Schönswetter *et al.* 2002). Instead, the biogeographical patterns suggested successive colonization or vicariance. They also concluded that several high mountain taxa had originated from lowland forms (cf. Kropf *et al.* 2002, 2003). The use of neutral genetic markers mirrors historical processes such as bottlenecks, migration and genetic drift that have shaped the present-day genetic structure (Hewitt 1996, Taberlet *et al.* 1998), but do not reveal anything about other evolutionary processes, such as natural selection, influencing intraspecific variation in morphology or adaptation to different habitats. Thus, many intraspecific taxa may have evolved independently and many times in different mountain ranges (e.g. Kropf *et al.* 2003).

The Quaternary cold periods in Europe starting 2.4 Myr ago had a dramatic

influence on the distribution and differentiation of many plant species (Huntley & Briks 1983). The repeated contraction and expansion of species' ranges are considered one of the main causes of modification and reorganization of the genome structure within species (Hewitt 1996). The intraspecific differentiation was especially pronounced during the Pleistocene, and many extant lineages may have differentiated during the two most recent glacial periods (Comes & Kadereit 2003). During the last ice age (which ended about 15000 yr BP), glaciers covered large parts of northern Europe, including the whole of Scandinavia and northern Britain (Björck 1995, Svendsen *et al.* 2004). Two of the major mountain ranges in southern and central Europe, the Pyrenees and Alps, were almost entirely covered by ice caps, while the more eastern Carpathians and Balkans were less extensively glaciated (Charlesworth 1957, Messerli 1967). Between the main ice sheet in the north and the southern mountain ranges was a plain of permafrost covered by tundra and cold steppe (Hewitt 1999). Populations that inhabited the southern parts of Europe and Asia Minor, including the Iberian Peninsula, Italy, Turkey and Balkans survived where the climate was more favourable and recolonized northern areas as glaciers withdrew (Hewitt 1996, Taberlet *et al.* 1998). Parallel to this latitudinal migration, are fluctuations in the altitudinal distribution of mountain and alpine species in southern and central European mountain ranges (e.g. Kropf *et al.* 2003, Ronikier *et al.* 2008a). Some trees and shrubs could also have survived in pockets of favourable conditions in the southern parts of the steppe-tundra in eastern, central and south-western Europe (Willis *et al.* 2000, Carcaillet & Vernet 2001). Moreover, pollen from the steppe-tundra element was deposited close to the southern margins of the main ice cap during the last glacial maximum (Lang 1994). Species belonging to open habitats, e.g. *Helianthemum* were deposited in central Britain during the late glacial period, probably at the end of the Allerød Interstadial (12000 yr BP) and also in southern Scandinavia during the same period (Iversen 1944, Berglund 1966, Smith *et al.* 2005). Many temperate-adapted species may thus have survived the long, unfavourable Quaternary cold periods north of the southern European Peninsulas (Stewart & Lister 2001, Provan & Bennett 2008).

Several molecular studies have identified glacial refugia of mountain and alpine plants in the Alps and the Carpathians (Tribsch *et al.* 2002, Schönswetter *et al.* 2005, Ronikier *et al.* 2008a) and even confirmed the presence of periglacial refugia between the Scandinavian and the Alpine ice shields (Stewart & Lister 2001, Palmé & Vendramin 2002, Rendell & Ennos 2002, Ronikier *et al.* 2008b, Hedrén 2009, Huck *et al.* 2009). An individual species may have its own phylogeographic history (Taberlet *et al.* 1998), but species sharing similar ecological factors and altitudinal distribution may be similarly affected by the Pleistocene, e.g. plant species of high mountains (Schönswetter *et al.* 2005) or northern latitudes (Brochmann *et al.* 2003). There are, however, so far few large-scale geographic studies of herbaceous plants and dwarf shrubs (but see, e.g. Comes & Abbott 2001, Abbott & Comes 2003, Griffin & Barret 2004, Fjellheim *et al.* 2006, Prentice *et al.* 2008, Hathaway *et al.* 2009), especially studies

of species with broad ecological amplitudes, e.g. species occurring from lowlands to high mountain environments (cf. Ronikier *et al.* 2008b).

European plants with a Mediterranean origin, such as the Cistaceae family, often have their main centre of diversity in the western part of the Mediterranean Basin. The genus *Cistus*, for instance, with a more or less restricted Mediterranean distribution, has the greatest number of species in the Iberian Peninsula and north-west Africa and a low number of species in the eastern Mediterranean region (Guzmán & Vargas 2005, 2009), indicating a differentiation of the genus proceeding from west to east. Likewise, the genus *Helianthemum*, which has a broader European distribution, shows an Iberian centre of species diversity despite the problematic circumscriptions of several species (Tutin *et al.* 1968). According to the most recent phylogeny of Cistaceae (Guzmán & Vargas 2009), two closely related species complexes of *Helianthemum* diverged during Pleistocene, *H. marifolium* and *H. oelandicum*. The current distribution of *H. marifolium* is restricted to the western Mediterranean region, while *H. oelandicum* occurs in the same region but has a wider geographical distribution, indicating a common west-Mediterranean origin of both species. The intraspecific taxonomy of both complexes has traditionally been based partly on hairiness of the leaves and this character shows a complicated pattern of variation (Tutin *et al.* 1968, Widén 1988).

In the present study we investigated how morphological variation, as expressed by current taxonomy, correlates with large-scale genetic divergence patterns as revealed by cpDNA haplotype distribution in *Helianthemum oelandicum* complex. The complex has mainly a European distribution with parallel differentiation in morphology in many mountain ranges of the European Alpine system. The species distribution extends from lowland to alpine altitudes and has marginal disjunct distribution in areas covered by the Scandinavian ice sheet during the Last Glacial Maximum. We investigate the distribution of cpDNA haplotypes in populations sampled across the main distribution of the species complex to determine whether the geographical distribution of cpDNA haplotypes could reveal any potential glacial refugia and postglacial migration routes.

Materials and Methods

The species complex

Members of the *H. oelandicum* complex (Cistaceae) are diploid (2n = 22), self-incompatible, insect and wind pollinated perennial dwarf shrubs. They are restricted to calcareous soils and limestones in open, lowland to alpine grasslands, often with exposed outcrops (Janchen 1907, Proctor 1956). The complex is distributed mainly in central and southern Europe, and it also extends into Turkey and the Caucasus. Marginal populations occur in the British Isles and in Scandinavia, where the complex is an important component in the alvar grasslands of the Baltic Island of Öland (Sterner 1936a&b). Isolated populations are also found in the Pinega area in northern Russia and in the Atlas mountain range in North Africa (Proctor 1956).

The complex comprises a variety of morphs that show complex variation in

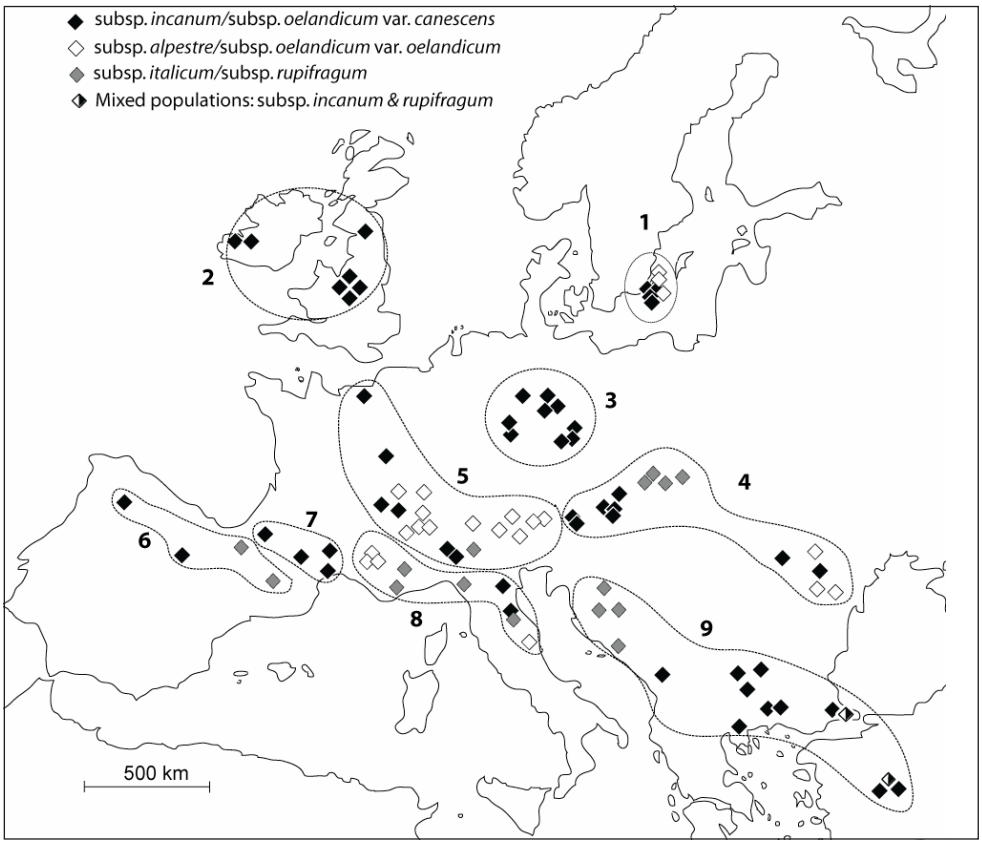

Figure 1. Location of the 91 populations of the *Helianthemum oelandicum* complex studied. Taxonomic affiliation is indicated by different symbols. The populations were grouped into nine regions for comparison of genetic diversity (see text for details).

both indumentum and flowering phenology (Janchen 1907, Sterner 1936a&b, Widén 1980, 2010). Three main types of trichomes are recognized in the Cistaceae (Grosser 1903). These are found in different combinations in the *H. oelandicum* species complex (Widén 1988): stellate hairs, bristles (or simple hairs) and glandular hairs. Indumentum may vary both within and between populations and shows considerable geographical variation even on the local scale (Widen 1988, 2010).

The species complex is characterized by two flowering phenologies; the concentrated flowering (CF) where the inflorescences are borne at the apex of the previous year's growth and the protracted flowering (PF) with inflorescences borne laterally on the current year's growth as well as at the apex of the previous year's growth. Flowering phenologies have been studied thoroughly on Öland, where CF plants flower early in the season (early June), while PF plants flower throughout the season with one flowering peak in early June from inflorescences born on

the previous year's growth and another peak in July-August with inflorescences born on the current year's growth (Widén 1980, 2010).

The taxonomy of the species complex in Europe has been treated in different ways. Janchen (1907) recognized five species: *H. canum* (L.) Baumg., *H. oelandicum* (L.) Willd., *H. italicum* (L.) Pers., *H. rupifragum* Kerner and *H. alpestre* (Jacq.) DC. Proctor in Flora Europaea (Tutin *et al.* 1968) divided the complex into two species based on the presence/absence of dense felt of stellate hairs on the abaxial surface of the leaves: *H. canum* with seven subspecies and *H. oelandicum* with five subspecies. Widén (2010) has revised the species-complex on Öland and recognized one subspecies *H. oelandicum* subsp. *oelandicum* with two varieties; var. *oelandicum* (plants with a CF phenology and without stellate hairs) and var. *canescens* (plants with a PF phenology and variable with respect to stellate hairs on the abaxial surface of the leaf).

In the present study, we treat the taxonomic subdivision of the complex as following. We suggest the presence of only one species in the complex: *H. oelandicum* with five subspecies in Europe. Plants from Öland belong to the subspecies *oelandicum*. Following Widén (2010) we distinguish between var. *oelandicum* (with the CF phenology) and var. *canescens* (with the PF phenology) based on the flowering phenology. The subspecies *alpestre* refers to an alpine taxon, usually with large flowers, from higher altitudes in the Alps, Pyrenees, Tatra Mountains, Carpathians and other high mountains in south-eastern Europe. The subspecies *rupifragum* is a taxon restricted to lower and intermediate altitudes in south-eastern Europe. The subspecies *italicum* (relatively small flowers) habituates low altitudes in the western and central Mediterranean areas. All morphs from continental Europe and the British Isles with a dense felt of stellate hairs on the

Table 1. Description of the four primer pairs and the corresponding restriction enzymes used for the PCR–RFLP in this study.

Primer pair	Fragment length (bp)	Restriction enzymes	TA (°C)	Extension time (min.)	Reference
trnK-trnK	2500	*EcoR1, Hinf1*	60	3	Demesure *et al.* (1995)
trnS–trnT	1500	*Hinf1*	57	2	Demesure *et al.* (1995)
trnT–psbC	3500	*EcoR1, Hinf1, Taq1, Tru1*	57	4	Dumolin–Lapegue *et al.* (1997)
trnT –psbD	1500	*EcoR1, Hinf1, Tru1*	57	2	In this study

lower side of the leaves were provisionally lumped together into the subsp. *incanum*, despite marked regional differences in the growth form, leaf and petal size, and indumentum (B. Widén unpublished). In several Mediterranean mountain areas morphs with and without stellate hairs on the lower side of the leaf may occur sympatrically (B. Widén & E. Soubani, personal observations). A sequence of two or three subspecies often occurs in distinct zones in many mountain ranges, usually in the order *italicum/rupifragum, incanum* and *alpestre* from low altitudes to the alpine zone, but also with *italicum/rupifragum* at intermediate altitudes. When tested, progeny from individual plants in mixed populations could be segregated based on the presence/absence of stellate hairs, showing hybridization and gene flow between morphs (B. Widén unpublished). Artificially produced hybrids between taxa in the species complex indicate weak crossing barriers between morphs in many but not all combinations (Widén 1986 and unpublished).

We included two other closely related species (cf. Guzmán & Vargas 2009) as reference material: *H. hymettium* (Greece) and *H. marifolium* (Spain). They belong to the same section (*Plectolobum*) as *H. oelandicum*.

Plant material

Sixty-seven populations were collected from natural populations by the first (BW) and third author (ES). The remaining 24 accessions were collected and provided by colleagues and or by botanical gardens (see Fig. 1, the Appendix). The material was sampled in the form of seeds and cuttings, which were sown and planted in pots outdoors in the experimental garden, Lund University. Most populations consisted of 20-25 cultivated individuals.

Morphological studies

Cultivated plants were classified according to the presence/absence of stellate hairs on the lower side of the leaves and whether the inflorescences appeared only on the previous year's growth (CF) or if the majority of inflorescences appeared on the current year's growth (PF). Scattered and rare inflorescences borne on the current year's growth were ignored. Each population was referred to one of the traditional taxa (see above). A few populations, which consisted of a mixture of morphs, were referred to the taxon of the majority of the plants in the population.

DNA extraction and PCR-RFLP

A total of 201 individuals from 91 populations of *H. oelandicum* (average n = 2 individuals/population) were used for genetic analysis. The number of investigated individuals per population was one in 9 populations, two in 71 populations and three or more in 11 populations. We also included three specimens of the reference species (*H. marifolium* and *H. hymetium*). Fresh leaves from all the plants were stored frozen in plastic bags at -80°C during the spring between 2004 and 2007. Due to the problems we encountered later with the extraction and amplification of DNA, we concentrated on including as many populations as possible but with few individuals in each. This approach will provide a clearer picture of the overall chloroplast DNA variation across the species natural range and provide an accurate estimate of gene polymorphism at

individual sites (Pons & Petit 1995).

Helianthemum is characterized by having a considerable amount of mucilage (secondary metabolites) in mature leaves (Mathe *et al.* 1976), which may interfere with DNA extraction, amplification and further digestion with restriction enzymes. It was not possible to remove all these compounds using the original CTAB-based protocol (Doyle & Doyle 1990). Therefore, we employed the protocol of Lodhi *et al.* (1994), which included two additional components: sodium chloride which removes gummy polysaccharides (resins) and polyvinyl polypyrrolidone which eliminates polyphenols. The polyphenoles are known as strong oxidizing agents that reduce the yield and quality of DNA. The Lodhi protocol has successfully produced DNA of high quality and quantity from other plant species including *Malus, Prunus, Rubus*, *Vitis* and *Ampelopsis* (Lodhi *et al.* 1994). Some minor adjustments were made to optimize the protocol for *Helianthemum*.

A pilot study was performed on a subset of 10 plants from different populations to test for amplification by universal primers. This screening included 19 universal chloroplast DNA primer pairs for amplification of the following cpDNA regions: *atpB-rbcL* (Chiang *et al.* 1998), *intron L, trnL-trnF* and *trnT-trnL* (Taberlet *et al.* 1991), *trnS-trnM, trnS-trnT, trnK-trnK, trnC-trnD, psaA-trnS, trnM-rbcL, trnD-trnT, trnH-trnK* and *psbC-trnS* (Demesure *et al.* 1995), *trnK-trnQ, rpoC1-trnC*, *trnT-psbC*, *trnM-psaA* and *trnF-trnV* (Dumolin-Lapegue *et al.* 1997), *rpl2–rps*19 (Weising & Gardner 1999) and six mitochondrial DNA universal primer pairs (Dumolin-Lapegue *et al.* 1997): [*nad4/2-nad4/3, nad5/1-nad5/2, nad7/2-nad7/3, nad7/3-nad7/4, nad1/2-nad1/3* and *nad4/2-nad4/2*]. Twelve regions were successfully amplified and tested for restriction polymorphism using eight restriction enzymes (*EcoR1, Hinf1, Tru1I, Taq1, AluI, RsaI, NdeII* and *CfoI*; Roche Applied Science). Three regions showed restriction variation among the populations and were therefore selected for the final screening of the whole material (see Table 1). However, due to the amplification difficulties we faced later in the study with the primer pair *trnT-psbC*, we designed a new reverse primer *psbD* (*5′-CAA AGG TTG TAC CTG TGA ACC A-3′*) based on the consensus sequences of the *psbD* gene of different plant species (i.e. *Nicotiana* and *Arabidopsis*) obtained from GenBank. The *psbD* gene is located directly downstream of the variable intergenic spacer present in the *trnT*-spacer-*psbD-psbC* region. The new primer pair *trnT-psbD* showed restriction polymorphism using three restriction enzymes: *EcoR1, Hinf1* and *Tru1* and was applied together with *trnT–psbC/Taq1* for screening of the remaining material.

The PCR reactions were carried out in a total volume of 10 ml containing: 6.8 ml distilled water, 1 ml (10x PCR) buffer, 0.8 ml (25 mM) $MgCl_2$, 0.2 ml (10 mM of each) dNTPs, 0.08 ml (25 pmol/ml) each of the forward and reverse primers, 0.24 ml (1unit/ml) *Taq* polymerase and 0.8 ml (14 ng/ml) DNA template. The PCR amplification was performed in a PTC-100 DNA thermal cycler. An initial of 4 minutes denaturation at 95°C was followed by 39 cycles of 92°C for 45 s, annealing at 57-60°C (depending on the primer pair; Table 1) for 45 s, and extension at 72°C for 2 to 4 minutes depending on the fragment length. All the PCR programs were concluded with a 10 min

extension at 72°C. The PCR products (10 ml) were digested with 10 ml restriction enzyme mixture (7.3 ml distilled water, 2 ml 10x buffer and 0.7 ml [10 units/ml] restriction enzyme) and incubated overnight at the optimum temperature of the restriction enzyme. The digested PCR products were then separated and visualized on 1.8% agarose gels stained with ethidium bromide and photographed under UV light.

Data analysis

The molecular data was scored as unordered multistate characters, where each primer-enzyme combination was considered a character and the different banding patterns were regarded as being character states. Since the chloroplast genome does not recombine in most flowering plants (Singh 2004), we defined a haplotype as a particular combination of restriction fragment patterns over all the regions studied. Populations were grouped into nine regions based on the presence of physical barriers such as mountain ranges (the Alps, Carpathians and Pyrenees) and water bodies (see Fig. 1, the Appendix): 1) Öland, Sweden; 2) the British Isles; 3) Germany and the Czech Republic; 4) the Alps and adjacent areas in northern Italy and eastern France; 5) the Carpathian Mountains in Romania and Slovakia and adjacent areas of Austria; 6) the Iberian Peninsula south of the Pyrenees; 7) southern France, north of the Pyrenees; 8) middle and north-east Italy; and 9) the Balkans, northern Greece and western Turkey.

Table 2. Band phenotype composition of the ten haplotypes identified in the present study. Letters A to G denote primer–enzyme combinations and numbers denote different banding patterns (A: *trnK–trnK/EcoR1*; B: *trnK–trnK/Hinf1*; C: *trnS–trnT/Hinf1*; D: *trnT–psbC (psbD)/EcoR1*; E: *trnT–psbC/Taq1*; F: *trnT–psbC (psbD)/Hinf1*; G: *trnT–psbC (psbD)/Tru1*). Haplotypes H1–H9 belong to *H. oelandicum*, whereas H10 is specific to *H. hymettium*. Np is the total number of populations in which each haplotype was found. Taxonomic distribution gives the total number of populations of a given taxon in which each haplotype was found. The percentage of a respective flowering phenology CF or PF is included per taxon.

	Polymorphic sites (primer–enzyme combinations)								Taxonomic distribution				
Haplotype	A	B	C	D	E	F	G	Np	*incanum*	*itali/rup*	*alpestre*	*oelandicum*	*canescens*
H1	1	1	1	1	2	2	2	32	20	1	6	1	4
H2	1	1	1	2	2	1	2	34	25	5	2	2	0
H3	1	1	1	1	2	1	2	13	1	5	7	0	0
H4	1	2	1	3	1	1	1	1	0	1	0	0	0
H5	1	2	1	1	2	1	1	2	2	0	0	0	0
H6	2	3	2	1	2	2	1	4	0	4	0	0	0
H7	3	2	2	1	2	2	2	1	1	0	0	0	0
H8	1	4	1	1	2	2	2	2	0	2	0	0	0
H9	1	2	2	1	2	1	2	1	0	0	1	0	0
H10*	1	2	2	1	2	1	3	2	0	0	0	0	0
CF%									98	86	100	100	
PF%									2	14			100

The hierarchical distribution of genetic diversity between/within the nine regions and between/within the six taxa was described by molecular variance (AMOVA). Calculations of F_{ST} were based on haplotype frequency and significance levels were tested by 10000 permutations. Absolute levels of haplotype diversity within each region and taxon were calculated as standard diversity based on haplotype frequencies.

The relationship between haplotypes was illustrated graphically by a minimum spanning network (MSN) such that the total number of changes in banding phenotypes between haplotypes was minimized. A Mantel test (Mantel 1967) was employed to test for associations between genetic distances expressed as pairwise haplotype frequencies between regions and geographic distances. Significance was tested by 10000 random permutations. AMOVAs and MSN were calculated with Arlequin 3.01 (Excoffier *et al.* 2005).

To test for the presence of a phylogeographic structure within the species, G_{ST} (based on differences in haplotype frequency between regions) and N_{ST} (based on differences between haplotypes) were calculated according to Pons and Petit (1996), and a test to determine whether N_{ST} was significantly greater than G_{ST} according to Burban *et al.* (1999) was performed with 10000 permutations using the software PERMUT 2 (http://www.pierroton.inra.fr/genetics/labo/software).

Results

Among the seven polymorphic primer-enzyme combinations, a total of nine haplotypes were identified in the *H. oelandicum* complex plus *H. marifolium* and one haplotype (H10) in *H. hymettium*. Haplotype definitions are given in Table 2. No variation in cpDNA was found within any of the populations examined.

The geographic distribution and relationships between cpDNA haplotypes

The distribution of cpDNA haplotypes was highly structured (see Fig. 2a, 2b). H2 was the most frequent haplotype (found in 34 populations) with the widest geographical distribution. It is distributed over the eastern part of Europe (i.e. Romania, Macedonia and Bulgaria), central Europe (i.e. Italy, the Czech Republic, eastern Austria and Slovakia) and in Turkey, as well as middle and northern part of the Great Alvar on Öland in northern Europe. H1 was the second most frequent (present in 32 populations), and was distributed over the western part of Europe (i.e. the British Isles and France), central Europe (i.e. Switzerland and Germany) as well as in the southern part of the Great Alvar on Öland. H3 is restricted to the Alps in Austria and to the northern part of Italy. H4 and H5 are confined to Spain. H6, which differs markedly from the previously mentioned haplotypes, was restricted to the western coast of the former Yugoslavia: Croatia, Bosnia & Herzegovina and Montenegro. H7 is a rare haplotype found in an isolated population in southern France. H8 and H9 are also rare haplotypes found in two populations in northern Italy and in one population in

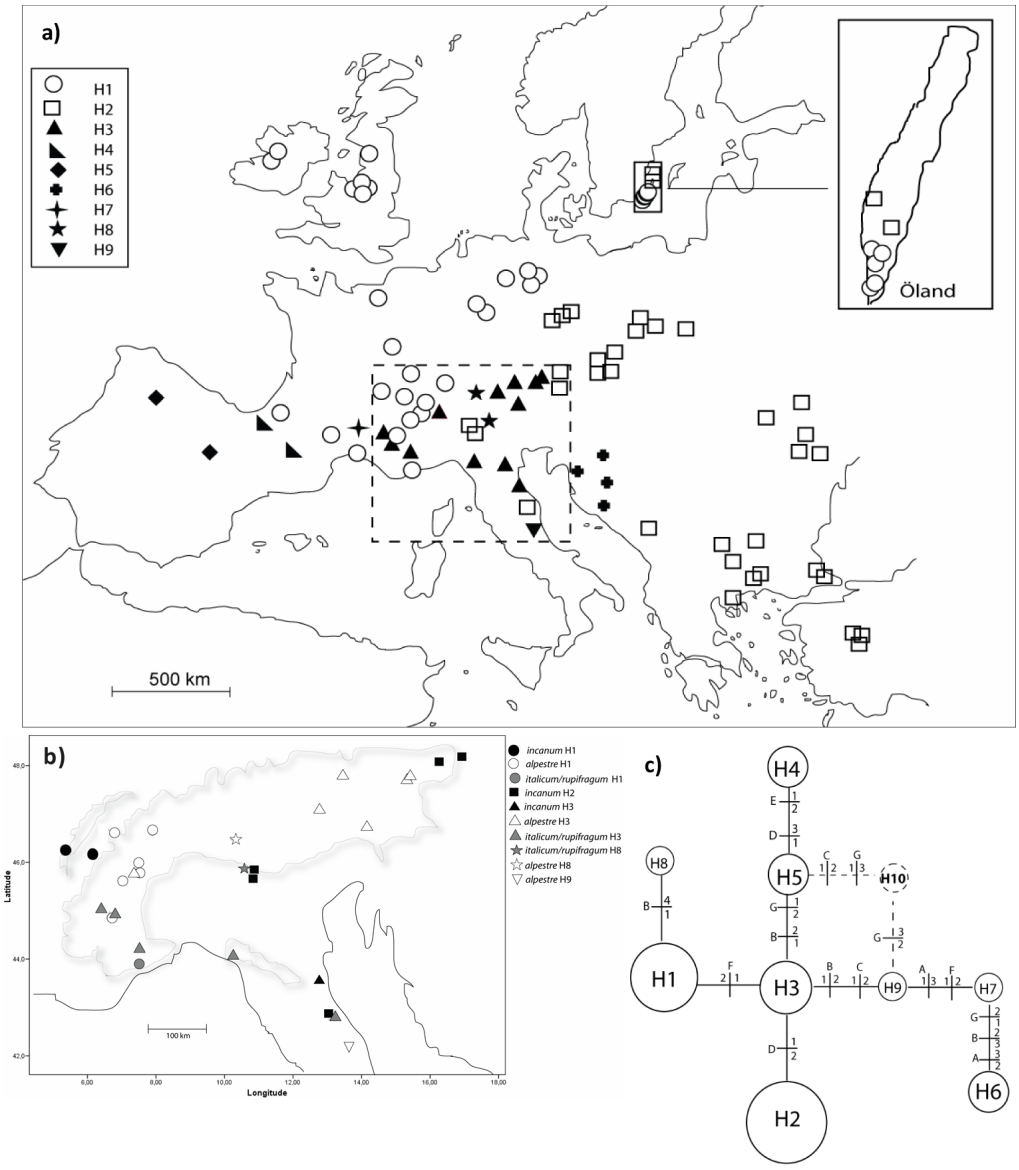

Figure 2. a) Distribution of the nine cpDNA haplotypes identified in the *H. oelandicum* complex in Europe and Turkey. The taxonomic affiliation of haplotypes in the Alps and northern Italy (framed by dotted line) is given as Fig. 2b. b) Map of the Alps and northern Italy showing the locations of the sampled populations of the *Helianthemum oelandicum* complex. The shape of the symbols denotes the haplotypes and shading denotes the taxonomic affiliation. c) Minimum spanning network summarizing the relationship between the nine cpDNA haplotypes detected in the *H. oelandicum* complex. H10 was only present in *H. hymettium*. The size of the circles is proportional to haplotype frequency. The superimposed lines correspond to mutation sites with character state inferences based on the results given in Table 2.

central Italy, respectively. H10 is restricted to *H. hymettium* in southern Greece and was found in two populations.

The MSN connecting all the cpDNA haplotypes is given in Fig. 2c. If it is hypothesized that the network can be rooted by the *H. hymettium* haplotype H10, it can be deduced that haplotypes H1, H2 and H8 are derived from H3, H4 is derived from H5 and that H7 and H6 are derived in sequence from H9. However, the support for this scenario is low and the position of H10 is ambiguous.

Chloroplast DNA diversity

A substantial amount of haplotype differentiation between the nine regions was detected in the complex (F_{ST} = *0.38*, *p<0.0001* in Table 3). However, A weak but significant genetic differentiation was observed between the six taxa (F_{ST} = *0.167*, p<0.0001, see Table 3). In Table 4, the highest amount of gene diversity (*HS = 0.7*) was observed in region seven; southern France north of the Pyrenees and the lowest (*HS = 0*) in region one; Öland, Sweden. The subspecies *alpestre* acquired the highest amount of gene diversity (*HS = 0.757*), whereas subsp. *canescens* had the lowest (*HS = 0*). The Mantel test revealed a slight, but insignificant association between differentiation in haplotypes and geographic distances between the regions (r = 0.341, *p = 0.071*). The test for a phylogeographic structure within the *H. oelandicum* complex in Europe was significant (*p = <0.001*), as N_{ST} (0.409) was significantly higher than G_{ST} (0.362).

Chloroplast DNA haplotypes vs. morphological/taxonomical polymorphism

When flowering phenology was examined in cultivation, the majority of populations were characterized by a concentrated flowering (CF) phenology, i.e. producing inflorescences only on the previous year's growth (see the Appendix). A regular production of inflorescences on the current year's growth as well as a small fraction of the inflorescences on the previous year's growth (PF phenology) was detected only in subsp. *oelandicum* var. *canescens* and a few continental populations (see below). However, a number of populations showed a tendency to produce scattered inflorescences on the current year's growth the year after re-potting (data not shown).

The subspecies *incanum* is the most frequent taxon (represented by 49 populations) and covers almost the entire distribution range of the complex. The taxon maintains a uniform CF flowering phenology (except for one population in southern France, see the Appendix) and a dense cover of stellate hairs on the lower side of the leaf, despite the pronounced regional variation in growth form, size and shape of leaf and petal and in the distribution of indumentum on different parts of the plant (e.g. stellate hairs on the upper side of the leaf; B. Widén, unpublished). The taxon occurs in lowland and mountain habitats and is represented by five haplotypes; the two main haplotypes H1 and H2, and the more restricted H3 in the Alps, H7 in France and H4 in Spain (Fig. 1, 2, Table 2).

The subspecies *italicum* is restricted to lowland and mountain habitats in southern Europe (Italy, Spain and France). All of the eight populations in our sample are

characterized by the CF phenology, except for one population in Italy that has the PF phenology.

Table 3. Partitioning of genetic diversity (AMOVA) between/within regions and between/within taxa of *H. oelandicum* based on haplotype frequency.

Source of variation	df	Sum of squares	Variance components	% variation
Between regions	8	31.97	0.346	38.08*
Within regions	83	46.82	0.564	61.92
Total	91	78.80	0.911	
Between taxa	5	12.51	0.148	16.67*
Within taxa	85	63.02	0.741	83.33
Total	90	75.53	0.889	

* ($p<0.0001$)

Table 4. Calculations of standard diversity indices {number of polymorphic loci (L), haplotypes (H), populations (P)} and estimation of gene diversity (HS) for each of the nine regions and six taxa.

Region	L	H	P	HS
1	0	1	7	0
2	2	2	7	0.476
3	2	2	9	0.500
4	3	4	14	0.582
5	1	2	21	0.428
6	2	2	4	0.666
7	7	3	4	0.700
8	4	4	10	0.644
9	6	2	15	0.419
Taxon				
alpestre	7	5	17	0.757
italicum	5	5	10	0.755
incanum	6	5	49	0.582
rupifragum	6	2	8	0.571
var. *oelandicum*	2	2	3	0.666
var. *canescens*	0	1	4	0

Four haplotypes were found in this taxon; H1, H2, H3 and H5 (Fig. 1, 2, Table 2).

The subspecies *italicum* is restricted to lowland and mountain habitats in southern Europe (Italy, Spain and France). All of the eight populations in our sample are characterized by the CF phenology, except for one population in Italy that has the PF phenology. Four haplotypes were found in this taxon; H1, H2, H3 and H5 (Fig. 1, 2, Table 2).

The subspecies *rupifragum* is characterized by a CF phenology (i.e. seven populations) except for one population in Slovakia. This taxon is mainly a mountain plant that displayed two haplotypes; H2 and H6 (Fig. 1, 2, Table 2).

The subspecies *alpestre* is an alpine plant; inhabiting high altitudes in i.e. the Alps and the Carpathians mountain ranges, but it occurs also in middle altitude mountain habitats. All the nineteen populations in our sample have the CF phenology but differ from the rest of the taxa in having fairly larger flowers (1.5 to 2 times larger). Four haplotypes are found in this taxon: H2 is found in the Carpathians and H1 and H3 in the Alps, while H9 is found in the Apennine Mountains (Fig. 1, 2, Table 2).

The two allopatric varieties of subsp. *oelandicum*, var. *canescens* in four populations and var. *oelandicum* in one population share H1 in the southern part of the Great Alvar on Öland. However, the two populations of var. *oelandicum* from the northern part of the Great Alvar have H2. The sharing of H1 in the two varieties occurs in the populations sampled at a distance of a few hundred metres on each side of a distinct hybrid zone between the two varieties (Widén 1980) (Fig. 1, 2, Table 2).

The three most common cpDNA hap-

lotypes (H1, H2 and H3) comprised the whole range of morphological variation in the complex. For instance, plants with H1 are assigned to subsp. *incanum*, *alpestre*, *italicum*, *oelandicum* var. *oelandicum* and *oelandicum* var. *canescens*. Among H2 plants we find subsp. *incanum*, *alpestre*, *rupifragum* and *oelandicum* var. *oelandicum*, while H3 comprises subsp. *incanum*, *alpestre* and *italicum* (Fig. 1, 2, Table 2, Appendix). Other cpDNA haplotypes sampled from more than one population were either restricted to one taxon (H5; subsp. *incanum*, H6; subsp. *rupifragum*) or to two taxa (H8; subsp. *alpestre* and *italicum*). H4 is found in two populations of subsp. *italicum* and one population of the closely related *H. marifolium* in Spain (see the Appendix). The two rare haplotypes found in only one population each belong to subsp. *incanum* (H7) and subsp. *alpestre* (H9).

Discussion

CpDNA haplotype diversity in the H. oelandicum complex

We have analysed a large number of samples covering the natural range of the species distribution apart from some marginal areas (e.g. southern Italy, North Africa and the Caucasus). No intra-population cpDNA haplotype polymorphism was found. Our results will thus give a fairly accurate view of the geographical distribution of cpDNA diversity of the complex, despite the small size of each population sample (cf. Pons & Petit 1995). The AMOVA analysis of genetic diversity (based on cpDNA haplotype frequency) was carried out on a regional scale. There was a substantial cpDNA differentiation between and within regions (Table 3), and N_{ST} was significantly higher than $G_{ST;}$ indicating the presence of a phylogeographic structure within the *H. oelandicum* complex in Europe. We thus concluded that: (1) differentiation between regions may be due to historical events i.e. glacial refugia and postglacial recolonization (see below), and (2) gene flow between regions is restricted, probably because they are well separated by barriers or long distances. The latter conclusion is supported by the fact that rock roses are currently more or less confined to areas with open, calcareous bedrocks, which have an island-like distribution.

The origin of the H. oelandicum complex

The geographical distribution of the cpDNA haplotypes within the *H. oelandicum* complex shows a distinct pattern. The number of haplotypes is greatest in the south-western part of the geographical area covered by the complex (Spain and the Alps surroundings), with a pronounced decrease in the numbers of haplotypes in the northern and eastern parts of the distribution range (Fig. 2a). Haplotype H4 is found in *H. oelandicum* and the west-Mediterranean *H. marifolium* which indicates a common origin of these two species in the Iberian Peninsula during the Pleistocene (cf. Guzmán & Vargas 2009). Following the origin of an Iberian *H. oelandicum,* subsequent differentiation of the species may have proceeded from west to east and north during several cycles of glaciations. Thus, *H. oelandicum* shows a typical Cistacean differentiation pattern with a west-Mediterranean origin and decreasing diversity from west to east (cf. Guzmàn & Vargas 2005, Guzmàn *et al.* 2009).

Phylogeography, putative refugia and migration routes

Some of the rare haplotypes found in the complex deviate from the more common ones (Fig. 2c) and may represent ancient lineages that arose early in the history of the species. The two distinct haplotypes H4 and H5 (Fig. 2a) have so far been found only in Spain. They presumably survived several cycles of glaciation in the Iberian Peninsula, but did not expand to the rest of Europe as they could not disperse across the Pyrenees. Similar patterns have been observed in other species, e.g. the black alder *Alnus glutinosa* (King & Ferris 1998). H6 is quite distinct (Fig. 2a, 2c), and has only been found in a deglaciated area along the coast of the Adriatic Sea. It may have been present in this region throughout the Pleistocene and may represent an old evolutionary lineage that had been separated from the *H. oelandicum* lineages early on, since the plants with chloroplast haplotype H6 have some unique morphological traits not found in the complex (B. Widén and E. Soubani, unpublished).

Most haplotypes were found in the Italian peninsula south of the Alps (the three frequent haplotypes and two rare ones). H8 was found in two populations in northern-central Italy; in an area that Schönswetter *et al.* (2005) identified as a southern-alpine peripheral refugium for mountain plants between Lake Como and the Dolomites. It is reasonable to suggest that H8, which differs in only one site (i.e. one mutation), evolved from the widespread H1 and persisted in the southern Alps. H9, which differs from H3 by two polymorphic sites, was found in a single population in central Italy. Haplotype H7 was only found in one population in southern France. Since H7 is not directly related to any of the common haplotypes, it may represent an ancient refugium that persisted through several ice ages in this area.

The H3 lineage is confined to the regions surrounding the Alps and to middle Italy. The presence of potential glacial refugia on calcareous bedrock along the border of the Alps has been suggested for several mountain plants (Schönswetter *et al.* 2005). However, the relatively poor sampling of *H. oelandicum* from the Alps does not allow for a comprehensive assessment of the genetic subdivision in the area. Thus, the disjunct distribution of H3 in the western and eastern parts of the Alps (Fig. 2b) is probably due to a lack of samples in the middle-northern parts of the Alps rather than a true disjunction. Multiple refugia in the Alps, on the other hand, are known for several species (cf. Stehlik *et al.* 2002). The northern boundary between H3 and the two dominant haplotypes (H1 and H2) may suggest an earlier arrival of H1 and H2, which may have blocked the further northward expansion of H3 as suitable habitats may already have been occupied at the end of the last ice age (Taberlet *et al.* 1998). Previous studies based on cpDNA polymorphisms in *Fagus sylvatica* (Demesure *et al.* 1996), produced similar results, where Italian haplotypes did not contribute to the northern expansion. A contact zone between adjacent populations of H1 and H3 is evident in the south-western part of the Alps. This contact zone coincides with a well-known phyto-geographical border and suggests that the biogeographic history of the two haplotypes in this region is related to the Alpine glaciations. It has been suggested in other studies that

the south-western Alps is a potential contact zone (e.g. Barton & Hewitt 1981, Flanagan *et al.* 1999, Lugon-Moulin *et al.* 1999, Kropf *et al.* 2002, Petit *et al.* 2003), and that it constitutes one of the major suture zones in Europe (Taberlet *et al.* 1998, Hewitt 2000).

Our data also suggest a contact zone between the H2 and H3 lineages in the eastern part of the Alps, although the sampling of populations was not dense enough to infer the exact location of the zone. However, H2 has also been detected in two populations in the southern Alps and in one in central Italy, which may indicate a south-west postglacial expansion of this haplotype from nearby areas in northeast Italy or a long-term presence in this area.

As shown by the MSN (Fig. 2c), H3 is closely related to H1 and H2, and differs only by a single step from each. H3 may thus be the common ancestor of the two dominant haplotypes: H1 characterizing a western, central and northern lineage, and H2 characterizing an eastern, central and northern lineage. These two lineages meet in central Europe north of the Alps and on Öland in the Baltic. It is reasonable to assume that H1 persisted in one or several potential refugia in areas north of the Pyrenees and west/north-west of the Alps, and expanded to Scandinavia and western Europe during or after the last ice age (cf. Fig. 2a). A similar re-colonization pattern has been demonstrated for the herbaceous plant *Meum athamanticum* (Huck *et al.* 2009). Abundant pollen records of *Helianthemum* spp. close to the retreating Scandinavian ice shield (Berglund 1966) may suggest that the H1 lineage survived the last ice age elsewhere in the permafrost area of western Europe (cf. Soubani *et al.* submitted). Periglacial refugia have been suggested for several temperate animal and plant species (e.g. Englbrecht *et al.* 2000, Stewart & Lister 2001, Hänfling *et al.* 2002, Deffontaine *et al.* 2005, Gum *et al.* 2005, Nieberding *et al.* 2005, Pinceel *et al.* 2005, Bhagwat & Willis 2008, Ronikier *et al.* 2008b).

The H2 lineage may have recolonized the eastern part of central and northern Europe from potential refugia in the Balkans; such a lineage has also been demonstrated in the common beech *Fagus sylvatica* (Demesure *et al.*1996), silver fir *Abies alba* (Konnert & Bergmann 1995), and *Hippophae rhamnoides* (Bartish *et al.* 2006). However, the H2 lineage cover a large geographic area in eastern Europe and Asia Minor and the detected level of cpDNA polymorphism does not allow for a precise localization of putative refugia. Ice-free, exposed calcareous bedrocks in the tundra and cold steppe areas of central Europe may well have served as refugia during the last glacial maximum as indicated by pollen records of *Helianthemum* from late glacial time in southern Bohemia (Kunes *et al.* 2008).

The distribution of the two most frequent haplotypes on Öland may suggest an earlier arrival of H1, which colonized the southernmost of the Great Alvar, followed by a rapid expansion of H2 over the middle and northern part of Öland. This view is reinforced by the fact that the southernmost part of the island emerged before the northern parts after the last ice age (Königsson 1968, see also Soubani *et al.* submitted). *H. oelandicum* is one of the most conspicuous species on the Great Alvar forming continuous populations over large areas (Sterner 1936 a&b, Königsson 1968, Bengtsson *et al.* 1988). The Great Alvar is so far the only area

where we can trace introgression between different haplotype lineages of *H. oelandicum* (cf. Soubani *et al.* submitted).

Taxonomy and morphological variation

The traditionally recognized taxa in the *H. oelandicum* complex are based on few morphological characters (stellate hairs, flowering phenology and to some extent leaf and petal size), but also on altitudinal distribution in Europe. All taxa show considerable morphological variation in the growth form, shape of leaf, petal, capsule, inflorescence and seed and in the distribution of indumentum on different parts of the plant (Janchen 1907, Sterner 1936 a&b, Proctor 1957, Davies 1975, Widén 1980, 1988, 2010). All these traits are stable in cultivation (B. Widén personal observation), only the proportion of inflorescences borne on the current year's growth shows phenotypic plasticity (cf. Widén 1980). When continental populations have been examined in cultivation, most have been found to have the CF phenology, while only a few have the PF phenology. However, plants with a small proportion of inflorescences on the current year's growth were found in cultivated populations derived from scattered areas throughout the continental distribution (B. Widén personal observation). Thus, these populations may be heterozygous for the genes governing flowering phenology. It follows that populations fixed for either flowering phenology may have been derived on repeated occasions from populations variable for this character. Accordingly, populations characterized by the same flowering phenology may not necessarily be linked to each other. A full account of flowering phenology in the *H. oelandicum* complex will be published elsewhere (B. Widén in prep.).

There is a remarkable parallel distribution of taxa in the mountain ranges of the European Alpine system. The different taxa are often distributed in altitudinal zones where populations may be allopatric or parapatric, but sometimes also sympatric (B. Widén, personal observation). It is also important to point out the similarity between the parallel distribution of taxa in the European Alpine system and the parapatric distribution of the two varieties of subsp. *oelandicum* on Öland (Widén 1980, 1988, 2010, Soubani *et al.* submitted). The varieties *oelandicum* and *canescens* cover the variation in indumentum and flowering phenology traditionally used to delimit taxa in the whole complex.

Based on the morphological variation of the complex, there are various indications of hybridization and introgression between taxa. On Öland, where detailed studies have been performed, a narrow hybrid zone between the distribution of *oelandicum* and *canescens* has been established (Widén 1980, 1988 and unpublished, Soubani *et al.* submitted). Janchen (1907), who published the most detailed account of morphological variation within the complex, pointed out that many intermediates occur between taxa in continental Europe. He mentioned that certain varieties of *H. italicum* and *H. rupifragum* were difficult to distinguish from *H. canum* apart from the lack of stellate hairs on the lower side of the leaves. Rare intermediate or deviating individuals can occur in natural populations (B. Widén, personal observation). When seeds from individual plants in a polymorphic Turkish population of subsp. *incanum* were sown in cultivation, the progeny seg-

regated into two categories; with and without stellate hairs on the lower side of the leaves (B. Widén and E. Soubani, personal observation). Preliminary results from crosses suggest that this character is determined by one gene with the allele for a felt of stellate hairs on the lower side of the leaf being recessive (B. Widén in prep.). Davies (1975) found it difficult to base the taxonomy of the complex on the presence/absence of stellate hairs on the lower side of the leaf. Janchen (1907) also found it difficult to distinguish between the three species *H. italicum, H. rupifragum* and *H. alpestre*. He even suggested that *H. alpestre* could have arisen from either *H. italicum* or *H. rupifragum* as a result of adaptation to the climate variation in the alpine region.

Artificial crosses in cultivation indicate increasing crossing barriers with spatial distances between populations irrespective of taxonomic affinities (Widén 1986 and B. Widén unpublished). Preliminary results imply that crosses between the unique haplotypes (H4, H5 and H6) and frequent ones (H1, H2 and H3) will give rise to relatively sterile F1 progeny.

The closely related species *H. marifolium* was represented by one population in eastern Spain, sharing the CF phenology, bristles and haplotype (H4) with subsp. *italicum* from the same region. This pattern could be the result of hybridization between the two closely related species (Guzmán & Vargas 2009), but must be verified by a larger sample size. However, the cordate-shaped leaves of *H. marifolium* as well as the presence of stipules in the inflorescences make this taxon easily distinguishable from *H. oelandicum* subsp. *italicum* (B. Widen & E. Soubani, personal observations).

Mechanisms shaping the morphological and genetic architecture of the H. oelandicum complex

Our understanding of the postglacial history of closely related taxa can be confounded by evolutionary processes that influence the partitioning of genetic variation within and among populations (e.g. Ferris *et al.* 1993). Natural hybridization and introgression can have different effects on uniparentally inherited, non-recombining gene segments, such as the chloroplast genome, and biparentally inherited nuclear genes (Rieseberg & Wendel 1993, Soltis & Soltis 1995). Molecular studies of closely related taxa have revealed different phylogenetic patterns inferred from chloroplast DNA and nuclear genes or the phenotypic expression of nuclear genes (Soltis & Kuzoff 1995, Wolfe & Elisens 1995, Comes & Abbott 2001). Processes shaping the genetic architecture of plants with disjunct geographical distributions can be either long-distance dispersal (e.g. Schönswetter *et al.* 2002, 2004, 2006, Tribsch *et al.* 2002, Kropf *et al.* 2006) or recent inter- or postglacial vicariance (Comes & Kadereit 2003, Kropf *et al.* 2006, 2008, Ehrich *et al.* 2007).

The phylogeographic histories of numerous European plant and animal species with disjunct distribution in European mountain ranges have been published in recent years (see Schmitt 2009 for an overview). Many of these species have a distribution comparable to a terrestrial 'island' system surrounded by unsuitable lowland environments (cf. Kropf *et al.* 2008). The *H. oelandicum* complex has such an 'island' distribution pattern, where the species is currently confined to

open calcareous bedrocks. *H. oelandicum* lacks a mechanism for long-distance dispersal; the capsules open in moist weather and the seeds are shaken off by the wind or washed away by rain, travelling only short distances (B. Widén, personal observation). Dispersal may have been enhanced during repeated cycles of glaciation, during which the complex may have had a wider distribution in lowland areas. However, a long-distance dispersal of subsp. *alpestre,* for instance, between mountain ranges in Europe is not probable during the last glacial period or postglacially.

We suggest a scenario of inter- and/or postglacial vicariance to explain the parallel evolution of morphological variation in the three most frequent chloroplast haplotypes H1 H2 and H3. The evolution of morphological variation and therefore taxa may be of recent origin. Guzmán *et al.* (2009) found rapid radiation of leaf traits in the *Cistus salviifolius* lineages in Mediterranean areas as a form of adaptation to different environments. Based on molecular clock estimates, they suggested that speciation events in some lineages took place approximately 40 000 years BP. Similar rapid differentiation in the *H. oelandicum* complex would explain the parallel morphological patterns shown by different cpDNA lineages.

As a consequence of range expansion and migration of *H. oelandicum*, potential contact or meeting zones between cpDNA haplotype lineages may have been established in ice-free areas. At least in one region, the Baltic island of Öland, we can date this contact to postglacial times and find evidence of hybridization and introgression between cpDNA haplotype lineages (Soubani *et al.* submitted). We cannot exclude hybridization and introgression between lineages in contact zones in the Alps, since our sampling in this area was not dense enough to detect such processes.

Conclusions

The distribution of cpDNA haplotype lineages of the *H. oelandicum* complex shows a distinct geographic pattern with three widespread, but allopatric lineages. The morphologically homogenous cpDNA lineages H4, H5 and H6 may represent older evolutionary lineages that never managed to re-colonize areas beyond their glacial or interglacial refugia. If we consider the 'European Alpine System' it reveals a pronounced parallel differentiation in morphology from lowland to high alpine altitudes that have evolved in three of the most frequent haplotype lineages. The distribution of haplotypes may be due to old dispersal and divergence patterns in the complex, whereas the morphological groups recognized as traditional taxa have arisen independently in many mountain areas as adaptation to specific environmental conditions, e.g. related to altitude. Our results also support periglacial refugia for the complex north of the alpine glacial shields and probably also in central-east Europe. We conclude that the genetic differentiation in *H. oelandicum* depends primarily on vicariance patterns in a west – eastern direction established during Pleistocene, and secondarily on postglacial migration patterns in south – north direction.

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Appendix. Population code, country, coordinates, altitude, taxon, distribution of the two main morphological characters [(i) flowering phenology (FP): CF: concentrated FP, PF: protracted FP and (ii) indumentum (ind.): presence/ absence of stellate hairs (ST/–) or glabrous (G) leaves], cpDNA haplotype (H), number of individuals/population (N) and the main collectors: BW: Björn Widén, ES: Eman Soubani, LE: Lennart Engstrand, MP: M.C.F. Proctor and IM: Isabelle Mateu. BD: Bundesgärten, Wien, Austria. MB: Montpellier botanical garden, France. LG: Station Alpine du Lautaret, Grenoble, France. JB: Jardin Botanique 21033 Dijon–Mairie, France. MNHN: Musée national d'Histoire naturelle–Cultures, France. UWB: Universität Würzburg botanical garden, Germany. PB: Parco Nazionale Gran Paradiso, Giardino Botanico, Italy. BGM: Botanischer Garten Marburg, Italy. GBAR: Giardino Botanico Alpino Rezia, Italy. GBAP: Giardino Botanico Alpino Paradisia, Italy. GBB: Gradina Botanica Al. Borza, Romania. CJB: Conservatoire et Jardin Botaniques, Switzerland. HBB: Hortus Botanicus Bernensis, Switzerland.

Pop	Country	Latitude / Longitude	Altitude	Taxon	FP	Ind.	H	N	Collector
458	Austria	47°50′ N/13°27′ E	1500	*alpestre*	CF	–	H3	2	*BW*
459	Austria	47°40′ N/15°18′ E	1800	*alpestre*	CF	–	H3	2	*BW*
460	Austria	47°45′ N/15°21′ E	1600	*alpestre*	CF	–	H3	2	*BW*
463	Austria	47°40′ N/12°46′ E	2000	*alpestre*	CF	–	H3	2	*BW*
450	Austria	48°12′ N/16°54′ E	200	*incanum*	CF	ST	H2	2	*BW*
452	Austria	48°30′ N/16°12′ E	300	*incanum*	CF	ST	H2	2	*BW*
453	Austria	48°00′ N/16°54′ E	470	*incanum*	CF	ST	H2	2	*BW*
1079	Austria	46°43′ N/ 4°10′ E	2500	*alpestre*	CF	–	H3	2	*BD*
1080	Austria	48°48′ N/16°56′ E	500	*incanum*	CF	ST	H2	2	*BD*
2047	Austria	48°48′ N/16°56′ E	500	*incanum*	CF	ST	H2	2	*BD*
2091	Bulgaria	41°58′ N/24°55′ E	400	*incanum*	CF	ST	H2	2	*RN*
703	Bulgaria	42°36′ N/23°36′ E	1100	*incanum*	CF	ST	H2	2	*BW*
704	Bulgaria	42°24′ N/23°51′ E	1000	*incanum*	CF	ST	H2	2	*BW*
705	Bulgaria	42°00′ N/24°48′ E	600	*incanum*	CF	ST	H2	2	*BW*
706	Bulgaria	42°45′ N/24°39′ E	1600	*incanum*	CF	ST	H2	2	*BW*
401	Czech Republic	50°00′ N/14°18′ E	300	*incanum*	CF	ST	H2	2	*BW*
402	Czech Republic	49°54′ N/14°12′ E	400	*incanum*	CF	ST	H2	2	*BW*
403	Czech Republic	50°30′ N/14°21′ E	200	*incanum*	CF	ST	H2	2	*BW*
211	England	54°19′ N/2°45′ W	200	*incanum*	CF	ST	H1	2	*BW*
214	England	53°18′ N/3°21′ W	100	*incanum*	CF	ST	H1	1	*BW*
215	England	53°18′ N/3°23′ W	100	*incanum*	CF	ST	H1	2	*BW*
216	England	53°15′ N/3°36′ W	100	*incanum*	CF	ST	H1	2	*BW*
218	England	53°18′ N/3°41′ W	100	*incanum*	CF	ST	H1	1	*BW*
1023	France	44°24′ N/3°45′ E	900	*incanum*	CF	ST	H7	2	*BW*
2083	France	43°50′ N/3°42′ E	300	*incanum*	CF	ST	H1	4	*MB*
502	France	43°17′ N/2°14′ E	200	*incanum*	PF	ST	H1	4	*BW*
503	France	42°50′ N/0°30′ E	1200	*incanum*	CF	ST	H1	2	*BW*
506	France	46°12′ N/6°54′ E	1300	*incanum*	CF	ST	H1	2	*BW*

2040	France	44°50′ N/6°39′ E	2000	*italicum*	CF	–	H3	2	*LG*
1092	France	45°00′ N/ 6°24′ E	2200	*italicum*	CF	–	H3	2	*LG*
1105	France	47°28′ N/ 4°56′ E	500	*incanum*	CF	ST	H1	2	*JB*
2039	France	44°51′ N/ 6°42′ E	2600	*alpestre*	CF	–	H1	2	*LG*
2068	France	49°00′ N/ 1°36′ E	200	*incanum*	CF	ST	H1	2	*MNHN*
500	France	43°50′ N/ 7°30′ E	900	*italicum*	CF	–	H1	2	*BW*
301	Germany	51°54′ N/11°42′ E	200	*incanum*	CF	ST	H1	2	*BW*
302	Germany	51°19′ N/11°20′ E	200	*incanum*	CF	ST	H1	2	*BW*
304	Germany	51°18′ N/11°45′ E	200	*incanum*	CF	ST	H1	2	*BW*
305	Germany	51°17′ N/11°39′ E	200	*incanum*	CF	ST	H1	1	*BW*
1102	Germany	49°58′ N/ 9°45′ E	300	*incanum*	CF	ST	H1	2	*UWB*
325	Germany	49°54′ N/ 9°48′ E	200	*incanum*	CF	ST	H1	2	*BW*
657	Greece	38°10′ N/23°40′ E	900	*H. hymettium*	CF	–	H10	1	*BW*
659	Greece	38°48′ N/23°38′ E	1000	*H. hymettium*	CF	–	H10	1	*BW*
3009	Greece	41°54′ N/24°54′ E	1300	*incanum*	CF	ST	H2	1	*LE*
204	Ireland	53°36′ N/ 9°22′ W	200	*incanum*	CF	ST	H1	1	*MP*
207	Ireland	53°60′ N/ 9°22′ W	200	*incanum*	CF	ST	H1	1	*MP*
1078	Italy	45°45′ N/ 7°20′ E	2600	*alpestre*	CF	–	H3	2	*PB*
476	Italy	43°32′ N/12°45′ E	1000	*incanum*	CF	ST	H3	2	*BW*
478	Italy	42°51′ N/13°36′ E	1300	*incanum*	CF	ST	H2	1	*BW*
491	Italy	45°42 ′N/10°45′ E	300	*incanum*	CF	ST	H2	1	*BW*
477	Italy	42°48′ N/13°54′ E	700	*italicum*	PF	–	H3	2	*BW*
480	Italy	44°00′ N/10°15′ E	1100	*italicum*	CF	–	H3	2	*BW*
482	Italy	44°12′ N/ 7°30′ E	700	*italicum*	CF	–	H3	2	*BW*
490	Italy	45°51′ N/10°36′ E	700	*italicum*	CF	–	H8	2	*BW*
1110	Italy	45°38′ N/ 7°20′ E	2300	*alpestre*	CF	–	H1	2	*PB*
1111	Italy	42°28′ N/13°34′ E	1600	*alpestre*	CF	–	H9	2	*BGM*
2012	Italy	46°29′ N/10°17′ E	2000	*alpestre*	CF	–	H8	2	*GBAR*
2021	Italy	45°47′ N/ 7°28′ E	2000	*alpestre*	CF	–	H1	2	*GBAP*
493	Italy	45°51′ N/10°48′ E	400	*italicum*	CF	–	H2	2	*BW*
753	Romania	46°54′ N/25°54′ E	1500	*alpestre*	CF	–	H2	2	*BW*
754	Romania	45°30′ N/25°54′ E	1600	*alpestre*	CF	–	H2	2	*BW*
751	Romania	45°48′ N/25°42′ E	600	*incanum*	CF	ST	H2	2	*BW*
1088	Romania	45°31′ N/25°12′ E	2000	*alpestre*	CF	–	H2	2	*GBB*
752	Romania	46°48′ N/23°12′ E	700	*incanum*	CF	ST	H2	2	*BW*
423	Slovakia	48°33′ N/17°33′ E	200	*incanum*	CF	ST	H2	2	*BW*
410	Slovakia	49°00′ N/19°48′ E	1500	*rupifragum*	CF	–	H2	2	*BW*
411	Slovakia	48°54′ N/19°00′ E	1400	*rupifragum*	CF	–	H2	2	*BW*
414	Slovakia	49°00′ N/21°00′ E	500	*rupifragum*	PF	–	H2	3	*BW*
416	Slovakia	49°15′ N/19°00′ E	1200	*rupifragum*	CF	–	H2	2	*BW*

1029	Spain	42°53′ N/5°34′ W	1200	*incanum*	CF	ST	H5	2	*IM*
552	Spain	41°36′ N/2°20′ W	1000	*incanum*	CF	ST	H5	2	*BW*
550	Spain	42°30′ N/0°30′ W	1300	*italicum*	CF	–	H4	2	*BW*
1026	Spain	39°30′ N/0°30′ W	200	*H. marifolium*	CF	ST	H4	2	*IM*
556	Spain	41°30′ N/1°48′ W	900	*italicum*	CF	ST	H4	2	*BW*
1001	Switzerland	46°00′ N/7°30′ E	2400	*alpestre*	CF	–	H1	2	*CJB*
1002	Switzerland	46°40′ N/6°30′ E	2200	*alpestre*	CF	–	H1	2	*CJB*
1030	Switzerland	46°39′ N/7°54′ E	2000	*alpestre*	CF	–	H1	2	*HBB*
1021	Switzerland	56°15′ N/5°24′ E	1300	*incanum*	CF	ST	H1	4	*CJB*
611	Macedonia	42°00′ N/21°30′ E	500	*incanum*	CF	ST	H2	2	*BW*
603	Croatia	43°36′ N/16°24′ E	600	*rupifragum*	CF	–	H6	1	*BW*
605	Bosnia & Herz.	43°54′ N/17°54′ E	1000	*rupifragum*	CF	–	H6	2	*BW*
609	Montenegro	43°21′ N/17°57′ E	1200	*rupifragum*	CF	–	H6	2	*BW*
610	Montenegro	42°24′ N/18°48′ E	900	*rupifragum*	CF	–	H6	2	*BW*
p11	Sweden	56°19 N/16°21′ E	20	*canesense*	PF	ST	H1	8	*BW*
p133	Sweden	56°17′ N/16°21′ E	20	*canesense*	PF	–	H1	5	*BW*
P2	Sweden	56°15′ N/16°21′ E	20	*canesense*	PF	–	H1	2	*BW*
PI	Sweden	56°15′ N/16°25′ E	20	*canesense*	PF	–	H1	2	*BW*
p113	Sweden	56°19′ N/16°27′ E	20	*oelandicum*	CF	–	H1	8	*BW*
p27	Sweden	56°23′ N/16°30′ E	20	*oelandicum*	CF	G	H2	9	*BW*
p36	Sweden	56°34′ N/15°40′ E	20	*oelandicum*	CF	–	H2	2	*BW*
T10	Turkey	39°34′ N/29°27′ E	900	*incanum*	CF	ST	H2	5	*ES*
T13	Turkey	41°38′ N/27°36′ E	300	*incanum*	CF	ST	H2	2	*ES*
T 9	Turkey	39°20′ N/29°15′ E	1000	*incanum*	CF	mix	H2	4	*ES*
T11	Turkey	39°35′ N/29°27′ E	800	*incanum*	CF	mix	H2	2	*ES*
T12	Turkey	41°35′ N/27°44′ E	200	*incanum*	CF	ST	H2	4	*ES*


Paper II

Colonization, establishment and introgressive hybridization between two postglacial immigration lineages of *Helianthemum oelandicum* (Cistaceae) on the Baltic Island, Öland, Sweden: evidence from chloroplast DNA haplotype and indumentum data

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Keywords: PCR-RFLP, cpDNA haplotypes, *Helianthemum*, variety, flowering phenology, indumentum, Öland, introgression.

Abstract

Helianthemum oelandicum subsp. *oelandicum* on the Baltic island of Öland is represented by two endemic and allopatric varieties, differing in flowering phenology and indumentum. The variety *oelandicum* is widely distributed, while var. *canescens* is restricted to small areas in the southernmost part of Öland. Only two, allopatrically distributed, cpDNA haplotypes were detected in the present study. The border between the distributional areas of the two haplotypes in southern Öland is distinct and cuts across extensive, more or less continuous populations of var. *oelandicum*. This border coincides with marked differences in the frequency of hairy and glabrous plants. We conclude that the *oelandicum* variety, with the chloroplast haplotype H1, has arisen by directional flow of nuclear genes governing morphological recognition of var. *oelandicum* into populations of var. *canescens* ("pollen swamping").

Introduction

During the last ice age (ended about 15000 years BP), Fennoscandia was covered with a thick sheet of ice. A woodless tundra vegetation with a typical Arctic climate dominated areas south of the major ice shield, extending from the southern British Isles in the west to central and northeast Russia in the east (Berglund 1966, Björck 1995a). As the main ice sheet retreated from Fennoscandia, plants and animals recolonized the region from different directions and source areas. Hence, Fennoscandia is populated by a mixture of taxa that have survived in potential nonglaciated refugia in Russia and Europe and recolonized Fennoscandia (including the Baltic islands of Öland and Gotland) when the conditions became favourable after the last ice age (Hewitt 1999). Various recolonization patterns have been suggested in numerous studies of plants (i.e. lineages and source areas)

based on spatial genetic patterns in present-day populations (e.g. Taberlet *et al.* 1995, Ferris *et al.* 1998, Nordal & Jonsell 1998, Jaarola *et al.* (1999), Berglund & Westerbergh (2001), Tyler (2002), Vainio & Väinölä (2003), Malm & Prentice 2005, Bartish *et al.* 2006, Nordström & Hedrén 2008, Ronikier *et al.* 2008).

A significant outcome of species expansion and contraction during and after the last ice age is the formation of hybrid zones and contact zones. Many animal and plant species have been split into genetically and morphologically distinct groups in their glacial refugia. When these taxa met and admixtured, hybrid or contact zones were formed. Such zones may be stable over time, possibly because the different migrant populations have become adapted to different environmental conditions in geographically separated refugia (Nichols & Hewitt 1994, Hewitt 2001).

Phylogeographic studies of plant species often use data from chloroplast DNA (cpDNA) to localize potential glacial refugia and reconstruct postglacial migration routes, as well as to identify postglacial hybridization (e.g. Arnold *et al.* 1991, Kron *et al.* 1993, Ferris *et al.* 1998, Grivet & Petit 2002, Palmé *et al.* 2003, Petit *et al.* 2003a, Griffin & Barrett 2004, Hathaway *et al.* 2009, Hedrén 2009). The level of cpDNA polymorphism detected is, however, often too low to reconstruct precise patterns of past evolutionary processes (cf. Holderegger *et al.* 2002, Griffin & Barrett 2004, Bartish *et al.* 2006). To understand the adaptation of colonizers to a novel climate, a new physical environment and a mixture of species, nuclear genes or preferably the phenotypic expression of nuclear genes must be taken into consideration.

Here, we have chosen the Baltic island of Öland and the *Helianthemum oelandicum* complex for a detailed phylogeographic study. The island's bedrock is made up of Ordovician limestone pavements. The southern part of the island is dominated by the Great Alvar, a flat limestone plateau, which is to a large extent fully exposed or covered by a thin layer of weathered soil. The Great Alvar is about 300 km^2, and is a more or less treeless steppe characterized by an extremely flat, shallow topography (Königsson 1968). The Great Alvar may be subjected to extreme drought in summer, frost perturbation in winter and water-logging in autumn and spring. The plants and animals living there are particularly well adapted to this unique and unpredictable environment (Sterner 1936a, Königsson 1968, Lundegårdh *et al.* 1994). As a result, a number of exotic plant species characterized by isolated disjunct populations on Öland and different geographical distributions in Europe are found there. These plants belong to several phytogeographic groups described by Sterner (1948); a Siberian group (e.g. *Potentilla fruticosa*), a south-eastern European group (e.g. *Viola elatior*), a southern European group (e.g. *Fumana procumbens*), a south-western European group (e.g. *Hedera helix*), a northern European group (e.g. *Lychnis alpina*) and an endemic group (e.g. *Helianthemum oelandicum*). Sterner (1948) pointed out that these plant species may be relicts that have survived the repeated glacial cycles of the Late Pleistocene in ice-free refugia.

Helianthemum oelandicum represents one of the few non-arboreal plants with good postglacial pollen records from southern Scandinavia. Berglund (1966)

presented pollen maps showing the distribution of *H. oelandicum* at the end of the last ice age (see also Iversen 1944). He concluded that the species was abundant in open habitats around the margin of the Fennoscandian ice sheet in southern Scandinavia; i.e. in Denmark and southern and south-eastern Sweden (Blekinge). Today, the species is restricted to the alvar habitats of Öland. As it is characterized by low competitive ability and is restricted to alvar areas, *H. oelandicum* is likely to have been one of the pioneers that colonized Öland.

The landscape of Öland was open and dominated by the late Pleistocene tundra vegetation during the Preboreal time and early Boreal time (Königsson 1968). The pollen diagrams show that the Great Alvar was covered with forests during the warmer and more humid periods of Boreal and Atlantic periods and only a small amount of pollen from several herbaceous plant species such as *H. oelandicum* has been found during these periods (Iversen 1944, 1946). During the Subboreal time, the landscape opened and became treeless again, due to human activities (Königsson 1968). During the forested period, present-day alvar vegetation (e.g. *Artemisia* spp., *Juniperus* and *H. oelandicum*) had a limited and disjunct distribution in small open areas where survival was possible. As the forest cover declined due to human activities, the alvar-restricted herbaceous plants could again spread and colonize the forest-free areas (Königsson 1968). The present-day distribution of *H. oelandicum* on Öland is restricted to open grasslands, either on horizontal bedrocks with thin layers of weathered soils or shallow gravel deposits, or on bedrocks with open fissures and cracks, partly filled with soil (Sterner 1936a, Albertsson 1950, Königsson 1968, Bengtsson *et al.* 1988). The dominance of such habitats on the Great Alvar promotes the growth and colonization of continuous and large populations of *H. oelandicum* over thousands of square metres, making it one of the most conspicuous and important plants on the Great Alvar today.

A recent study of the phylogeography of the *H. oelandicum* complex (Widén *et al.* submitted) revealed strong geographic structuring of chloroplast haplotypes within the complex in Europe. These chloroplast haplotypes do not correlate with the taxonomic subdivision or the morphological variation within the complex; rather, several chloroplast lineages cover most of the morphological variation within the complex, indicating the parallel evolution of morphological traits in mountain areas of the European Alpine System (Widén *et al.* submitted). Two main postglacial lineages contributed to the northward expansion into Scandinavia, one from potential refugia southwest of the Scandinavian ice sheet and the other from south-eastern refugia (either from the Balkans or from the eastern part of Europe). These two lineages meet on Öland where the species has a disjunct distribution; well separated from the nearest populations in central Europe and the British Isles. The morphology of *H. oelandicum* on Öland covers the whole range of variation traditionally used to distinguish taxa within the complex (Sterner 1936b, Widén 1980, 1988, 2010). The morphological variation (Widén 1980, 1988) and distribution of cpDNA haplotypes on Öland (Widén *et al.* submitted), suggest occurrence of introgressive hybridization between postglacial lineages. Altogether,

this makes Öland an ideal area to study the evolutionary processes shaping the morphological and genetic differentiation of an early postglacial colonizer in the areas of the former Scandinavian ice sheet (B. Widén in prep.)

In the present study, we investigated the fine-scale genetic structure of the Öland rock rose *Helianthemum oelandicum* on Öland. The aims of the study were: (i) to investigate the chloroplast DNA variation of the species on Öland, (ii) to compare the genetic pattern with the geographic distribution of morphological variation and (iii) to interpret the spatial distribution of both genetic and morphologic variation in the light of available pollen records, evolutionary processes and the species' life history traits.

Materials and Methods

The subspecies

Helianthemum oelandicum subsp. *oelandicum* is a diploid, self-incompatible, wind-pollinated, long-lived (Fröberg *et al*. 2009) dwarf shrub belonging to a species complex – awaiting a modern revision – mainly found in southern and central Europe, exhibiting a disjunct distribution on Öland (Tutin *et al.* 1968). The subspecies has a disjunct distribution on Öland and is represented by two endemic varieties, var. *oelandicum* and var. *canescens* (Widén 2010). The two varieties differ in their flowering phenology (Widén 1980, 2010). Whereas var. *oelandicum* has a short concentrated flowering period in early June, var. *canescens* has an extended flowering period from June till October with one peak in June and another in July-August (Widén 1980). The differences in flowering phenology depend on the shoot system of the species (see Fig. 18 in Widén 2010). The variety *oelandicum* has inflorescences borne on the previous year's growth, which gives rise to the very concentrated flowering (CF) in early June, while the majority of inflorescences in var. *canescens* are borne on the current year's growth giving rise to a mid- and late-summer flowering (PF). A small proportion of the inflorescences in var. *canescens* is borne on the previous year's growth and flowering is less notable than the flowering of var. *oelandicum* in June (Widén 1980). Thus, the two varieties have an overlapping flowering period in June.

Both varieties show a variation in indumentum (Widén 1988). Variety *oelandicum* varies from glabrous (hairless) to hairy plants with bristles on leaves, sepals and inflorescences. Variety *canescens* varies from plants with bristles to plants with both bristles and a dense cover of stellate hairs on the lower side of the leaf. The two varieties are allopatrically distributed; var. *canescens* is restricted to the southernmost part of Öland, while var. *oelandicum* is widely distribution over the Great Alvar and on isolated alvars in the middle and north of the island (Fig. 1). However, in the southernmost part of the Great Alvar, where the two varieties meet, a narrow hybrid zone has been established (Widén 1980, 1988 and unpublished). The habitat of var. *canescens*, at least in areas where the two varieties meet, tends to be drier and better drained than the habitat of var. *oelandicum* (cf. Sterner 1936a, Widén 1988), giving rise to an ecological differentiation of the two varieties.

Preliminary results from ongoing

crossing analyses indicate various dominance relationships between the character states of both indumentum and flowering phenology. The inheritance of these character states seems to be governed by one or few nuclear genes (B. Widén, unpublished). For instance, F1 progeny in crosses between CF and PF plants bear most inflorescences borne on the previous year's growth and only a few on the current year's growth, giving rise to a pronounced early peak in flowering in June and a reduced peak of flowering in July-August.

Sampling of plant material

Based on earlier knowledge of the geographic distribution of morphs and varieties of *H. oelandicum* subsp. *oelandicum* on Öland (Widén 1980, 1988, 2010), 123 sites were sampled. Sampling was particularly dense in areas that had shown variation in morphology in previous studies (Widén 1980, 1988). Sites were mostly separated by more than 100 metres and the exact position was determined with a GPS. Within a site (10 x 10 m), five plants (in some cases as many as 10 plants) were sampled randomly for DNA-extraction and morphological analysis. In the following, all plants from a site are considered a "population", although it should be observed that the distribution of subsp. *oelandicum* in many alvar areas on Öland is largely continuous and the neighbourhood size of the species is unknown.

Figure 1. The distribution of the two varieties of *H. oelandicum* on Öland according to Sterner (1936b) and Widén (1980, 1988). The distribution of the var. *canescens* is restricted to the southernmost part of the Great Alvar and small alvars (shaded black). Var. *oelandicum* is common in the remaining parts of Öland (shaded grey) both on the Great Alvar and in small alvars north of the Great Alvar. The two varieties are spatially separated by a narrow hybrid zone.

Table 1. Description of the four primer pairs and the corresponding restriction enzymes used for the PCR-RFLP in this study.

Primer pair	Fragment length (bp)	Restriction enzymes	TA (°C)	Extension time (min.)	Reference
trnK–trnK	2500	*EcoR1, Hinf1*	60	3	Demesure *et al.* (1995)
trnS–trnT	1500	*Hinf1*	57	2	Demesure *et al.* (1995)
trnT–psbC	3500	*Taq1*	57	4	Dumolin-Lapegue *et al.* (1997)
trnT–psbD	1500	*EcoR1, Hinf1, Tru1*	57	2	Widén *et al.* submitted *

DNA extraction and PCR-RFLP analysis

A total of 302 individuals representing 123 populations (average n = 2 individuals/population, but sometimes as many as 10 plants/population) were used for DNA extraction and PCR-RFLP analysis (see the Appendix). Total genomic DNA was extracted following the CTAB-based extraction protocol (Doyle & Doyle 1990) with some minor adjustments according to Lodhi *et al.* 1994 to optimise the protocol for *Helianthemum*. Based on Widén *et al.* (submitted), four primer pairs that showed considerable variation in the PCR-RFLP were used for screening of the whole material (Table 1). The PCR reactions were carried out in a total volume of 10 µl containing 6.8 µl distilled water, 1 µl (10x PCR) buffer, 0.8 µl (25 mM) $MgCl_2$, 0.2 µl (10 mM of each) dNTPs, 0.08 µl (25 pmol/µl) each of the forward and reverse primers, 0.24 µl (1 unit/µl) *Taq* polymerase and 0.8 µl (14 ng/µl) DNA template. The PCR amplification was performed in a PTC-100 DNA thermal cycler. An initial 4 minutes denaturation at 95°C was followed by 39 cycles at 92°C for 45 s, annealing at 57-60°C for 45 s, and extension at 72°C for 2 to 4 minutes depending on the fragment length. All the PCR programs were ended with 10 minutes extension at 72°C. The PCR products (10 µl) were then digested with 10 µl restriction enzyme mixture (7.3 µl distilled water, 2µl 10x buffer and 0.7 µl [10 units/µl] restriction enzyme) and incubated over night at the optimum temperature of the applied restriction enzyme. The digested PCR products were then separated and visualized on 1.8% agarose gels stained with ethidium bromide and photographed under UV light.

Morphological studies

A total of 615 plants (n = 5 individuals/population) were preserved as dried specimens and studied thoroughly. Taxonomic designation and variation in hairiness were determined for each specimen. It has been reported in rare cases that a few individuals of var. *oelandicum* may develop scattered inflorescences on the current year's growth in autumn, especially in years with rainy periods in this season. Three such individuals were found during sampling. They were transplanted to the experimental garden at Lund University, but have so far shown no tendency to flower from the current year's growth in cultivation. The plants were classified ac-

Table 2. Haplotype, banding patterns (I, II) detected in seven primer-enzyme combinations (A: *trnK-trnK/EcoR1*, B: *trnK-trnK/Hinf1*, C: *trnS-trnT/Hinf1*, D: *trnT-psbD/EcoR1*, E: *trnT-psbC/Taq1*, F: *trnT-psbD/Hinf1* and G: *trnT-psbD/Tru1*), N: number of populations and frequency of taxon per haplotype. Each banding pattern consists of several digested fragments (bp), and x denotes the frequency of a particular digested fragment.

	Primer-enzyme combinations							N	Frequency of taxa	
	A	B	C	D	E	F	G		*oelandicum*	*canescens*
H1	I	I	I	I	II	II	II	66	53	13
	900	450(2x)	692	500	1500	750	501			
	692	320(2x)	290	250	450(2x)	500	489			
	600	242(2x)	270		242(2x)	250	320			
H2	I	I	I	II	II	I	II	61	61	0
	900	450(2x)	692	600	1500	750	501			
	692	320(2x)	290	250	450(2x)	500	489			
	600	242(2x)	270		242(2x)		320			

cording to two categories of indumentum variation pattern: (i) presence/absence (1/0) of bristles (BR) on the leaf and (ii) presence/absence (1/0) of a dense cover of stellate hairs (ST) on the lower side of the leaf.

Data analysis

The molecular data was scored as multistate characters where each primer-enzyme combination was considered a character and the different banding patterns (as a result of differences in the size and numbers of fragments) as character states (Table 2).

We followed several approaches for the analysis of geographical distribution of cpDNA haplotypes. Separate maps were constructed to plot (i) the distribution of haplotypes with taxon specification, (ii) the distribution of bristles with taxon specification and (iii) the distribution of stellate hairs with taxon specification. The scatter plots were performed using the computer program SPSS 15.0.

A Mantel test implemented in PAST software (http://folk.uio.no/ohammer/past/) based on Euclidean distance with 10000 randomization replicates was performed to test for a total of six correlations between genetic vs. geographic distances, genetic vs. bristles characteristics, genetic vs. stellate hairs characteristics, taxa vs. genetic, taxa vs. bristles characteristics and taxa vs. stellate hairs characteristics. A Monmonier's Maximum Difference algorithm analysis was performed in "Alleles In Space" software (*AIS,* Miller 2005) to identify major zones of haplotype differentiation on Öland.

Results

No populations in the hybrid zone between the two varieties in the southernmost part of the Great Alvar (Fig. 1) were polymorphic with respect to flowering phenology. All populations were thus re-

ferable to a distinct variety. Accordingly, 65 of the investigated individuals (11%) had the PF phenology and thus belong to var. *canescens* and the rest (89%) had the CF phenology (var. *oelandicum*).

Two cpDNA haplotypes were identified: H1 and H2 as earlier revealed in Widén *et al.* (submitted). The 13 populations of var. *canescens* were fixed for H1. Of the 110 populations of var. *oelandicum*, 49 populations contained H1, 57 populations contained H2, and four showed a mixture of the two chloroplast haplotypes (Table 2, Appendix).

The two haplotypes are mainly allopatric with a narrow, U-shaped southeast-northwest/southwest-northeast contact zone in the southern part of the Great Alvar, extending from the village of Segerstad in the east to the village Smedby in the west (Fig. 2). H1 is mainly found in the central and southern part of the Great Alvar, in a disjunct area in the northern part of the Great Alvar, and in two isolated alvars in the southernmost part of Öland (Fig. 2). H2 has a more continuous distribution extending from the central part of the Great Alvar to the northern part of the island, except for the small area (Resmo) which is fixed for H1 in the northern part of the Great Alvar (Fig. 2). The Monmonier's Maximum Difference algorithm identified one boundary in the middle part of the Great Alvar (Fig. 2). This boundary shows a strong change in the genetic composition in relation to space, and splits Öland into two segments; a southern-middle and middle-northern part.

The only variety found on the Great Alvar north of the southernmost part is var. *oelandicum* (Fig. 1), which has a relatively continuous distribution in the central part of the Great Alvar across the contact zone of the two haplotypes. The western part of the contact zone is very distinct; populations displaying different haplotypes sometimes separated by as little as 100 m. But the eastern part of the contact zone is more diffuse (Fig. 2). No mixed populations with respect to haplotype composition were found in the contact zone.

The two varieties differed from each other in the frequency of plants with bristles (Fig. 3, Table 3). A total of 166 of the 550 investigated individuals of var. *oelandicum* (30.2%) were glabrous (see the Appendix), but all individuals of var. *canescens* had bristles. Nine populations of var. *oelandicum* were monomorphic with only glabrous plants. Most of these were found in the distributional area of H2 (Fig. 2, Fig. 3). In the southern part of the Great Alvar, across the contact zone, a clear relationship between the distribution of haplotypes and variation in indumentum was observed (Fig. 3, Table 3). Hairy plants with H1 dominated the southern and western parts of the contact zone while glabrous plants with H2 dominated the north and east of the zone.

Most plants with a dense indumentum of stellate hairs on the lower side of the leaf belonged to var. *canescens* (47.7% of the var. *canescens* sample). However, only two populations of var. *canescens* were monomorphic for a dense cover of stellate hairs (see the Appendix) and they were found in the northern part of the distributional area of the variety (Fig. 4). Seven individuals (six populations) of var. *oelandicum* had a dense cover of stellate hairs (1.3% of the var. *oelandicum* sample). Five of these had haplotype H1 and two had haplotype H2 (Fig. 4, Appendix).

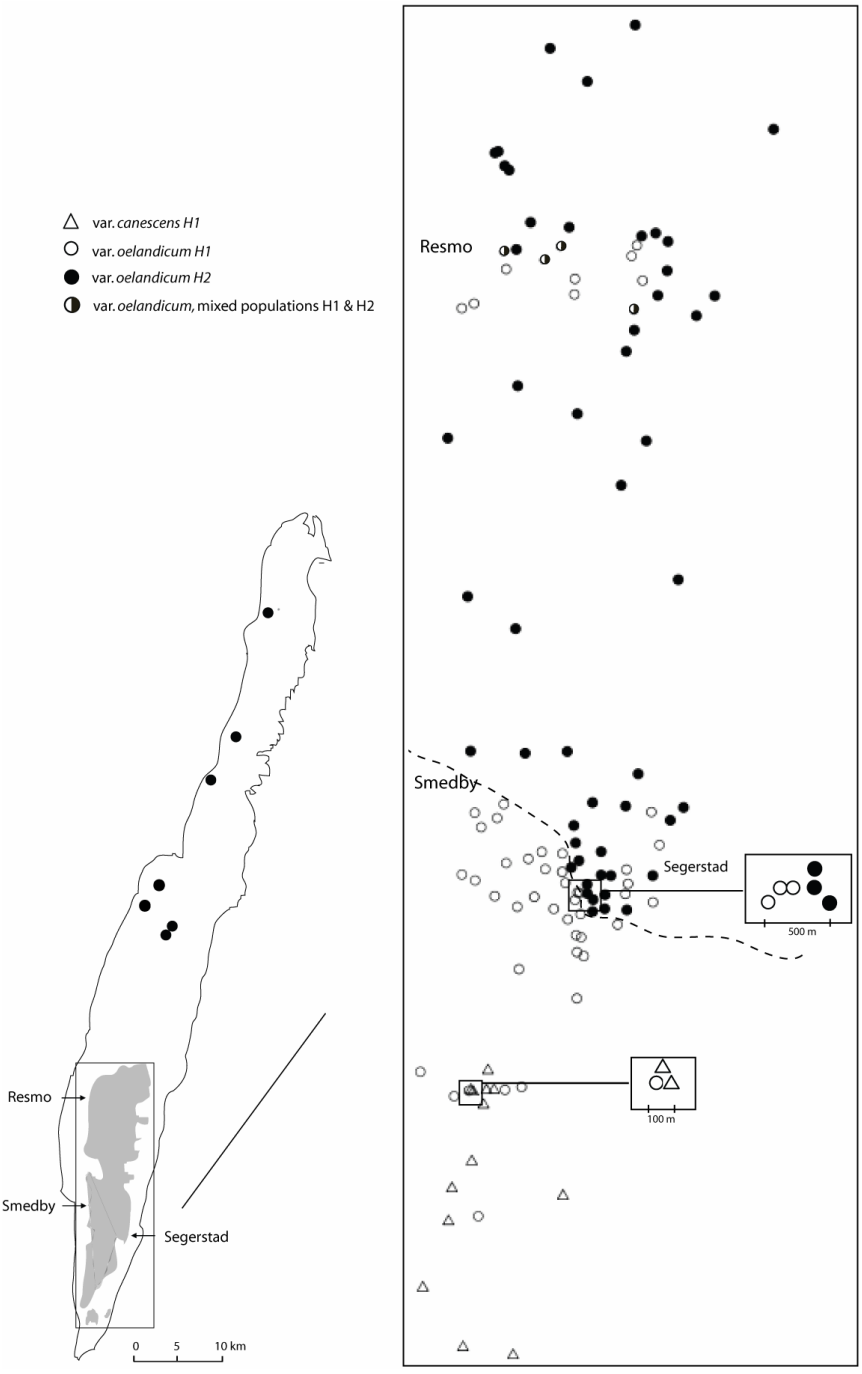

Figure 2. The distribution of the two cpDNA haplotypes on Öland combined with taxa specification (var. *oelandicum* and var. *canescens*). The symbols represent the populations analysed in this study. The rectangular area represents the Great Alvar and two alvars south of the Great Alvar (see Fig. 1). The dashed line shows the genetic boundary detected in southern Öland using Monmonier's Maximum Difference algorithm.

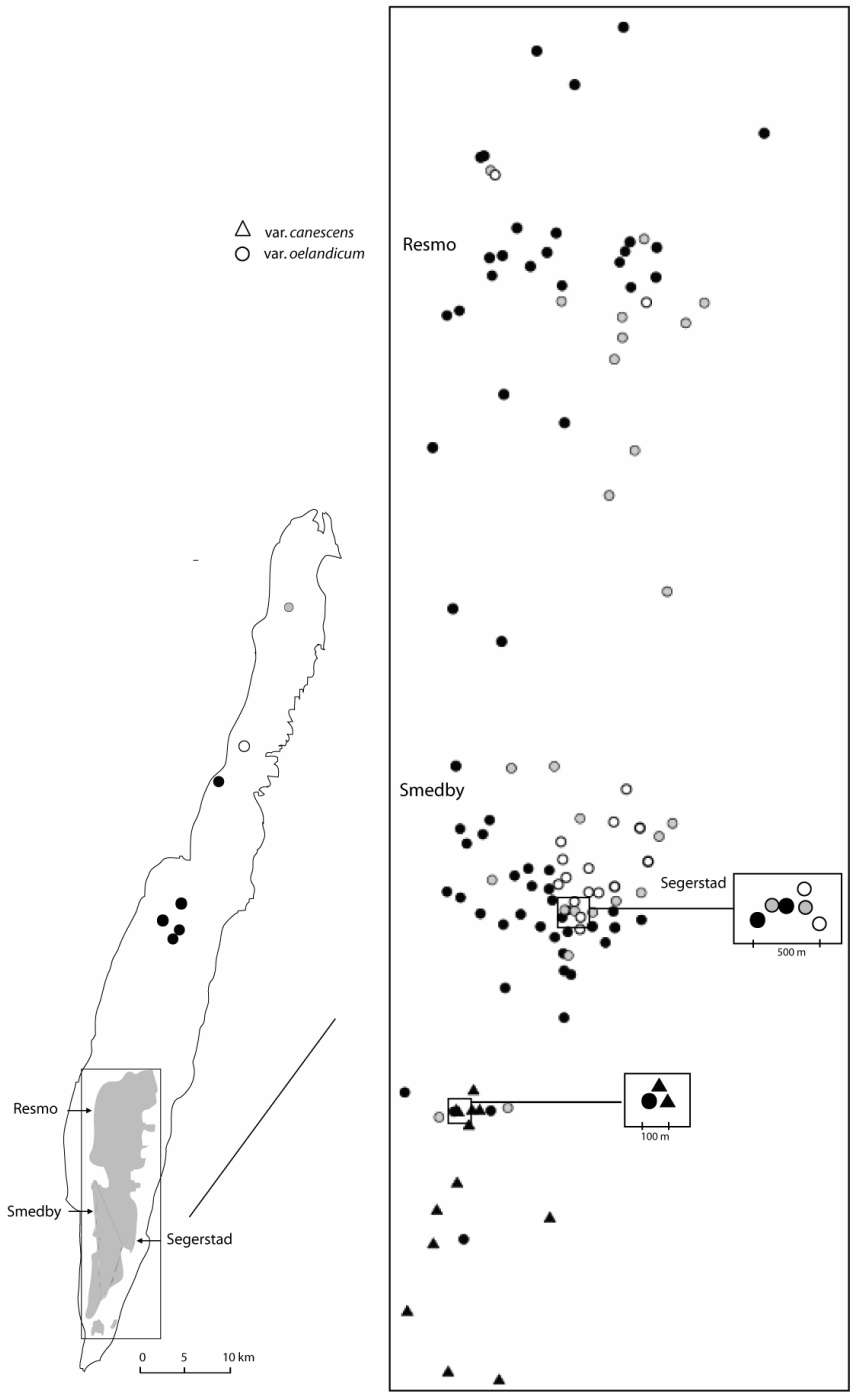

Figure 3. The distribution of taxa combined with the presence of bristles. Filled symbols denote the presence of bristles in 4-5 individuals per population, shaded symbols denote the presence of bristles in 2-3 individuals per population, and empty symbols indicate the presence of 0-1 individuals per population with bristles.

Table 3. The correlation coefficients and p-values for different distance matrixes performed by the Mantel test implemented in PAST.

Matrix distances	Correlation coefficient	P value
Genetic *vs.* geographic	r = 0.1025	*p < 0.05*
Genetic *vs.* bristles	r = 0.1276	*p < 0.05*
Genetic *vs.* stellate hairs	r = 0.0163	*p < 0.05*
Taxa *vs.* genetic	r = 0.0339	*p < 0.05*
Taxa *vs.* bristles	r = -0,0868	*p = 0.991*
Taxa *vs.* stellate hairs	r = 0.7488	*p < 0.05*

Figure 4. The frequency of plants with stellate hairs on the underside of the leaves in populations in the southernmost part of the Great Alvar and two small alvar populations in southern Öland (cf. Fig. 1). Only populations with plants that have stellate hairs are shown north of the area indicated by the rectangle.

Discussion

cpDNA and morphological diversity

In this study we found a distinct pattern in the geographic distribution of cpDNA haplotypes that differs from the distribution of morphological traits. Hence the distribution of cpDNA haplotypes in subsp. o*elandicum* on Öland does not coincide with the taxonomic entities distinguished in the species complex (Sterner 1936b, Tutin *et al.* 1968, Widén 2010). The same phenomenon has recently been detected across the entire distribution range in Europe (Widén *et al.* submitted).

The two chloroplast haplotypes, H1 and H2, already identified by Widén *et al.* (submitted), have an allopatric distribution on Öland. Only in the disjunct distribution area of H1 in the northern part of the Great Alvar do the two haplotypes occur in mixed populations (Fig. 2). These mixed populations seem to be at the margin of the northern and eastern part of this H1-disjunct area. Sampling was dense across the contact zone between the two haplotypes in the southern part of the Great Alvar due to prior knowledge of the variation in indumentum density (Widén 1988), and differences in topography and habitat between the eastern and western parts of the Great Alvar in this particular region (see the physiognomic map of the Great Alvar of Öland in Königsson 1968). The bedrock to the east and north of the contact zone is uniform over large areas, being dominated by weathered soils and very shallow gravel deposits, whereas the central part of the Great Alvar to the west of the contact zone is characterized (to some extent) by diverse habitats on deeper gravel deposits. The western boundary between the distribution of H1 and H2 in the central part of the Great Alvar is very sharp despite the fact that *H. oelandicum* occurs in large, more or less continuous populations across the contact zone (B. Widén, personal observation). The eastern part of the U-shaped contact zone close to the eastern margin of the Great Alvar, on the other hand, is more diffuse and is characterized by a mosaic distribution of *H. oelandicum* habitats (B. Widén, personal observation).

Several studies of the morphological variation in *H. oelandicum* have revealed a pronounced geographic structure of morphological diversity on Öland (the present study and Widén 1980, 1988, 2010; see also Du Rietz 1923, Sterner 1936b). The most conspicuous morphological variation of *H. oelandicum* on Öland is the flowering phenology, which is the basis for the separation of the two varieties (Widén 2010). The geographic distribution of the two varieties is allopatric, and they are either separated by habitats free from any *H. oelandicum* or by a narrow hybrid (Widén 1980, 1988). Hybridization between the two varieties is possible due to the overlapping flowering periods in June (B. Widén, unpublished results). However, the two varieties remain ecologically separated with both a spatial and a partially temporal barrier to gene exchange, which may explain the distinct morphological character states on each side of the hybrid zone (B. Widén, unpublished results).

One of these character states is the presence of a felt of dense stellate hairs on the underside of the leaves which is almost completely limited to var. *canescens*, occurring only sporadically in var. *oelandicum* (Widén 1988). A trait with a

more or less continuous variation is the presence of bristles. The glabrous plants are restricted to var. *oelandicum*, but glabrousness is observed in plants carrying either one of the two haplotypes. However, glabrous plants are most common in the area of chloroplast haplotype H2, and the difference in frequency of glabrous plants across the contact zone is striking (Fig. 2, Fig. 3, see also Fig. 11 in Widén 1988). A large, continuous area (several square kilometres) north-east of the contact zone has a high frequency of glabrous plants (>90%). Small areas with a high frequency of glabrous plants can also be found in regions dominated by hairy plants (cf. Sterner 1936a). Areas with populations exhibiting a high frequency of glabrous plants have in common long waterlogged periods, especially during snow melting in the spring (B. Widén, personal observation).

Origin of morphs with H1

Pollen diagrams (Berglund 1966) and the distribution of the cpDNA lineage H1 (Widén *et al.* submitted) indeed suggest that *H. oelandicum* with H1 survived the last glacial period in the periglacial landscape that existed north-west of the Alpine and south-west of the Scandinavian ice shields. A growing number of studies suggest the presence of refugia for temperate plant and animal species in the north and north-west of the Alps (Englbrecht *et al.* 2000, Stewart & Lister 2001, Hänfling *et al.* 2002, Palmé & Vendramin 2002, Rendell & Ennos 2002, Willis & van Andel 2004, Deffontaine *et al.* 2005, Gum *et al.* 2005, Nieberding *et al.* 2005, Pinceel *et al.* 2005, Kotlik *et al.* 2006, Mráz *et al.* 2007, Bhagwat & Willis 2008, Ronikier *et al.* 2008, Hedrén 2009, Huck *et al.* 2009). As the main ice sheet retreated, plants with the H1 haplotype must have colonized the ice-free regions near the edge of the ice sheet and eventually reached Scandinavia. We have no evidence of which variety (var. *oelandicum* or var. *canescens*) survived the last glacial period and immigrated to Öland from the south-west. The distribution of extant populations of the *H. oelandicum* complex with H1 closest to Öland is found in disjunct populations in central Germany and in Britain and Ireland (Widén *et al.* submitted, see also Fig. 1 in Proctor 1956). All these populations belong to the *incanum* group, i.e. plants with a dense cover of stellate hairs on the underside of the leaves, and all plants have the CF phenology (Widén *et al.* submitted). This combination of characters is almost absent on Öland. Present-day populations with a clear PF phenology occur in southern France (H1, with stellate hairs) and eastern Slovakia (H2, without stellate hairs). We therefore hypothesize that the Öland population characterized by H1 either survived in a discrete refugium to the south of the Scandinavian ice sheet, migrated to Öland and subsequently became extinct from its refugium and then started to hybridize with another lineage on Öland, or that the Öland population has become segregated from a genetically and morphologically variable lineage in connection with its recolonization, perhaps as the result of adaptation to the specific alvar habitats on Öland.

Origin of var. oelandicum with H2

Two scenarios could be suggested for potential glacial refugia of *H. oelandicum* with the chloroplast haplotype H2. Ro-

nikier *et al.* (2008) discussed a northward migration of *Pulsatilla vernalis* from a periglacial refugium in German and Polish lowlands south of the Scandinavian ice shield. Such a migration route could be possible for *H. oelandicum*, but there is little support for this scenario based on the present morphological diversity of the species complex. Present-day populations of *H. oelandicum* belonging to the H2 lineage closest to that seen on Öland are found in the Prague region of the Czech Republic. These populations have a character combination, stellate hairs and CF phenology, that is almost absent on Öland today (Widén *et al.* submitted). An interesting contemporary *Helianthemum* population (recognized as *H. pinegense*) with a character combination resembling var. *oelandicum* has been found in the Penegia region in northern Russia (Meusel *et al.* 1978); having leaves without stellate hairs and CF phenology, but differing in leaf shape and petal size (B. Widén and E. Soubani, personal observations, see also Sterner 1936b). Unfortunately, the chloroplast haplotype of this population is not known. However, available data suggest an alternative late glacial migration route from a refugium to the vicinity of the eastern margin of the retreating ice sheet. Large areas of the present southern Baltic Sea were ice-free land around 11000 BP and may have served as stepping-stones for migration to Öland (Björck 1995b).

Postglacial migration, colonization and present-day population processes on Öland

Two hypotheses could explain the origin of the variation in flowering phenology of the *H. oelandicum* complex on Öland (cf. Widén 1980). Either the two varieties immigrated to Öland separately, at different times or along different routes, or the variation originated *in situ* on Öland by transformation of the PF strategy to the CF strategy (or vice versa), combined with spatial differentiation by natural selection in a heterogeneous environment. Local origin of either PF or CF on Öland is unlikely, based on the widespread distribution of the PF and CF strategies in continental Europe. Simultaneous arrival of the two varieties is also unlikely, because of the lack of barriers to gene flow between them (see below). We thus postulate that the two varieties expanded from their potential refugia in western and southeastern/eastern Europe, and arrived on Öland separately. Pollen records demonstrate that *Helianthemum* was abundant in Denmark and in the southernmost part of Sweden (Blekinge) in the first ice-free areas in the Older Dryas, *c.* 10500 BP (Iversen 1944, Berglund 1966). At that time, Öland was still submerged by the Baltic Ice Lake (Björck 1995b).

The first records of *Helianthemum* pollen from Öland are from the Younger Dryas, *c.* 8500 BP (Berglund 1966) and the Preboreal period, *c.* 8000 BP (Königsson 1968), at which time central Öland had risen above sea level. Although sceptical of the possibility of discriminating between pollen of different *Helianthemum* species, Königsson (1968) reported abundant pollen grains from central Öland during the Preboreal time, when northern Öland was still submerged by the Baltic Ice Lake (Björck 1995b). According to Königsson (1968) there are records of *Helianthemum* pollen later (during the early Boreal time) on the southern part of Öland.

We suggest a scenario in which var.

canescens with the western cpDNA lineage (H1) arrived during the Younger Dryas period (*c.* 8500 BP) in the northern part of the present Great Alvar, and expanded southwards as more of the island rose above sea level. At that time open arctic tundra vegetation prevailed on Öland (Königsson 1968). Var. *oelandicum* with the eastern haplotype (H2) must have arrived in central Öland soon after the first migration wave. This lineage was the only one to expand northwards when northern Öland rose above sea level (Björck 1995b). We do not know how well-adapted the two lineages were to the postglacial climatic and habitat conditions on Öland. However, var. *oelandicum* seems to be better adapted to the current climatic conditions on Öland than var. *canescens*. Seed set is more predictable in CF plants because they often complete fruit production before the frequent summer drought, while the main flowering of PF plants coincides with the dry season (Widén 1980, and unpublished results). It is probable that hybridization between the two separate lineages was initiated soon after they met on Öland, and such a process would explain the present distributions of haplotypes and the haplotype sharing of the two varieties. This early hybridization must have given rise to the introgression of nuclear genes from var. *oelandicum* to the cytoplasm of var. *canescens* – an assumption consistent with the current hybridization pattern in their natural habitat (Widén unpublished results).

The present-day gene flow (and probably also the historical gene flow) between the two flowering phenologies is mainly unidirectional in this wind-pollinated species; from var. *oelandicum* with a CF) to var. *canescens* with a PF (B. Widén, unpublished results). The CF strategy creates a massive pollen cloud during a few weeks in June, and the relatively few simultaneous flowers of the plants exhibiting the PF strategy in mixed populations (i.e. in the present-day hybrid zone between the two varieties) will receive pollen mainly from CF plants. The reverse pollen flow is less probable due to the minority of PF flowers. Consequently, the majority of F1 progeny will possess the cytoplasm from PF plants and since most of their inflorescences are borne on the previous year's growth, this will give rise to a flowering peak in June, coinciding with the flowering peak of one of the parents (CF plants). Thus, most back-crossings of F1 plants in mixed populations will be by pollen from plants exhibiting the CF strategy (i.e. var. *oelandicum*). If plants of var. *oelandicum* (with H2) entered populations of var. *canescens* (with H1) early in the history of the species on Öland, natural selection may have favoured early flowering, reinforcing the introgression of CF genes into the cytoplasmic background of H1, i.e. "pollen swamping" (Potts & Reid 1988; Petit *et al.* 2003b). Since CF plants are better adapted than PF plants to the present-day climate on Öland, as they avoid flowering and fruiting during the frequent summer drought, they have successively invaded all available habitats, irrespective of chloroplast haplotype. The fact that PF plants still occur in the southernmost part of the Great Alvar may be due to natural selection favouring different flowering strategies in a heterogeneous environment in that area. Alternatively, the contemporary occurrence of PF plants on Öland may only represent a temporal stage in the hybridization and introgression between the two varieties

under current climatic conditions.

Pollen records show that after the initial periods of open vegetation, a forest period prevailed on Öland during the wetter Boreal and Atlantic periods (Königsson 1968). The vegetation on the Great Alvar was denser than today, and the amount of *Helianthemum* pollen declined, suggesting that *H. oelandicum* was restricted to small areas of open bedrock (Königsson 1968). The habitats of *H. oelandicum* may have been further restricted by higher annual precipitation, which may have created large alvar lakes or areas of temporal pools and swampy vegetation. The low ridges of gravel deposits on the horizontal bedrock, which dip slightly to the east (Königsson 1968), may in fact have dammed large areas in the eastern part of the Great Alvar before human activities drained them. We thus suggest that *H. oelandicum* survived the Boreal and Atlantic periods in small isolated populations restricted to areas with well-drained, open bedrock.

The levels of *Helianthemum* pollen continued to be low in the central part of Öland, where contemporary populations of *H. oelandicum* are restricted to small alvar areas in a mosaic of forests, grazed pastures and arable fields (Fig. 1). On the Great Alvar, on the other hand, the level of *Helianthemum* pollen increased dramatically in the Subboreal period, and it has continued to be abundant ever since (Königsson 1968). This increase in pollen coincided with an increase in human impact on the landscape, which gave rise to more open vegetation on the Great Alvar (Königsson 1968). The distribution of *H. oelandicum* expanded and the present-day geographical pattern of chloroplast haplotypes/flowering strategies became established. Both variation in flowering strategies and variation in indumentum of *H. oelandicum* may reflect adaptation to local drainage conditions, partly created by the human activities on the Great Alvar. We can still trace the original hybridization between var. *canescens* and var. *oelandicum* in the morphological variation. Plants with a felt of stellate hairs on the underside of the leaves are rare in var. *oelandicum* on the Great Alvar far north of the present distribution of var. *canescens* (Fig. 3, and Fig. 1 in Sterner 1936b). Furthermore, scattered individuals of var. *oelandicum* produce few inflorescences on the current year's growth in the autumn (this study, Widén 1980, Fig. 1 in Sterner 1936b). If the high frequency of individuals of var. *canescens* without stellate hairs originated from introgression of nuclear genes from var. *oelandicum*, or by natural selection acting on an originally polymorphic colonizer, has still to be investigated.

Conclusions

The most plausible scenario for the postglacial immigration of *H. oelandicum* into Öland is the migration of two lineages, var. *canescens* (chloroplast haplotype H1) and var. *oelandicum* (chloroplast haplotype H2), along the same route but at different times. An early stage of hybridization and introgression of nuclear genes from var. *oelandicum* to var. *canescens* created three morphological/cytoplasm lineages: var. *canescens* (with haplotype H1), var. *oelandicum* (with haplotype H1) and var. *oelandicum* (with haplotype H2). A long period of forest vegetation on Öland during the Boreal and Atlantic periods restricted the habitats of *H.*

oelandicum to small isolated areas of open bedrock where the species survived. The variety *oelandicum* (with haplotype H2) survived in northern and middle Öland, as well as in various areas of the northern and eastern parts of the Great Alvar. Var. *oelandicum* (with haplotype H1) survived in a small area of the northern Great Alvar (the Resmo area) and in the southern part of the Great Alvar. The variety *canescens* (with haplotype H1) survived in the southernmost part of the Great Alvar and in the small alvar areas south of the Great Alvar. When the vegetation became open again, due to human impact in the Subboreal period, *H. oelandicum* expanded and the distinct pattern of present-day diversity was established. The present geographical pattern of diversity in hairiness and flowering phenology may reflect migration history, a long period of restricted distribution and natural selection in different habitats on Öland.

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Appendix. Population code, latitude, coordinates (Swedish grid (SG) x, y), haplotype, flowering phenology: concentrated flowering phenology (CF), protracted flowering phenology (PF), frequency of glabrous plants (G), frequency of plants with stellate hairs (ST) in all the five individuals within a population and no. of individuals investigated per population for the PCR-RFLP.

Population code	SG-x	SG-y	Haplotype	Flowering phenology	G	ST	N
P 2	6236210	1540520	1	PF	0	1	2
P 1	6236444	1539083	1	PF	0	0	2
P 3	6238179	1537932	1	PF	0	2	2
R137	6240110	1538666	1	PF	0	2	2
Sebberneby	6240260	1539521	1	CF	0	2	2
Eketorp	6240849	1541951	1	PF	0	2	2
R 133	6241067	1538767	1	PF	0	1	5
Sebberneby north	6241853	1539334	1	PF	0	2	2
R115	6243503	1539671	1	PF	0	5	2
R110	6243753	1538829	1	CF	3	0	2
R411	6243878	1539373	1	PF	0	1	2
R211	6243912	1539251	1	CF	1	1	4
P11	6243926	1539757	1	PF	0	4	8
R311	6243931	1539312	1	PF	0	3	2
R112	6243933	1539973	1	PF	0	5	2
R113	6243938	1540287	1	CF	1	0	8
R114	6244022	1540765	1	CF	2	0	2
Albrunna	6244463	1537861	1	CF	0	0	3
R109	6244500	1539800	1	PF	0	3	2
Träby	6246600	1542350	1	CF	0	0	2
Tingstenen	6247450	1540690	1	CF	0	1	2
Träby 1	6247832	1542546	1	CF	0	0	2
Träby 2	6247944	1542353	1	CF	0	0	2
Träby 4	6248379	1542471	1	CF	2	0	2
Träby 3	6248440	1542327	1	CF	0	0	2
Segerstad 8	6248750	1543518	1	CF	0	0	2
Segerstad 4	6248901	1542090	1	CF	0	0	3
Segerstad 9	6249052	1542455	1	CF	0	0	2
Segerstad 29	6249128	1542799	2	CF	4	0	2
Segerstad 5	6249174	1543783	2	CF	0	0	2
Segerstad 3	6249206	1541686	1	CF	0	0	2
Segerstad 25	6249210	1543146	2	CF	1	0	2
Klinta 5	6249267	1540639	1	CF	0	0	2
Segerstad 7	6249400	1544530	1	CF	0	0	2
Segerstad 10	6249467	1542295	1	CF	1	0	2
Segerstad 27	6249476	1542817	2	CF	4	0	2
Klinta 7	6249556	1541128	1	CF	1	0	2

Klinta 3	6249577	1539993	1	CF	1	0	2
R 83	6249615	1543157	2	CF	3	0	2
Segerstad 23	6249641	1543739	1	CF	1	0	2
Segerstad 30	6249655	1542649	2	CF	3	1	2
Segerstad 11	6249673	1542429	1	CF	1	0	2
Segerstad 12	6249681	1542365	1	CF	3	1	2
Segerstad 31	6249917	1542643	2	CF	4	0	2
Segerstad 1	6249938	1543816	1	CF	3	0	2
P 26	6249960	1542018	1	CF	1	0	4
Klinta 2	6250036	1539431	1	CF	0	0	2
Segerstad 16	6250175	1544523	2	CF	2	0	2
Segerstad 24	6250176	1543326	2	CF	4	0	2
Segerstad 26	6250192	1543042	2	CF	5	0	2
Klinta 1	6250206	1539051	1	CF	0	0	2
Segerstad 17	6250282	1541928	1	CF	1	0	2
Segerstad 15	6250350	1543772	1	CF	4	0	2
Klinta 9	6250364	1541443	1	CF	1	0	2
Segerstad 2	6250413	1542192	2	CF	4	0	2
Klinta 4	6250541	1540322	1	CF	2	0	2
P 27	6250613	1542410	2	CF	5	0	10
Klinta 6	6250662	1540957	1	CF	0	0	2
Segerstad 13	6250816	1541935	1	CF	1	0	2
Klinta 8	6250866	1541342	1	CF	1	0	2
Segerstad 14	6250873	1543044	2	CF	5	0	2
Segerstad 20	6251066	1544717	1	CF	5	0	2
Segerstad 18	6251126	1542310	2	CF	5	0	2
Klinta	6251583	1539606	1	CF	0	0	3
Segerstad 32	6251634	1542261	2	CF	5	0	2
Segerstad 19	6251786	1545027	2	CF	3	0	2
Smedby 3	6251849	1540062	1	CF	0	0	2
Smedby 2	6252002	1539420	1	CF	0	0	2
Segerstad 21	6252027	1544487	1	CF	5	0	2
Mellbykant	6252159	1545407	2	CF	3	0	2
Segerstad 22	6252198	1543749	2	CF	5	0	2
Smedby 1	6252254	1540253	1	CF	0	0	2
Segerstad 28	6252299	1542801	2	CF	3	0	2
Mellby alvar	6253137	1544106	2	CF	4	0	2
Storåsen	6253739	1540866	2	CF	2	0	2
Tresocknar	6253789	1542075	2	CF	3	0	2
R 93	6253800	1539300	2	CF	0	0	2
Penåsa	6257360	1540590	2	CF	0	0	2
Kastlösa	6258300	1539218	2	CF	0	1	2
Hulterstad	6258792	1545256	2	CF	2	0	2
Gösslunda SV	6261539	1543620	2	CF	2	0	2

Gösslunda	6262828	1544342	2	CF	2	0	2
Bredinge	6262911	1538649	2	CF	0	0	3
Bredinge east	6263617	1542361	2	CF	0	0	2
Bårby	6264430	1540653	2	CF	0	0	2
Bårby källa 2	6265436	1543767	2	CF	3	0	2
Bårby källa 1	6266055	1543994	2	CF	3	0	2
Kritmossen	6266473	1545779	2	CF	2	0	3
Möckeln south	6266642	1543986	mix	CF	2	0	2
Mysingehög	6266690	1539050	1	CF	0	0	2
Mysinge north	6266827	1539401	1	CF	0	0	2
Frösslunda SV	6267050	1546303	2	CF	2	0	2
Möckeln east 1	6267064	1544669	2	CF	4	0	2
Möckel SV	6267093	1542278	1	CF	3	0	2
Möckelmossen	6267497	1544235	1	CF	0	0	2
Resmo	6267546	1542293	1	CF	0	0	5
Möckeln east 2	6267785	1544937	2	CF	0	0	2
Resmo SO	6267828	1540321	1	CF	0	0	5
Resmo 3	6268093	1541404	mix	CF	0	0	3
Möckeln west 3	6268212	1543919	1	CF	1	0	2
Resmo NO	6268342	1540251	mix	CF	1	0	5
Resmo 1	6268403	1540615	2	CF	0	0	3
Resmo 4	6268490	1541870	mix	CF	0	0	2
Möckeln west 2	6268521	1544071	1	CF	1	0	1
Möckeln east 3	6268633	1544963	2	CF	0	0	2
Möckeln west 1	6268792	1544208	2	CF	0	0	3
Möckeln north 1	6268879	1544607	2	CF	3	0	2
Resmo 5	6269046	1542131	2	CF	0	0	2
Resmo 2	6269192	1541021	2	CF	0	0	3
Vickleby A (P36A)	6270711	1540405	2	CF	5	0	2
Vickleby B (P36B)	6270836	1540281	2	CF	3	0	2
Vickleby C (P36C)	6271213	1540000	2	CF	0	0	2
P 36	6271253	1540094	2	CF	0	0	2
Ekelunda	6271901	1547989	2	CF	0	0	2
Karlevi 2	6273290	1542650	2	CF	0	0	2
Karlevi 1	6274257	1541580	2	CF	0	0	1
Kalkstad	6274935	1544023	2	CF	0	0	2
Odens flisor	6293808	1549974	2	CF	0	0	2
Noaks ark	6294363	1550280	2	CF	0	0	2
Räpplinge	6298880	1548707	2	CF	1	0	2
Djupvik	6320525	1560747	2	CF	0	0	2
Sandvik	6325369	1564603	2	CF	5	0	2
Mensalvaret	6344499	1569704	2	CF	3	0	2

Phylogeography of the European rock rose *Helianthemum nummularium* (Cistaceae); incongruent patterns of differentiation in chloroplast DNA and morphology

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Keywords: Cistaceae**,** *Helianthemum*, Pleistocene, chloroplast DNA, microsatellites, phylogeography, shape analysis, moment invariants.

Abstract

Helianthemum nummularium is a morphologically variable species that has been subdivided into several subspecies, primarily based on indumentum characters. We investigated four of these subspecies for variation in chloroplast DNA and leaf and petal shape in Europe. Three size-variable mononucleotide repeat regions were amplified by means of species specific primers and 18 combined haplotypes were identified. The highest haplotype diversity was found in the Alps and the surrounding lowland areas, whereas marginal areas such as northern Europe and south-eastern Balkans had low diversity. Most of the common haplotypes were shared between subspecies and showed a geographic structuring across the species range. Leaf and petal shape descriptors could not differentiate between subspecies. It is concluded that the poor correspondence between chloroplast haplotype distribution and subspecies circumscription is due to multiple origins of morphologically similar morphs (grouped into taxonomic subspecies) in different parts of the distribution range of the complex.

Introduction

It is generally considered that levels of intraspecfic genetic variation (expressed by molecular and morphological characters) and the spatial structuring of the variation could be shaped by various factors, including species life history traits as well as interactions with physical environment and other organisms. Furthermore, genetic variation patterns could be affected by contemporary processes such as gene flow and genetic drift and historical events such as the Pleistocene glaciations. Specifically, the impact of Quaternary climatic fluctuations on within-species variation patterns has received a lot of attention especially in the temperate regions (e.g. Taberlet *et al.* 1998, Hewitt 2000, Petit *et al.* 2003).

Early phylogeographic studies described a general pattern of species restricted to

isolated populations or refugia in southern Europe (e.g. the Iberian Peninsula, Italy and the Balkans) during the cold glacial cycles. And at the onset of warm interglacial periods, these species expanded unidirectional out of these refugia into the ice-free uncolonized territories (Taberlet *et al.* 1998, Hewitt 1999, Petit *et al.* 2003). However, a growing number of studies have also indicated the existence of periglacial refugia in relatively northern areas in the periglacial zones (Stewart & Lister 2001). This pattern applies to various trees, including the common beech (Demesure *et al.* 1996) and the black alder (King & Ferris 1998), as well to some herbaceous plants, such as *Pulsatilla vernalis* (Ronikier *et al.* 2008) and *Meum athamanticum* (Huck *et al.* 2009).

The separation of the refugial populations has presumably led to increased diversification of populations by the combined action of genetic drift, restricted gene flow, and accumulation of mutations as a result of long periods of persistence and adaptation to environmentally favoured habitats in refugial areas. Following range expansion, the refugial populations would be expected to retain some of their unique characteristics, whereas a subset of the genetic material would have been transmitted with the expanding populations. The highest genetic diversity would be found at middle latitudes where genetic lineages from different refugia met and intermixed. However, populations at northern latitudes would be expected to contain less genetic diversity. Overall, genetic diversity is expected to decrease towards the north (Bennett *et al.* 1991, Hewitt 1996).

Based on comparative phylogeographic studies, it has become clear that many species have unique recolonization patterns differing in timing, rate and route of migration, depending on species-specific sets of environmental and ecological requirements (Huntley & Birks 1983, Huntley 1991, Taberlet *et al.* 1998, Hewitt 1999).

Chloroplast DNA has been widely used in plant phylogeographic studies to reconstruct the migration history after the last ice age (Dumolin-Lapégue *et al.* 1997a, King & Ferris 2000, Grivet & Petit 2002, Petit *et al.* 2002, 2003). Chloroplast DNA (cpDNA) is a haploid, multicopy, nonrecombining genome which is transmitted by seeds in most angiosperms (Wolfe *et al.* 1987). Commonly employed techniques such as the PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) may display insufficient polymorphism in phylogeographic studies below species level. An alternative to the PCR-RFLP is the use of chloroplast DNA microsatellite markers (Goldstein & Pollock 1997). These microsatellites are often highly polymorphic and have been utilized to explore patterns of inter- and intrapopulation variation of different plant species (Provan *et al.* 1999, Parducci *et al.* 2001, Grivet & Petit 2002, Petit *et al.* 2002, Provan & Campanella 2003, Nordström & Hedrén 2009). The usefulness of cpDNA microsatellites for phylogeographic studies has been reviewed by Provan *et al.* (2001) and Petit & Vendramin (2007).

Molecular studies based on cpDNA markers in species complexes commonly reveal variation patterns that are better explained by geography than taxonomy (e.g. morphology), in contrast to the nuclear markers (Petit *et al.* 2002). Sharing of cpDNA haplotypes among subspecies have been previously detected in the *Helianthemum oelandicum* complex (Widén *et al.* submitted) as well as in many other

groups (Rieseberg & Soltis 1991, King & Ferris 2000, Dobes *et al.* 2003, Palme *et al.* 2004, Fehrer *et al.* 2007, Hedrén *et al.* 2008, Hathaway *et al.* 2009). This phenomenon can be explained by (i) homoplasy or incomplete lineage sorting of chloroplast genomes from a polymorphic ancestor (Estoup *et al.* 1995, Jarne & Lagoda 1996, Provan *et al.* 2001, Bänfer *et al.* 2006) and/or (ii) gene flow or introgression among sympatric taxa (Rieseberg & Soltis 1991, Wendel & Doyle 1998, Linder & Rieseberg 2004, Palme *et al.* 2004). For example, in *Helianthemum oelandicum,* (Widén *et al.* submitted), parallel morphs classified as subspecies have evolved in separate mountain ranges in central and southern Europe.

This study investigates the taxonomic complex of *Helianthemum nummularium* (Cistaceae). The complex consists of evergreen, diploid (2n = 20), insect-pollinated and out-breeding dwarf shrubs that produce multiple racemes of yellow or pink flowers in early summer. It favours dry open grasslands, rocky places and meadows. The species complex has a wide distribution in Europe but disjunct populations can be found in Turkey, the Caucasus and northern Iran (Widén 2010). The morphological variation in *H. nummularium* is complex and especially the indumentum characters that have been considered to be taxonomically important (cf. Janchen 1909). According to Flora Europaea (Tutin *et al.* 1968), the *H. nummularium* complex consists of eight subspecies of which five display yellow flowers; *H. nummularium* (L.) Mill. subsp. *glabrum* (WDJ Koch) Wilczek, subsp. *grandiflorum* (Scop.) Schinz & Thell, subsp. *nummularium* (L.) Mill., subsp. *obscurum* (Čelak.) Holub and subsp. *tomentosum* (Scop.) Schinz & Thell. The subspecies *nummularium* and subsp. *tomentosum* are very similar in morphology and both have a dense cover of stellate hairs, at least on the lower side of the leaf. The subspecies *tomentosum* has larger leaves and petals and is considered to be an alpine taxon (Janchen 1909), whereas subsp. *nummularium* has more a diffuse distribution; occupying lowlands to alpine altitudes. The subspecies *obscurum,* subsp. *grandiflorum* and subsp. *glabrum* are all characterized by the absence of a dense cover of stellate hairs on the lower side of the leaf. The subspecies *glabrum* has glabrous (hairless) leaves, whereas subsp. *obscurum* and subsp. *grandiflorum* are characterized by the presence of long bristles on the leaves. The subspecies *grandiflorum* has considerably large petals, and the sepals are glabrous between the ribs as opposed to subsp. *obscurum*.

According to Tutin *et al.* (1968), Meusel (1978) and Hultén & Fries (1986), the complex has a wide distribution in almost all of Europe except for the extreme north. The subspecies *nummularium* is widespread in Europe. The subspecies *obscurum* is distributed in central Europe and parts of eastern and southern Europe and extends northwards into Sweden and Denmark. The complex shows the greatest diversity in mountain areas of southern and central Europe, where several subspecies can be found in the vicinity of each other, such as subsp. *tomentosum,* subsp. *glabrum* subsp. *grandiflorum,* subsp. *obscurum* and subsp. *nummularium.*

The broad objective of the present study was to describe the phylogeographic structure of the *H. nummularium* complex in Europe. Specific objectives were to: (1) develop a polymorphic cpDNA

marker system appropriate for inference about the species´ postglacial history; (2) analyse variation in two independent morphological characters, leaf and petal shape, across the taxonomic subdivision of the *H. nummularium* complex; and (3) integrate the results from both molecular and morphological data and interpret the findings in the light of evolutionary processes and the species´ life history traits.

Materials and Methods

Plant material

A total of 115 wild populations were collected in the form of seeds between 2000 and 2007 by colleagues and European botanical gardens across the species range in Europe (see the Appendix, Fig. 1). The seeds were sown in pots in the experimental garden, Lund University in 2006 and 2007. One to eighteen plants were obtained from each population. The taxonomic designation of all plants was determ–

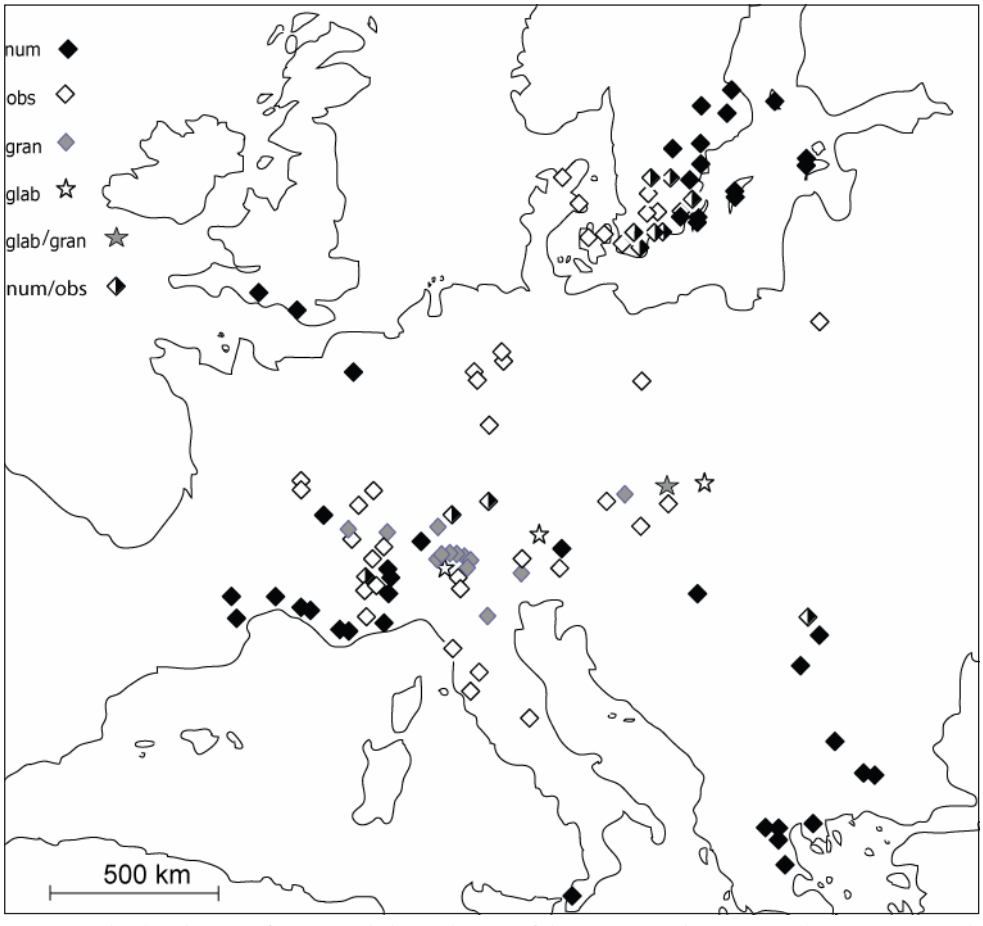

Figure 1. The distribution of 115 sampled populations of the *H. nummularium* complex in Europe. Each symbol represents a population with taxa specification according to Flora Europaea (Tutin *et al.* 1968), num: *nummularium,* obs: *obscurum*, gran: *grandiflorum*, glab: *glabrum*.

ined from the dried (cultivated) reference specimens based on the morphological characters described in Flora Europaea (Tutin *et al.* 1968). The five subspecies were arranged into two groups based on the absence/presence of a felt of stellate hairs on the lower side of the leaf. Group (1) included subsp. *glabrum*, subp. *obscurum* and subsp. *grandiflorum* which have no felt of stellate hairs on the lower side of the leaf. These three subspecies were distinguished from each other based on the previously described morphological characters (see introduction). Group (2) included subsp. *nummularium* and subsp. *tomentosum* which have a felt of stellate hairs. However, we were not able to distinguish between subsp. *nummularium* and subsp. *tomentosum* based on the size of leaf and petal (as they overlapped broadly) or any other character. We therefore joined the two subspecies into one; subsp. *nummularium*.

For the study of cpDNA polymorphism, a total of 275 plants representing 98 populations (average n = 3 plants/population) of the *H. nummularium* complex was screened. Since the main aim of the study was to investigate the large-scale geographic pattern of chloroplast variation within the complex, the choice was made to include as many populations as possible rather than many samples from each population (Magni *et al.* 2005).

For the multivariate morphometric analyses, two leaves and three petals per plant (average n = 7 plants/population) were preserved as dried specimens during summers 2007 and 2008. A total of 586 plants representing 96 populations were screened for leaf shape variation and 309 plants representing 77 populations for petal shape variation. Seventeen populations were included in the morphometric analysis, but not in the molecular analysis (see the Appendix).

Table 1. List of the cpDNA microsatellite primers designed and applied in this study.

Primer pair	Primer sequence (5'-3')	TA (°C)	Type	Fragment length (bp)
mtrnSf-trnGr	F: CCATTTCGAAAATTGGAGAGA R: CGCATTAACAATACGAAACTATAGA	57	polyT	128-131
mtrnL2-trnF	F: AAT GGGCATCGGAATACCA R: CATTTTACGAGA GGGCTTGG	57	polyT	213-218
mtrnL5-trnL3	F: AAT GGGATTGAATGGCTTTG R: TCCTGGAGTGAAAGGGTTGA	57	polyA	237-243

DNA extraction, PCR-RFLP and cpDNA microsatellite

Total genomic DNA was extracted from fresh, dried or frozen leaves by the CTAB method (Doyle & Doyle 1990) with adjustments according to Lodhi *et al.* (1994). A pilot PCR-RFLP study was done over a subset of 20 plants; covering the variation in taxonomy and geography, for testing universal primers. This screening included 21 universal cpDNA primer pairs for amplification of the following cpDNA regions: the *trnL intron*, *trnL–trnF*, *trnT–trnL* (Taberlet *et al.* 1991), *trnS–trn*M, *trnS–trnT*, *trnK*, *trnC–trnD, psaA–trnS*, *trnM–rbcL, trnD–trnT, trnH–trnK*, *psbC–trnS* (Demesure *et al.* 1995), *trnK–trnQ, rpoC–trnC*, *trnT–psbC*, *trnM–psaA, trnF–trnV, trnV–rbcL*, *trnC–rpoC* (Dumolin-Lapegue *et al.* 1997b), *atpB–rbcL* (Chiang *et al.* 1998), *trnS–trnG* (Hamilton 1999), *rpl2–rps*19 (Weising & Gardner 1999) and six mitochondrial mtDNA universal primer pairs for amplification of mtDNA regions: *nad4/2–nad4/3, nad5/1–nad5/2, nad7/2–nad7/3, nad7/3–nad7/4, nad1/2–nad1/3* and *nad4/2–nad4/2* (Dumolin-Lapegue *et al.* 1997b). Twelve regions were successfully amplified and further tested for restriction polymorphism using eight restriction enzymes (*EcoR1, Hinf1, Tru1I, Taq1, AluI, Rsa I, NdeII and CfoI*; Roche Applied Science).

Three noncoding cpDNA regions, (i) the *trnL–trnF* intergenic spacer, (ii) *trnL* intron (*trnL5-trnL3*) (Taberlet *et al.* 1991) and (iii) *trnS–trnG* intergenic spacer (Hamilton 1999) were selected for sequencing and later surveyed for polymorphic microsatellite loci. Three polymorphic mononucleotide (A/T) loci were detected and subsequently amplified by a set of three *Helianthemum* specific primer pairs designed in the Primer 3 program (Rozen 2000) (see Table 1). The PCR reactions were carried out in a total volume of 10 µl containing 6.8 µl distilled water, 1 µl (10x PCR) buffer, 0.8 µl (25 mM) $MgCl_2$, 0.2 µl (10mM of each) dNTPs, 0.08 µl (25 pmol/µl) each of the forward and reverse primers, 0.24 µl (1 unit/µl) *Taq* polymerase and 0.8 µl (14 ng/µl) DNA template. The PCR amplification was performed in a PTC-100 DNA thermal cycler. An initial 4 minutes of denaturation at 95°C was followed by 30 cycles at 92°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 1 minute. The PCR programs were ended with 10 minutes extension at 72°C. The PCR products were then mixed with appropriate size standards to determine the size of the amplified fragments. Dye-labelled fragments were separated on an ALF Express II DNA analyzer (GE Healthcare), and the size of fragments was determined by the ALFwin Fragment Analyzer 1.03.01 software (GE Healthcare).

Morphometry

Leaf and petal contours were captured by a video camera connected to a computer and an analogue-to-digital converter. The shape of leaf and petal was scored based on the moment invariants (MOM) in the ARBO program described by White *et al.* (1988). A canonical variates analysis (CVA, Dunn & Everitt 1982) was performed on the moment invariants of leaf and petal using the software package SPSS 15.0 to calculate Wilks´ lambda (Λ) values of within taxa (Λ_{TAX}) and within populations (Λ_{POP}) variations. The pro-

Table 2. Allele compositions of the 18 cpDNA haplotypes found within *H. nummularium*. Total number of individuals (N) and distribution among subspecies (absolute counts) are given, *num*: *nummularium, obs*: *obscurum*, *gran*: *grandiflorum*, *glab*: *glabrum*.

Haplotypes	*mtrnSf-trnGr*	*mtrnL2-trnF*	*mtrnL5-trnL3*	N	*num*	*obs*	*gran*	*glab*
H1	128	216	241	29		20	9	
H2	128	217	241	26	1	24	1	
H3	128	217	242	16	8	8		
H4	129	216	241	36	11	13	8	4
H5	129	217	241	77	22	45	10	
H6	129	217	242	24	21	3		
H7	129	218	241	6	6			
H8	130	213	237	3	3			
H9	130	214	241	3			3	
H10	130	215	243	1	1			
H11	130	216	242	3	3			
H12	130	217	241	10	5	2	3	
H13	131	216	241	7	3		1	3
H14	131	217	241	22	4	14	1	3
H15	128	218	241	1	1			
H16	129	214	239	3		3		
H17	129	215	243	5	5			
H18	129	216	242	3	3			

portion of the total variance attributable to differentiation between taxa was calculated as $1–\Lambda_{TAX}$, the proportion of the total variance attributable to differentiation between populations within taxa as $\Lambda_{TAX}–\Lambda_{POP}$, and the proportion of the total variance attributable to differentiation between individuals within population as Λ_{POP} (Runyeon & Prentice 1997, Rosquist & Prentice 2001). Two plots (leaf and petal shape) based on the first two canonical variates (CV1 and CV2) were produced to illustrate the differentiation between populations and taxa. To tests for any association between taxon and the size of leaf and petal, an ANOVA was performed using CV1 as the dependent variable. Linear regressions was performed to test for any association between altitude and CV1 for both leaf and petal shape.

Genetic analysis

Allele sizes were scored at each chloroplast locus and combined into multilocus haplotypes as chloroplast DNA does not recombine (Singh 2004). Alleles of the three cpDNA microsatellite loci were treated as ordered characters assuming that mutations primarily follow a stepwise mutation model (Ohta & Kimura 1973).

The completeness of haplotype sampling across the range of *H. nummularium* was estimated by Stirling probability distribution and Bayes' theorem (Dixon 2006) assuming even haplotype frequen-

cies and random sampling.

A hierarchical analysis of molecular variance (AMOVA) was performed to describe the partitioning of genetic diversity between taxa, between populations within taxa and within populations. Calculation of F_{ST} was based on haplotype frequency and significance level was tested with 10000 permutations. Twelve populations with mixed subspecies were assigned to the most frequent taxon in the population. Absolute levels of haplotype diversity within each subspecies was calculated as standard diversity based on haplotype frequencies To illustrate relationships between haplotypes, the sum of squared R_{ST} distances between pairs of haplotypes (Slatkin 1995) were used to construct a minimum spanning network (MSN) in Arlequin version 3.1 (Excoffier *et al.* 2005).

To test for the presence of a phylogeographic structure within the species according to Pons & Petit (1996), G_{ST} (based on differences in haplotype frequency between populations) was compared to R_{ST} (the sum of squared differences between haplotypes). Calculations were performed in PERMUT 2 (http://www.pierroton.inra.fr/genetics/labo/software) and significance was estimated from 10000 permutations according to Burban *et al.* (1999).

A Mantel test implemented in PAST version 1.85 (Hammer *et al.* 2001) was performed to test for correlation between genetic and geographic distances. Significance was estimated from 10000 permutations. Twenty-three populations with mixed haplotypes were omitted from the Mantel test.

Results

Two molecular techniques, the PCR-RFLP and cpDNA microsatellites were performed in this study to describe cpDNA variation of *H. nummularium* complex in Europe. However, the PCR-RFLP did not reveal any clear polymorphism within *H. nummularium* complex in Europe, in contrast to cpDNA microsatellites.

Haplotype composition and relationship

The three microsatellite loci yielded a total of 15 size variants (alleles), including 1–6 alleles per locus among the 275 plants. The allelic combinations produced 18 haplotypes (see Table 2). Using Dixon's (2006) method, the probability of haplotype sampling completeness [$P(n = x)$] was 1.0; suggesting that all potential haplotypes were completely sampled in this study. The majority of the populations (76.5%) harboured one haplotype, 22.4% of the populations harboured two haplotypes and only one population harboured three haplotypes (see the Appendix)The MSN (Fig. 2a) summarizes the genetic distances between the 18 cpDNA haplotypes. The frequent haplotypes H1, H2, H3, H4, H5, H6, and the rare ones H7 and H15 are separated from each other by one mutational R_{ST} distance. The rare haplotypes H10, H11, H17 and H18 are separated from each other by 1-2 R_{ST} differences. H12, H13 and H14 are separated from each other by one R_{ST} difference whereas the rare haplotypes H8, H9 and H16 are distantly related (5-6 R_{ST} differences).

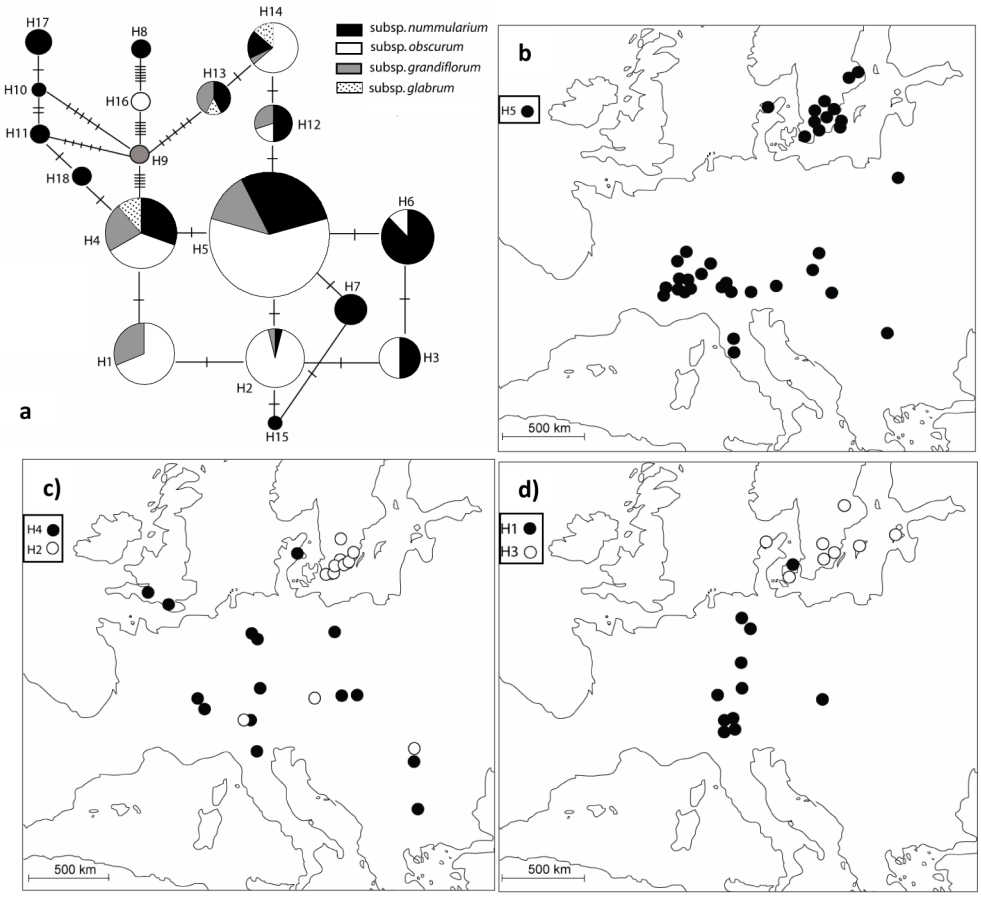

Figure 2. a) Minimum spanning network of the 18 cpDNA haplotypes detected in *H. nummularium*. The size of each pie diagram is proportional to the number of individuals carrying the particular haplotype and the sectors report the haplotype relative frequency in different subspecies. The frequency of hash lines on the connection lengths is proportional to the squared R_{ST} size difference between a haplotype pair. b-d) Distribution of the most common cpDNA haplotypes of *H. nummularium* in Europe (see each panel for details).

The geographic distribution of cpDNA haplotypes

The distribution of cpDNA haplotypes mainly overlapped across the species range in Europe. High haplotype diversity was detected in southern Europe whereas the northern areas displayed less haplotype diversity (see Fig. 2, 3, Table 2). The two most frequent haplotypes were H5 (present in 77 plants), which largely covered the species range in Europe, but was absent from the British Isles and north-western continental Europe (Fig. 2b), and H4 (present in 36 plants), which was found in northern, central and southern Europe, but was absent from Sweden and Estonia (Fig. 2c). The third most common haplotypes were H1, H2, H6,

H14, H3 and H12 that occurred in 29, 26, 24, 22, 16 and 10 plants, respectively. H2 was mainly concentrated to southern Sweden but was also found in three disjunct populations in Austria, Italy and Romania, respectively (Fig. 2c). H1 was found in a narrow zone extending from southern (Italy) into northern Europe (Denmark) and in one disjunct population in Slovakia (Fig. 2d). Both H3 and H6 were confined to Scandinavia and Estonia (Fig. 2d, Fig. 3). Two haplotypes were mainly found in areas surrounding the Alps; H14 which was mostly found in France, but also in Italy and Austria, and H12 which had a continuous distribution in Italy, France and Switzerland but was also found in a disjunct population in Finland. The remaining 11 haplotypes were rare and each occurred in less than eight plants. H8, H9 and H16 occurred in one population each (three plants), in Bulgaria, Austria and Italy, respectively. H7 (6 plants) and H15 (one plant) were restricted to Greece. H13 was found in three populations, in Belgium, Austria and Italy, respectively. Four of the rare haplotypes, H10, H11 and H18, were confined to the Pyrenees in south-western France (see Fig. 3) and H17 was confined to south-eastern France.

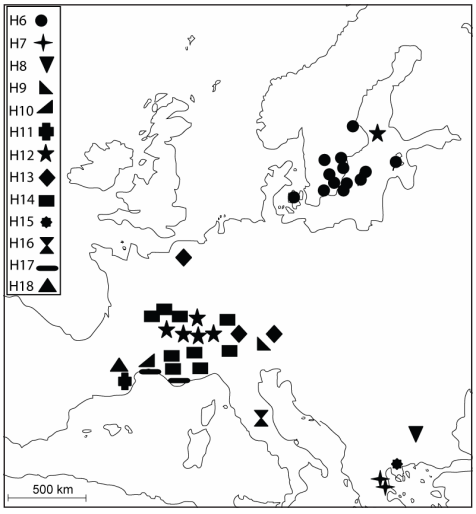

Figure 3. A distributional map of 13 haplotypes, H6-H18 of the *H. nummularium* complex in Europe.

Table 3**.** Analysis of molecular variance (AMOVA)

Source Of variation	d.f.	Sum of squares	Variance Components	Percentage of variation
Between subspecies	3	15.948	0.06157	8.09
Between populations within subspecies	92	161.711	0.59289	77.88
Within populations	172	18.367	0.10678	14.03
Total	267	196.026	0.76124	

F_{ST} *= 0.859, P-value< 0.0001*

Table 4. Calculations of standard diversity indices [number of haplotypes (H), individuals (N)] and estimation of gene diversity (HS) for each subspecies.

Taxon	H	N	HS
nummularium	15	97	0.8761
obscurum	9	132	0.8080
grandiflorum	8	36	0.8175
glabrum	3	10	0.7333

Genetic diversity of H. nummularium

A highly significant level of cpDNA differentiation was detected (F_{ST} = *0.859*, *P<0.0001*) with 77.8% of the total cpDNA variation found between populations within the four subspecies (see Table 3). The subspecies *nummularium* acquired the highest amount of haplotype diversity (*HS = 0.876*) whereas subsp. *glabrum* had the lowest haplotype diversity (*HS = 0.733*). There was a significant phylogeographic structure within the *H. nummularium* complex in Europe, as R_{ST} (0.919) was significantly (*P = <0.05*) higher than G_{ST} (0.782). The Mantel test showed a weak but significant correlation (*r = 0.093, p = 0.013<0.05*) between geographic and genetic distances between the populations.

Relationships between cpDNA haplotypes and taxonomy

The MSN in Fig. 2a showed no correspondence between cpDNA haplotypes and taxonomy. Several divergent haplotypes were shared among the four subspecies. For example, H4, H5 and H14 were shared between four subspecies. H12 was shared between three subspecies; subsp. *nummularium*, subsp. *obscurum* and subsp. *grandiflorum*. H13 was also shared between three subspecies; subsp. *nummularium*, subsp. *grandiflorum* and subsp. *glabrum.* Haplotypes H2, H3 and H6 were found in subsp. *nummularium* and subsp. *obscurum* whereas H1 was found in subsp. *obscurum* and subsp. *grandiflorum*. The remaining haplotypes (H7, H8, H9, H10, H11, H15, H16, H17 and H18) were each confined to a single subspecies.

Morphometric analysis of leaves and petals

The Wilks´ lambda (Λ) values were significant (*p<0.001*) for leaf shape differentiation within taxa (Λ_{TAX} = 0.946) and within populations (Λ_{POP} = 0.219). The Wilks´ Λ values were also significant (*p<0.001*) for petal shape differentiation within taxa (Λ_{TAX} = 0.938) and within populations (Λ_{POP} = 0.169). The hierarchical partitioning of leaf and petal shape differentiation demonstrates that most of the differentiation was due to differentiation between populations within taxa, 72.7% for leaf shape descriptors and 76.9% for petal shape descriptors (Table 5). The within-population portion of differentiation in both leaves and petals was also considerable (21.9% and 16.9%, respectively), while the between-taxa portion of differentiation was relatively small (5.4%

and 6.2%, respectively).

Population centroids with populations specified to subspecies were plotted along the first two canonical variates (CVs) for leaf and petal shape descriptors, respectively (Fig. 4). The first and second CVs accounted for 39.6% and 23.6%, respectively, of the total variance in leaf shape and for 38.7% and 25.8%, respectively, of the total variance in petal shape. None of the analyses showed any differentiation pattern corresponding to taxonomic subdivision of the *H. nummularium* complex. Both analysis produced a rather mosaic pattern where the four subspecies were scattered.

CV1 was significantly correlated with the leaf size, but there was no significant differentiation between taxa in position along CV1 (neither in leaf nor petal data sets, data not shown). However, a significant correlation between altitude and CV1 was observed for the leaf shape data ($r = 0.394$, $p<0.001$), but not for the petal shape data ($r = 0.157$, $p = 0.130$).

Discussion

The absence of any PCR-RFLP polymorphism in *H. nummularium* is probably due to the lower mutation rate of the chloroplast spacer regions on average as compared to the cpDNA microsatellites. The mutation rate for the "polymerase slippage" (e.g. insertion/deletion) of a repeat unit in a microsatellite region is higher than for point mutations or indels causing PCR-RFLP variation (Jarne & Lagoda 1996, Goldstein & Pollock 1997, Provan *et al.* 2001, Palmé & Vendramin 2002). Low or insufficient intraspecific polymorphism obtained by the PCR-RFLP has been found in several plant species, e.g. *Hordeum vulgare* (Provan *et al.* 1999) and *Fraxinus excelsior* (Heuertz *et al.* 2004).

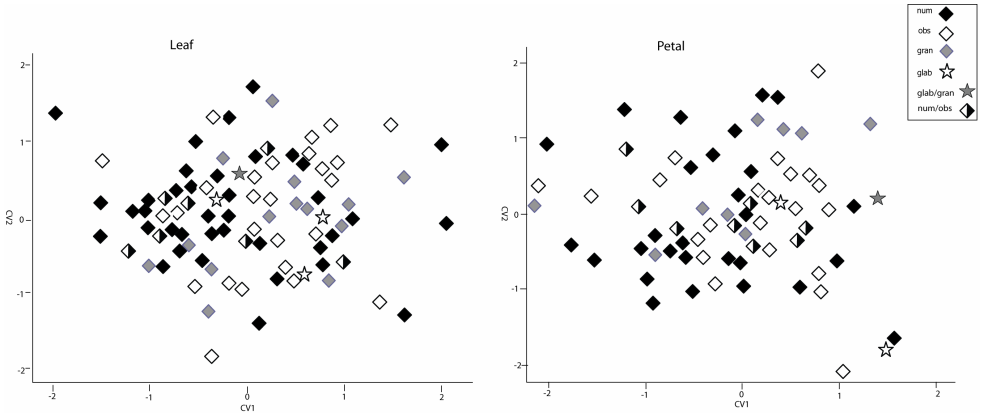

Figure 4. Canonical variates analysis (CVA) of morphometric shape analysis data (moment invariants) in the European *H. nummularium* using population as a grouping variable. Left panel: CVA based on leaf shape descriptors including 96 populations and 586 plants (n = 1172). The first and second CVs accounted for 39.6% and 23.6%, respectively, of the total variance. Right panel: CVA based on petal shape descriptors including 77 populations and 309 plants (n = 927). The first and second CVs accounted for 38.7% and 25.8%, respectively, of the total variance. Each symbol represents a population centroid with taxon specification, num: *nummularium,* obs: *obscurum*, gran: *grandiflorum*, glab: *glabrum*.

Table 5. Hierarchical partitioning of leaf and petal shape variation in *H. nummularium*.

Source of variation	Leaf	Petal
Between subspecies	5.4	6.2
Between populations within subspecies	72.7	76.9
Within populations	21.9	16.9

The phylogeography and migration history of the H. nummularium complex in Europe

In contrast to the PCR-RFLP, the analysis of cpDNA microsatellites detected high haplotype diversity and demonstrated that there is a complex pattern of genetic differentiation of *H. nummularium* in Europe. The Alps and the surrounding lowland areas constituted the centre of diversity with a total of 11 haplotypes. Peripheral areas, on the other hand, (e.g. northern Europe and south-eastern Balkans), displayed less chloroplast haplotype diversity. The presence of high genetic diversity in the Alps can either be explained by primary diversity (e.g. in surrounding refugia), and/or by secondary admixture via dispersal between different genetic lineages of taxa with refugia located elsewhere (Taberlet *et al.* 1998, Petit *et al.* 2003). The diversity pattern in *H. nummularium* indicates a west-Mediterranean origin of the species, a pattern typical for many members of the Cistaceae (Guzmán & Vargas 2009, Widén *et al.* submitted).

Most of the cpDNA haplotypes displayed overlapping distributions in Europe. Still, six of the most frequent haplotypes showed more or less distinct geographical distributions. A total of ten rare haplotypes were found, and seven of these were restricted to the Mediterranean regions. The two closely related haplotypes H3 and H6 have a restricted occurrence and are well established in Scandinavia and Estonia; suggesting the presence of a potential refugium in eastern Europe (e.g. Russia, not covered in this study) in which these haplotypes may have survived (cf. Van Rossum & Prentice 2004). The restricted distribution of certain regional haplotypes in northern Europe has also been detected in Alnus glutinosa (King & Ferris 1998) and Silene dioica (Hathaway et al. 2009). Haplotypes H12 and H14 are closely related and are mainly found in the Alps and surrounding areas. H12 is concentrated mainly in western Alps, whereas H14 has a wider distribution in areas surrounding the Alps. The distribution of H12 and H14 suggests the presence of potential glacial refugia in the Alps and the surrounding area. Several phylogeographic studies have identified similar glacial refugia of plants surrounding the Alps (Tribsch *et al.* 2002, Schönswetter et al. 2005). Surprisingly, H12 was also found in one plant individual on Åland, Finland and this may suggest two scenarios: (i) a long distance dispersal; subspecies obscurum was first recorded in southern Finland in 1951 (out of its natural range in Europe) and persisted to the late 1950's. It probably arrived accidentally to southern Finland during the Second World War (Widén 2010) and/or (ii) perhaps more likely, occurrence of an independent mutation from the widespread haplotype H5.

Haplotypes H5, H4, H2 and H1 were the most frequent haplotypes and widely distributed across the species´ range. They are genetically closely related and

their distributions overlap considerably in southern, central and northern Europe. The sampling design and the screening of cpDNA microsatellites in the present study do not allow precious insights into the exact locations or properties of the putative refugial populations, or the colonization routes of the frequent haplotypes in *H. nummularium* complex. However, we infer that at least three main refugia located in southern Europe (e.g. north and south of the Alps) and south-eastern Europe (e.g. the Balkans) should have contributed to the northward expansion of *H. nummularium* after the last ice age. These refugia must have contained the haplotypes H1, H2, H4 and H5, which subsequently met and admixtured in several contact zones during range expansion into ice-free territories. It is also possible that H1, H2, H4 and H5 may have survived in refugia located in the ice-free tundra areas close to the periglacial zone; south of the main ice sheet. H5 and H2 were absent from western continental Europe and the British Isles, but present in eastern Europe and Scandinavia, which support an eastern immigration lineage to Scandinavia. In contrast, the absence of H1 and H4 from Sweden (even though both are present in Denmark), supports a separate, south-western immigration route into western Scandinavia. It is possible that H1 and H4 represent a more recent immigration wave that arrived to Scandinavia when populations displaying other haplotypes such as H2, H3, H5 and H6 were already well established. Therefore, plants carrying H1 and/or H4 would be unlikely to become dominant and should be difficult to find.

Two closely related haplotypes are restricted to Greece, H7 (two populations) and H15 (one plant). It is thus likely that H15 has arisen from H7 in the same area. H8, H9 and H16 were rare and found in one population each in Bulgaria, Austria and Italy, respectively. They are distantly related in the MSN (separated by at least five R_{ST} steps) and are distantly related to the common H4. These rare haplotypes may represent ancient and relict putative refugia that remained isolated and trapped in the mountainous regions in Bulgaria, Austria and Italy, respectively, and may not have expanded further after the last ice age. H13 has a scattered disjunct distribution in three populations in Belgium, Austria and Italy, respectively. It is genetically closely related to H14 but distantly related to H9. The three haplotypes H10, H11 and H18 are restricted to the Pyrenees and did apparently not contribute to the northward expansion. A similar pattern has previously been observed in *Helianthemum oelandicum* (Widén *et al.* submitted) and *Alnus glutinosa* (King & Ferris 1998). Also, H16 in Italy and H17 in France are rare haplotypes that may represent ancient refugia. It is possible that they were hindered from northward expansion by the Alps and/or trapped by already established populations carrying other haplotypes e.g. H1, H5 and H14.

The AMOVA results show that approximately 78% of the total variation is due to differentiation between populations. The high population differentiation is probably due to long periods of isolation and accumulation of mutations in the putative refugia. The low cpDNA variation within populations indicates restricted gene flow by seeds between populations. The fact that RST is slightly greater than GST and that the highest proportion of the total variation was explained by va-

riation between populations, indicate the presence of a phylogeographic structure in the cpDNA haplotype distributions, such that closely related haplotypes have similar geographic distributions. The Mantel test indicated a significant but weak correlation between geographic and genetic distances. The finding that the six haplotypes present in Scandinavia (H1-H6) are all closely related is a clear example of this structure. Further examples are provided by the Greece haplotypes (H7 and H15) and the Pyrenees ones (H10, H11 and H18).

No correspondence between taxonomy and morphology

The examined four subspecies within the *H. nummularium* complex displayed wide zones of overlap in the CVA plots obtained from leaf and petal shape descriptors (Fig. 4). It can be assumed that these characters are representative of overall differentiation in morphology. Accordingly, the indumentum characters presently emphasized in the subdivision of *H. nummularium* into subspecies are poorly correlated to the overall morphology. For example, the two most common subspecies are subsp. *nummularium* and subsp. *obscurum,* which are distinguished on basis of presence/absence of a dense felt of stellate hairs on the lower side of the leaf, are scattered across the CVA plots and show no tendency of forming any clusters whatsoever. Presence/absence of stellate hairs is also not a discrete character since subsp. *obscurum* often has sparse, large stellate hairs on the lower side of the leaf (Soubani *et al.* in prep.). Mixed populations of subsp. *nummularium* and subsp. *obscurum* are common especially in northern Europe (Azzouzi *et al.* 1997, Soubani *et al.* in prep.). Preliminary results of crossing experiments between these two subspecies suggest that a recessive gene controls the presence of a felt of stellate hairs on the abaxial surface of the leaf (B. Widén unpublished). Progeny of individuals from mixed populations segregates for indumentum when tested in cultivation (B. Widén, personal observation). Obviously, the indumentums and leaf and petal shape variation pattern are more functionally and/or developmentally related than taxonomically (Rieseberg & Soltis 1991). For instance, the significant correlation between altitude and the size of leaf probably indicate selection pressures favouring larger leaves at higher altitudes.

No correspondence between taxonomy and cpDNA haplotypes

The MSN given in Fig. 2a does not show any correlation between taxonomy and cpDNA haplotype distribution or relationships. Accordingly, the subspecies do not represent different genetic lineages. All the four subspecies share several haplotypes with one another. For example, subsp. *obscurum*, the most frequent taxon in our sample (48% of the total), exhibits a substantial amount of haplotype diversity (nine haplotypes). The subspecies *nummularium* (35% of the total sample) displayed the highest haplotype diversity (15 haplotypes). The subspecies *grandiflorum* (13% of the total sample) included eight different cpDNA haplotypes. The subspecies *glabrum* was the least frequent taxa (represents 5% of the total sample) and comprised three cpDNA haplotypes.

The sharing of haplotypes among taxa may be due to homoplasy, introgression or incomplete lineage sorting from a poly-

morphic ancestor (Petit *et al.* 2002, Palmé *et al.* 2004, Heuertz *et al.* 2006). In this study, homoplasy may explain the disjunct distribution of H12 which is found in subsp. *nummularium* in Finland, and in three different subspecies in the Alps. However, homoplasy in *H. nummularium* is unlikely to be the major process shaping the present genetic variation because there is a geographic structure in the distribution of the most frequent cpDNA haplotypes (cf. Vogel *et al.* 2003). Assuming that haplotype divergence preceded "subspeciation", incomplete lineage sorting from a polymorphic ancestor may explain the sharing of cpDNA haplotypes among taxa. Haplotypes H1-H6 and H12-H14 are the most common ones, and probably represent ancient haplotypes that are likely to have been present in the common ancestor of *H. nummularium*. Some of the frequent haplotypes differ in geographic distribution in the different subspecies, which is another indication of incomplete lineage sorting. For example, H13 was found in three disjunct populations of three taxa, subsp. *nummularium* (Belgium), subsp. *grandiflorum* (Italy) and subsp. *glabrum* (Austria) (cf. Palme *et al.* 2004). Hybridization/introgression is considered to be an important evolutionary component in many plant groups, and the sharing of cpDNA haplotypes between species/subspecies is evident in some studies (Rieseberg & Soltis 1991). The tendency for populations of different subspecies to share the same haplotype in a given region may be explained by local introgression. Also, around 23% of the total number of populations were polymorphic for haplotypes, and 12 populations contained a mixture of subspecies (e.g. in Sweden, Slovakia, Germany, Austria, Romania and Italy) which may further indicate past and/or present introgression. For example, in Sweden where four of the common haplotypes (H2, H3, H5 and H6) dominate and overlap in distribution, they are common in both subspecies present, subsp. *nummularium* and subsp. *obscurum*. Accordingly, different subspecies from the same site tend to be more genetically similar to each other than to individuals of the same subspecies from other sites.

Taxonomic implications

The indumentum variation and size of leaf and petal have long been used to circumscribe and delimit taxa of the *H. nummularium* complex (e.g. Janchen 1909, Tutin *et al.* 1968). However, the indumentum variation seems to have a simple genetic background (B. Widén, unpublished) and the two morphometric character sets studied here (leaf and petal shape) do not show any significant divergence between the subspecies in cultivation. Neither the cpDNA haplotypes nor the morphometric analyses support the subdivision of *H. nummularium* into subspecies. Species complexes with intricate phylogenetic relationships (based on cpDNA), evolutionary history and intraspecific variation are common in vascular plants (Mereda *et al.* 2008). We conclude that the poor correspondence between chloroplast haplotype distribution and subspecies circumscription is due to multiple origins of morphologically similar morphs (grouped into taxonomic subspecies) in different parts of the distribution range of the complex. A similar phenomenon has been detected in *H. oelandicum* Widén *et al.* (submitted) that found no correlation between cpDNA haplotypes and traditional subspecies. Instead, parallel

morphs (subspecies) had evolved in different mountain ranges in central and southeastern Europe.

Conclusions

The geographical distribution of some rare cpDNA haplotypes in the Mediterranean area may indicate the existence of Pleistocene refugia with a long history in this region, whereas the distribution of common and closely related haplotypes could be used to infer putative migration routes and periglacial refugia during the last glacial cycles. The complex morphological variation patterns in *H. nummularium* are not correlated with the distribution of haplotypes and do not represent any phylogenetic groups in the species. Instead, the morphological diversity in the species appears to have been shaped by historical processes, e.g. Pleistocene expansion-contraction cycles, contemporary processes, e.g. hybridization and local introgression and adaptation to different habitats

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Appendix. Population code (Pop), country, locality, coordinate (longitude/latitude), taxon [subsp. *nummularium (num)*, subsp. *obscurum (obs),* subsp. *grandiflorum (gran)* and subsp. *glabrum (gla)*], cpDNA haplotype with frequency, total no. of individuals (N) surveyed for cpDNA polymorphism and the corresponded collector for each population: BW: Björn Widén, LE: Lennart Engstrand, RN: Rayna Natcheva, KI: Kerstin Isaksson, TR: Triin Reitalu, MC: Maarten Christenhusz, HC: Honor C. Prentice. Seventeen populations with no haplotypes data have been screened for morphometry but not for cpDNA polymorphism.

Pop	Country	Locality	Latitude/Longitude	Altitude	Taxon	Haplotype	N	Collector
2032	Austria	Steiermark, Lavamünder	46°37´N/14°48´E	500	*obs*	H5	2	Graz BG
2070	Austria	Weissensee, Techendorf	46°43´N/13°17´E	1400	*obs*	H5	3	HBU Universität Salzburg
2096	Austria	Schafberg, Saint Gilgen	46°47´N/13°25´E	1600	*gran*	H9	3	HBU Universität Salzburg
1093	Austria	Bodental, Ferlach	47°00´N/14°30´E	1000	*glab*	H13	3	Bundesgärten Wien, Alpengarten in Belvedere
2049	Austria	Kleinwalsertal	47°08´N/10°10´E	1400	*gran*	2 H5, 1 H14	3	Leisnig BG
2095	Austria	Salzburg, Gaisberg	47°47´N/12°56´E	1300	*num/obs*	2 H1, 1 H4	3	HBU Universität Salzburg
451	Austria	Hainburg an der Donau	48°08´N/16°56´E	100	*obs*	H2	3	BW
450	Austria	Hainberg ad. Donau	48°20´N/16°54´E	400	*num*	-	-	BW
2058	Belgium	Herentals	50°10´N/04°50´E	<50	*num*	H13	3	Bruxelles BG
2087	Bulgaria	Central Rhodope Mts, Assenovgrad	42°00´N/25°00´E	400	*num*	H8	3	RN
3000	Bulgaria	Mons Rhodopes, Prope urbem Assenovgrad	42°03´N/25°05´E	400	*num*	-	-	Bulgarian Academy of Sciences BG
2088	Bulgaria	Stara planina, Buchin Prohod	42°57´N/23°06´E	800	*num*	H4	3	RN
20	Denmark	Køge	55°27´N/12°10´E	<50	*num*	-	-	BW
26	Denmark	Själland, Vesterlynge	55°43´N/11°16´E	<50	*obs*	2 H3, 1 H6	3	BW
17	Denmark	Själland, Skibby	55°44´N/11°57´E	<50	*obs*	H1	3	BW
19	Denmark	Jylland, Glatved Strand	56°18´N/10°49´E	<50	*obs*	H4	3	BW
12	Denmark	Jylland, Sebbersund	56°58´N/09°33´E	<50	*obs*	2 H3, 1 H5	3	BW
3013	England	Salisbury, Swayne's Firs	50°59´N/01°54´W	<50	*num*	H4	5	KI
3012	England	Cissbury Ring	52°36´N/00°16´W	<50	*num*	H4	1	KI
2094	Estonia	Ösel, Varpe	58°10´N/22°10´E	<50	*num*	H6	3	TR
2002	Estonia	Raplamaa, Kohila	59°09´N/24°45´E	<50	*num*	H3	2	TR
3014	Finland	Åland, Seglinge	60°12´N/20°41´E	<50	*num*	H12	1	MC
2082	France	Hautes-Pyrénées	42°03´N/00°09´W	900	*num*	H11	3	Herbario Musei Parisiensis

503	France	Pyrénées, Gedre	42°48´N/00°05´E	1200	*num*	H18	3	BW
501	France	Massif de la Sainte Baume, Gemenos	43°25´N/05°60´E	750	*num*	-	-	BW
2084	France	Hérault, Murles	43°41´N/03°44´E	200	*num*	1 H10, 2 H17	3	Montpellier BG
2064	France	Castellars	43°48´N/07°30´E	400	*num*	-	-	Museum d'Histoire Naturelle, Paris
500	France	Alpes - Maritime, Col de Brouis	43°54´N/07°30´E	850	*num*	H17	3	BW
1091	France	Hautes-Alpes, Col du Lautaret	44°05´N/06°16´E	2000	*num*	H14	1	Station Alpine du Lautaret, Grenoble
2038	France	Hautes-Alpes, Lautaret	44°16´N/06°16´E	2100	*num*	H14	3	Grenoble BG
2080	France	Haute-Savoie Taninges,col de Ramaz	46°09´N/06°24´E	1600	*gran*	H12	2	Herbario Musei Parisiensis
2014	France	Haute-Savoie Taninges,col de Ramaz	46°10´N/06°34´E	1600	*obs*	H14	3	Museum d'Histoire Naturelle, Paris
2029	France	Mouthier Haute, Pierre	47°03´N/06°12´E	1000	*obs*	1 H4, 2 H5	3	Dijon-Mairie BG
1106	France	Gevrey, Chambertin	47°15´N/04°30´E	400	*num*	H12	3	Dijon-Mairie BG
2013	France	Yonne, Sormery	48°06´N/03°46´E	<50	*obs*	H14	2	Museum d'Histoire Naturelle, Paris
2079	France	Yonne, Sormery	48°13´N/03°42´E	300	*obs*	H14	4	Herbario Musei Parisiensis
2069	Germany	Trauchgau-Halblech	47°38´N/10°47´E	1000	*num/obs*	H1	3	Krefeld BG
2052	Germany	Breisgau, Hochschwarzwald	47°54´N/07°56´E	700	*obs*	H5	3	-
3011	Germany	Kallmünz	49°09´N/ 11°57´E	400	*obs*	H1	3	HC
2025	Germany	Harras, Eisfeld	50°25´N/10°51´E	1300	*obs*	H4	2	Marburg BG
2024	Germany	Schmalkalden, Meiningen	50°33´N/10°24´E	300	*obs*	H4	3	Marburg BG
2062	Germany	Elspe, Hochsauerland	51°17´N/18°23´E	500	*obs*	H4	3	Ruhr- Bochum University
2075	Germany	Sachsen-Anhalt, Welfesholz	51°38´N/11°33´E	200	*obs*	H1	4	Halle BG
1103	Germany	Sachsen-Anhalt, Halberstadt	51°53´N/11°02´E	100	*obs*	H1	2	Der Martin-Luther-University, BG
3008	Greece	Ossa, Lakeria	39°48´N/22°36´E	1700	*num*	H7	3	LE
3009	Greece	Asenovgrad, Dobrostan	41°54´ N/24°54´ E	1300	*num*	H15	1	LE
3006	Greece	Leptokarya-Anatolikos Olympus	39°55´N/22°32´E	700	*num*	-	-	LE
3005	Greece	Leptokarya-Anatolikos Olympus	40°02´N/22°30´E	700	*num*	-	-	LE
3004	Greece	Leptokarya-Anatolikos Olympus	40°03´N/22°30´E	700	*num*	H7	3	LE
2031	Hungary	Magyarország, Kiskunsági	46°53´N/19°18´E	100	*num*	H5	3	Vácrátót BG
2037	Italy	Pressidel Santaurio, Dinnammare, Messina	38°11´N/15°33´E	400	*num*	-	-	Catania BG

2048	Italy	Monte Aquila	42°25´N/13°34´E	1700	*obs*	H16	3	L'Aquila BG
1096	Italy	Grosseto	42°45´N/11°09´E	900	*obs*	H5	1	Alpino Rezia BG
1101	Italy	Casciano, Murlo	43°09´N/11°15´E	400	*obs*	H5	2	Di Siena University BG
480	Italy	Alpe, San Pellegrino	44°07´N/10°25´E	1100	*obs*	-	-	BW
1107	Italy	Benevello	44°30´N/08°04´E	2000	*obs*	H14	3	Alpino Paradisia BG
2011	Italy	Trentino, Vallaccia	45°19´N/11°34´E	2000	*gran*	H4	3	Alpino Rezia BG
2020	Italy	Salirod, Saint-Vincent Aosta	45°42´N/07°38´E	1000	*num*	H5	2	Alpino BG
2056	Italy	Chatillon Aosta	45°45´N/07°37´E	1100	*num*	H5	1	Alpino Paradisia BG
1108	Italy	Salirod, Saint-Vincent Aosta	45°45´N/07°40´E	1100	*num/obs*	H5	2	Alpino Paradisia BG
1109	Italy	Salirod, Saint-Vincent Aosta	45°45´N/07°40´E	1100	*num*	H5	3	Alpino Paradisia BG
2081	Italy	Val Ferret, Courmamayeur Aosta	45°48´N/07°03´E	1800	*obs*	H5	3	Herbario Musei Parisiensis
2016	Italy	Val Ferret, Courmamayeur Aosta	45°50´N/07°01´E	1800	*obs*	H14	1	Museum d'Histoire Naturelle-Cultures
2004	Italy	Santa Caterina, Valfurva	46°24´N/10°26´E	1800	*gran*	H5	3	Alpino Rezia BG
1097	Italy	Via Forni, Valfurva	46°24´N/10°31´E	2200	*gran*	H1	3	Alpino Rezia BG
2008	Italy	Via Forni, Valfurva	46°24´N/10°31´E	2200	*gran*	H1	3	Alpino Rezia BG
2085	Italy	Valdidentro, cancano	46°27´N/10°16´E	1800	*gran*	2 H1, 1 H13	3	Alpino BG
2007	Italy	Oga,Valdisotta	46°27´N/10°20´E	1500	*gran*	1 H2, 2 H5	3	Alpino Rezia BG
2006	Italy	Valdidentro, Valfurva	46°27´N/10°24´E	1500	*glab*	H14	3	Alpino Rezia BG
2086	Italy	Bormio	46°28´N/10°22´E	1200	*obs*	-	-	Alpino Rezia BG
1098	Italy	Passo di Foscagno, Livigno	46°29´N/10°12´E	2200	*obs*	H1	3	Alpino Rezia BG
2005	Italy	Valdidentro, cancano	46°29´N/10°17´E	1800	*gran*	H5	3	Alpino Rezia BG
2010	Italy	Livigno	46°32´N/10°08´E	2000	*gran*	H4	3	Alpino Rezia BG
2067	Poland	Suwatki, Wigry	54°05´N/23°04´E	200	*obs*	H5	2	-
2035	Romania	Strada Portile de Fier	45°46´N/23°33´E	200	*num*	H5	3	Al. Borza-Cluj BG
2034	Romania	Alba	46°09´N/23°33´E	1000	*num*	H4	2	Al. Borza-Cluj BG
2036	Romania	Posaga	46°27´N/23°22´E	700	*num/obs*	H2	3	Al. Borza-Cluj BG
2072	Slovakia	Dulovce-Kamenica	47°51´N/18°14´E	700	*obs*	H5	3	Bratislava BG
2076	Slovakia	Male Karpaty: Jelenia hora	48°25´N/17°15´E	500	*gran*	-	-	Bratislava BG

2071	Slovakia	Jelenie	48°25´N/19°52´E	300	*gran/glab*	1 H1, 2 H4	3	Bratislava BG
2051	Slovakia	Modranska	48°40´N/19°40´E	1000	*obs*	H5	3	Bratislava BG
2045	Slovakia	Slovensky Raj	48°48´N/20°12´E	1400	*glab*	H4	3	Halle BG
112	Sweden	Blekinge, Åkeholm	56°17´N/14°44´E	100	*obs*	H2	3	BW
132	Sweden	Blekinge, Eringsboda	56°26´N/15°22´E	100	*obs*	2 H3, 1 H6	3	BW
113	Sweden	Blekinge, Vilshult	56°21´N/14°22´E	100	*obs*	H5	3	BW
1087	Sweden	Gothem, Ekstakusten	57°09´N/18°00´E	<50	*num*	H6	3	BW
2078	Sweden	Gothem, Ekstakusten	57°09´N/18°00´E	<50	*num*	1 H3, 2 H6	3	BW
118	Sweden	Öland, Albrunna	56°19´N/16°24´E	<50	*num*	1 H5, 2 H6	3	BW
117	Sweden	Öland, Resmo	56°32´N/16°26´E	<50	*num*	2 H5, 1 H6	3	BW
6	Sweden	Östergötaland, Tryserum	58°08´N/16°37´E	<50	*num*	H6	3	BW
106	Sweden	Skåne, Åby	56°55´N/14°01´E	150	*num/obs*	1 H2, 2 H5	3	BW
105	Sweden	Skåne, Benestad	55°31´N/13°54´E	<50	*num/obs*	H2	3	BW
109	Sweden	Skåne, Genarp	55°35´N/13°24´E	500	*num/obs*	-	-	BW
111	Sweden	Skåne, Grödby	56°05´N/14°31´E	<50	*num/obs*	H2	4	BW
110	Sweden	Skåne, Lämmeström	55°29´N/13°23´E	<50	*obs*	1 H2, 2 H5	3	BW
128	Sweden	Skåne, Lyngsjö	55°55´N/14°04´E	<50	*num/obs*	H2	3	BW
3	Sweden	Småland, Älghult	57°00´N/15°34´E	200	*num/obs*	2 H5, 1 H6	3	BW
10	Sweden	Småland, Boanäs	58°41´N/13°16´E	200	*num/obs*	H2	3	BW
4	Sweden	Småland, Fagerhult	56°54´N/13°27´E	200	*num*	2 H5, 1 H6	3	BW
11	Sweden	Småland, Hågeryd	56°57´N/14°42´E	200	*obs*	H5	3	BW
8	Sweden	Småland, Klavreström	57°09´N/15°08´E	100	*num/obs*	H3	3	BW
116	Sweden	Småland, Kulla	56°42´N/15°48´E	100	*num*	1 H2, 1 H3, 1 H5	3	BW
24	Sweden	Småland, Marbäck I	60°05´N/17°10´E	<50	*num*	H6	3	BW
122	Sweden	Småland, Södra Vi	57°44´N/15°48´E	100	*num*	-	-	BW
129	Sweden	Småland, Spångenäs	57°35´N/16°05´E	100	*num*	-	-	BW
16	Sweden	Småland, Västerik	57°45´N/16°40´E	<50	*num*	H6	2	BW
108	Sweden	Småland, Vena	58°27´N/16°10´E	<50	*num*	-	-	BW

203	Sweden	Uppland, Halvbygda	60°07´N/17°57´E	<50	*num*	-	-	BW
206	Sweden	Uppland, Husby	59°24´N/17°11´E	<50	*num*	3 H3, 1 H5	4	BW
205	Sweden	Uppland, Knivstad	59°43´N/17°48´E	<50	*obs*	-	-	BW
202	Sweden	Uppland, Svinnö	60°08´N/18°29´E	<50	*num*	2 H5, 1 H6	3	BW
2065	Switzerland	Valais, rivedroite du Rhône	46°02´N/07°07´E	2000	*obs*	H5	3	Geneve BG
2022	Switzerland	Valais, rivedroite du Rhône	46°04´N/07°05´E	2000	*obs*	3 H5, 2 H12	5	De Lausanne BG
1031	Switzerland	Berner Oberland, Schyninge Platte	46°41´N/07°50´E	2000	*gran*	1 H4, 1 H12	2	Hortus BG
2068	Switzerland	Sagogn	46°47´N/09°12´E	1200	*num*	1 H5, 2 H12	3	Zürich BG

Postglacial history of the rock rose *Helianthemum nummularium* in Scandinavia: combined chloroplast DNA haplotype and indumentum suggests two postglacial immigration lineages

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Keywords: Helianthemum, microsatellites, Scandinavia, chloroplast DNA, indumemtum, polymorphism, morphometry, Pleistocene.

Abstract

Helianthemum nummularium has two subspecies in southern Scandinavia that differ in presence/absence of a dense felt of stellate hairs on the abaxial surface of the leaf. The subspecies *obscurum* (without a dense felt) occurs in southwest whereas subsp. *nummularium* (with a dense felt of stellate hairs) occurs in southeast Scandinavia. The distributions of the two subspecies overlap in a wide hybrid zone, where natural populations consist of a mixture of the two subspecies in various proportions. There is no significant difference in the leaf shape between the two subspecies although considerable differentiation in indumentum is observed across the hybrid zone. Twenty-seven populations displayed six chloroplast DNA haplotypes in Scandinavia. The geographic distribution of four of these haplotypes supports an eastern postglacial immigration lineage whereas the distribution of two others haplotypes supports a south-western postglacial immigration lineage.

Introduction

Scandinavia was covered with a thick ice sheet during the last (Weichselian) glaciation (Björck 1995). A woodless tundra vegetation with typical arctic climate dominated areas south of the Scandinavian ice shield. As the main ice sheet retreated from Scandinavia, different organisms recolonized the region from different directions and source areas when conditions became favourable (Hewitt 1999). A common consequence of repeated expansion and contraction of species ranges induced by the Pleistocene glaciations is the formation of contact or hybrid zones. When populations of a species representing different refugial areas, meet in the deglaciated areas, secondary contacts are established between the migrant populations. The hybrid zones are characterized by high genetic diversity with clinal transition between different morphs and genetic lineages (Endler 1977, Hewitt 2000, Hewitt 2001).

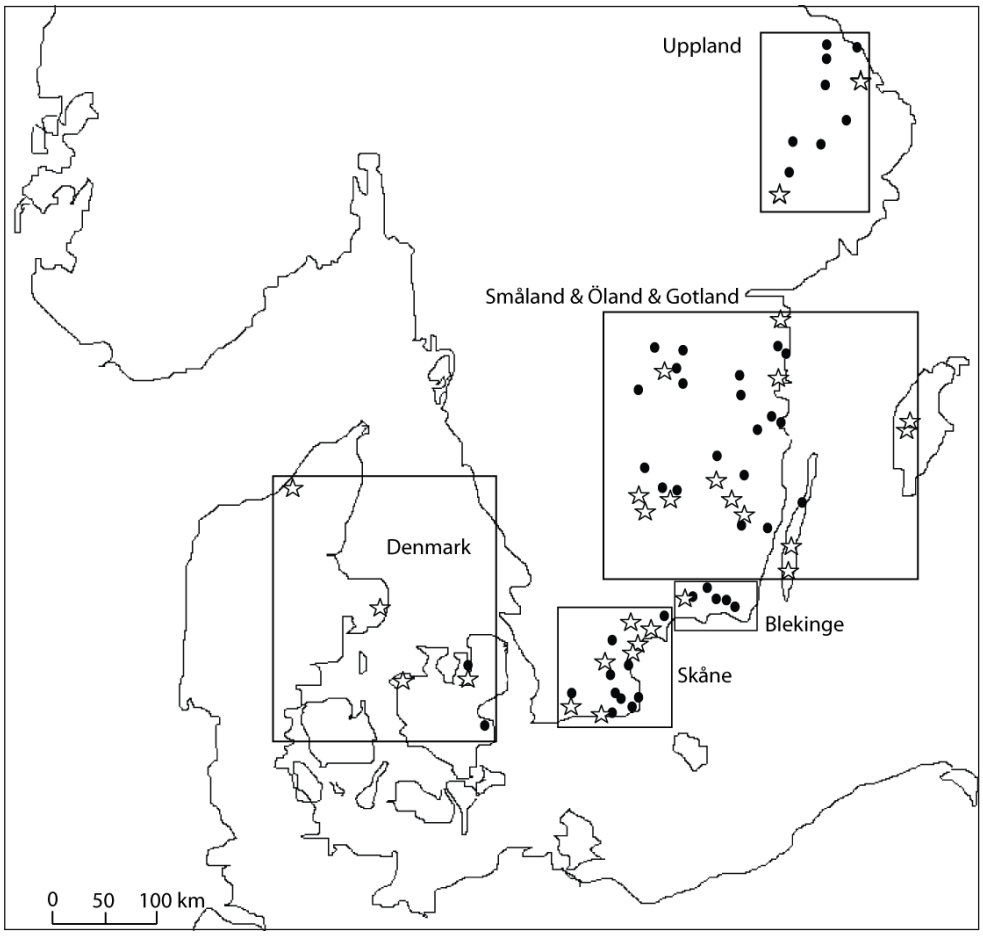

Figure 1. The distribution of 72 sampled populations of *H. nummularium* from Denmark and southern Sweden. Samples with star symbols were used for cpDNA microsatellite analysis.

Patterns and levels of genetic diversity in plants, allows for detection and estimation of past and present evolutionary processes shaping the present genetic structure. Historical events (i.e. the Pleistocene), the species ecological requirements (i.e. selection pressure) and life history traits are responsible for shaping the genetic variation in species. Also, interactions between different evolutionary processes such as genetic drift and gene flow account for the present genetic structure of different species. For example, gene flow, which occurs through seed and pollen dispersal, will increase the genetic variation within the exchanging populations and homogenize variation between populations, in contrast to genetic drift that usually causes genetic impoverishment within small population and differentiation between populations (Slatkin 1987, Hamrick & Godt 1990).

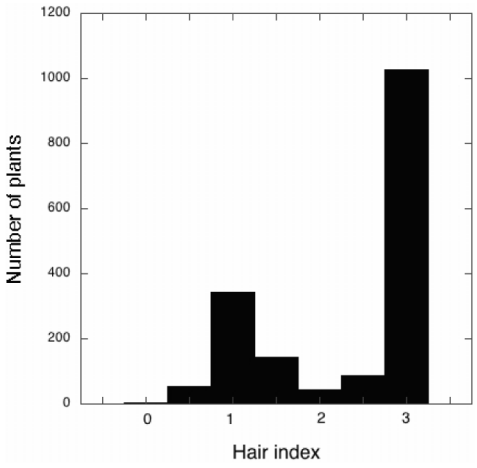

Figure 2. The distribution of plants (n = 1714) of *H. nummularium* based on hairiness in five regions in Scandinavia. Plants with hair index = 0 have with no stellate hairs on the abaxial surface of the leaf and plants with hair index = 3 have a dense cover of stellate hairs on the abaxial surface of the leaf (see text for further details).

Traditionally, taxonomy has been based on morphological characters supported by ecological factors of the species. Gottlieb (1984) pointed out that simple morphological difference such as presence/absence of a character (i.e. indumentum) in plants may have a simple genetic basis (one or few genetic factors). He also claimed that the genetic basis of morphological differentiation between species is similar to that of variation within species and cited numerous examples. Accordingly, the evolution of morphological characters may be associated with relatively simple changes in genes, e.g. genes coding for trichome production (i.e. Kärkkäinen & Ågren 2002, Kivimäki *et al.* 2007). On the other hand, many QTL studies show that most complex phenotypic traits are polygenic, but that the number and effect of genes differ across traits and species (Kalisz & Kramer 2008). For example, leaf shape variation pattern in *Antirrhinum majus*, has shown to involve at least 15 QTLs with small to moderate effect (Langlade *et al.* 2005).

Applications of automated image acquisition and statistical methods to analyse leaf shape variation have shown to be efficient in identification of plants (Hearn 2009) and also in detection of subtle differences between groups of plants (White *et al.* 1988, Weight *et al.* 2008). For example, Olsson *et al.* (2000) detected subtle leaf shape variation patterns separating closely related dogroses (Rosa sect. Caninae). Lönn and Prentice (1995) could separate between regional populations of *Hippocrepis ernerus* (Leguminosae) based on leaf shape variation pattern.

Chloroplast DNA (cpDNA) is maternally inherited in most flowering plants. It is transmitted only through seeds (seed-specific marker) with restricted gene flow and therefore shows higher potential for population differentiation and hence geographic structuring than nuclear genes that are transmitted also through pollen (Cavers 2003). Given these characteristics, cpDNA has been widely employed for detection of genetic differentiation and illustration of evolutionary processes and postglacial histories of many plant species (reviewed in Soltis *et al.* 1992). Microsatellites, or simple sequence repeats (SSRs), are hypervariable loci found in the nuclear and organelle DNA. In the chloroplast genome, microsatellites often consist of mononucleotide repeats, generally <15 base pairs in length. They are considered neutral markers and have been extensively applied in a broad range of studies (Provan *et al.* 2001). However, there are limitations to the application of cpDNA microsatellites. High genetic polymorphism and ho-

moplasy (identical by state and not by descent) may produce a complicated picture of the overall genetic variation, e.g. unresolved relationships between haplotypes. Consequently, limited inference about the species` postglacial history will be deduced (Estoup *et al.* 1995, Jarne & Lagoda 1996, Provan *et al.* 2001).

Helianthemum nummularium is an evergreen, dwarf shrub that belongs to Cistaceae. The species is out-breeding, insect pollinated and produces racemes of yellow flowers in early summer. It favours dry, sunny sites, open grasslands and meadows. It is decreasing in Scandinavia due to the change of land use, especially the cessation of grazing by cattle. The species has a wide distribution in Europe but disjunct populations can be found in Turkey, the Caucasus and northern Iran (Widén 2010). The morphological variation in *H. nummularium* is complicated and therefore the species is treated as a species-complex subdivided into eight subspecies in Flora Europaea (Tutin *et al.* 1968). In this paper we investigate the two subspecies found in Scandinavia, *H. nummularium* subsp. *nummularium* and *H. nummularium* subsp. *obscurum.* The subspecies *obscurum* has a south-western distribution (Denmark, the Swedish provinces: Skåne, western Blekinge and central Småland), while subsp. *nummularium* has a south-eastern distribution in Scandinavia and is distributed as far north as the Swedish province of Uppland and southern Finland. Both subspecies often occur in mixed populations in a wide zone in southern Sweden (Edqvist & Karlsson 2007, Widén 2010).

This study is a part of a wide-scale phylogeographic survey of the *H. nummularium* complex in Europe (Soubani *et al.* in prep.), and we ask if postglacial immigration from different directions can explain the present geographical distribution of the two subspecies of *H. nummularium* in Scandinavia. The objectives of this paper were (1) to examine the utility of cpSSR markers for assessing genetic variability within the species-complex in Scandinavia, (2) to investigate the geographic distribution of cpDNA haplotypes of *H. nummularium* in Scandinavia, and (3) to study possible correlations between cpDNA polymorphism and two morphological characters – one with a supposedly simple genetic background (indumentum) and one with an apparently polygenic background (leaf shape) – across the hybrid zone between the two subspecies in Scandinavia.

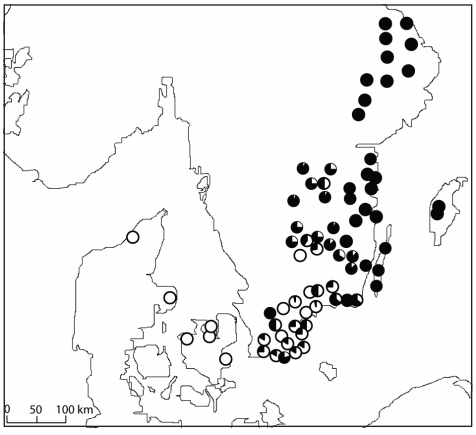

Figure 3. The pie diagrams describe the frequency of the subsp. *nummularium* and subsp. *obscurum* in the sampled populations. The white sectors refer to subsp. *obscurum* and the black sectors to subsp. *nummularium*.

Materials and Methods

Plant material

The material was collected from the provinces of Jylland and Själland in Denmark, and Skåne, Blekinge, Småland, Öland, Gotland, Östergötland and Uppland in Sweden by the third author. Based on the data obtained from recent floristic surveys (Fröberg 2006, Edqvist & Karlsson 2007, Tyler *et al.* 2007), a representative collection of 72 sites with subsp. *nummularium* and/or subsp. *obscurum* was visited. A random sample of individuals was collected from the entire area covered by *H. nummularium* at each site. The maximum sample size at each site was set to 40 individuals and the distance between samples was 1 to 10 meters (depending on population size), giving rise to sample sizes ranging from 5 to 39 (see Table 1). Each sample consisted of two vegetative shoots and seeds (if present). One shoot was dried and pressed for subsequent scoring of indumentum and from the other shoot; two undamaged opposite leaves (the largest leaf pair) were prepared for shape analysis. A collection of seeds covering the geographic range of the species in southern Scandinavia were sown in separate pots in the experimental garden at Lund University (Fig. 1). A total of 82 plants representing 27 populations (average n = 3 individuals/population) were surveyed for cpDNA polymorphism.

Morphometry: hair index and leaf shape

The diagnostic character distinguishing subsp. *nummularium* from subsp. *obscurum* is the presence of a dense cover of stellate hairs on the lower side of the leaf. Since there is a considerable variation in indumentum (Widén 2010), we scored and categorized the variation pattern based on the density of stellate hairs on the lower side of the leaf (well-developed young leaves from one shoot, the fourth or fifth leaf pair from the shoot-tip) accordingly, 0: no stellate hairs, 1: stellate hairs mainly on the mid-rib, 2: cover of large, sparse stellate hairs on the leaf surface (the leaf surface is green to the naked eye) and 3: a dense grey cover of stellate hairs all over the leaf surface. Individual samples can fall between these four categories and are thus given any of these scores 0, 0.5, 1, 1.5, 2, 2.5 or 3. We assigned plants with a hair index of 0–2 to subsp. *obscurum* and plants with hair index of 2.5–3 to subsp. *nummularium*. To test for significant differences in hairiness (hair indices) between regions, a Kruskal-Wallis test was performed in SPSS 15.0. Also, a linear regression test in SPSS 15.0 was performed between latitude and mean indumentum.

Table 1. Population code (pop. code), locality, country, coordinates (Swedish grid (SG) x, y), taxon specification (num: subsp. *nummularium*, obs: subsp. *obscurum*), cpDNA haplotype and number of individuals (N) per population used for morphometric analysis.

Pop. code	Locality	Country	SG-x	SG-y	Taxon	Haplotype	N
19	Jylland, Glatved Strand	Denmark	62,5	11,88	*obs*	H4	30
12	Jylland, Sebbersund	Denmark	63	11,08	*obs*	2 H3, 1 H5	6
20	Själland, Køge	Denmark	61,55	12,68	*obs*		17
17	Själland, Skibby	Denmark	61,8	12,6	*obs*	H1	35
18	Själland, Skuldelev	Denmark	61,85	12,6	*obs*		14
26	Själland, Vesterlynge	Denmark	61,8	12,16	*obs*	2 H3, 1 H6	31
105	Skåne, Benestad	Sweden	61,56	13,79	*num/obs*	H2	30
125	Skåne, Brantevik	Sweden	61,56	14,08	*num/obs*		14
107	Skåne, Everöd	Sweden	61,9	13,99	*num/obs*		37
109	Skåne, Genarp	Sweden	61,62	13,46	*num/obs*		12
102	Skåne, Gislöv	Sweden	61,52	14,03	*num/obs*		22
111	Skåne, Grödby	Sweden	62,18	14,19	*num/obs*	H2	39
104	Skåne, Kverrestad	Sweden	61,56	13,86	*num/obs*		35
128	Skåne, Lyngsjö	Sweden	62,03	13,91	*num/obs*	H2	36
110	Skåne, Lämmeström	Sweden	61,54	13,49	*num/obs*	1 H2, 2 H5	19
114	Skåne, Oderberga	Sweden	62,23	13,98	*obs*		22
127	Skåne, Onslunda	Sweden	61,66	13,89	*num/obs*		13
126	Skåne, Snoggedal	Sweden	61,64	13,92	*obs*		6
103	Skåne, Tosteberga	Sweden	62,1	14,16	*num/obs*		33
106	Skåne, Åby	Sweden	62,14	14,18	*num/obs*	1 H2, 2 H5	16
134	Blekinge, Askaremåla	Sweden	62,44	14,46	*obs*		7
132	Blekinge, Eringsboda	Sweden	62,56	14,72	*obs*	2 H3, 1 H6	9
121	Blekinge, Flyerud	Sweden	62,43	15,04	*num*		26
101	Blekinge, Flymen	Sweden	62,46	14,96	*num/obs*		25
133	Blekinge, Makrilla	Sweden	62,57	14,68	*num/obs*		8
120	Blekinge, Rödeby	Sweden	62,47	14,9	*num/obs*		30
113	Blekinge, Vilshult	Sweden	62,46	14,17	*num/obs*	H5	30
131	Blekinge, Åbyholm	Sweden	62,57	14,87	*num/obs*		6
112	Blekinge, Åkeholm	Sweden	62,4	14,34	*num/obs*	H2	31

118	Öland, Albunna	Sweden	62,44	15,39	*num*	1 H5, 2 H6	31
211	Öland, Djupvik	Sweden	63,2	15,6	*num*		0
117	Öland, Resmo	Sweden	62,67	15,43	*num*	2 H5, 1 H6	30
1087	Gothem, Ekstakusten	Sweden	63,48	16,55	*num*	H6	0
2078	Gothem, Ekstakusten	Sweden	63,48	16,55	*num*	1 H3, 2 H6	0
119	Småland, Adelöv	Sweden	64,32	14,32	*num/obs*		32
10	Småland, Boanäs	Sweden	63,34	14,29	*num/obs*	H2	34
9	Småland, Braås	Sweden	63,27	14,55	*num/obs*		32
23	Småland, Bredestad	Sweden	64,1	14,47	*num/obs*		30
4	Småland, Fagerhult	Sweden	63,34	14,87	*num*	2 H5, 1 H6	24
25	Småland, Forserum	Sweden	63,98	14,22	*num/obs*		30
11	Småland, Hågeryd	Sweden	63,14	14,32	*obs*	H5	5
2	Småland, Högsby	Sweden	63,4	15,12	*num*		27
8	Småland, Klavreström	Sweden	63,33	14,58	*num/obs*	H3	30
116	Småland, Kulla	Sweden	63	15,03	*num/obs*	1 H2, 1H3, 1 H5	31
115	Småland, Köpstaden	Sweden	62,97	15,03	*num/obs*		30
14	Småland, Loftahammar	Sweden	64,26	15,5	*num*		12
123	Småland, Mada	Sweden	64,29	14,52	*num/obs*		31
24	Småland, Marbäck I	Sweden	64,18	14,45	*num/obs*	H6	30
22	Småland, Marbäck II	Sweden	64,16	14,43	*num/obs*		30
1	Småland, Nottebäck	Sweden	63,29	14,64	*num/obs*		19
13	Småland, Nässja	Sweden	63,49	14,37	*num/obs*		12
21	Småland, Smedby	Sweden	62,82	15,23	*num*		26
129	Småland, Spångenäs	Sweden	63,85	15,16	*num*		32
122	Småland, Södra Vi	Sweden	64,1	15	*num*		31
108	Småland, Vena	Sweden	63,78	15,07	*num*		31
124	Småland, Vimmerby	Sweden	64	15	*num*		22
7	Småland, Virserum	Sweden	63,57	14,87	*num/obs*		27
130	Småland, Vånghult	Sweden	63,82	15,23	*num*		30
16	Småland, Västerik	Sweden	64,08	15,45	*num*	H6	30
15	Småland, Västra Ed	Sweden	64,3	15,44	*num*		30
3	Småland, Älghult	Sweden	63,17	14,97	*num/obs*	2 H5, 1 H6	30
204	Uppland, Berkinge	Sweden	66,95	16,22	*num*		5

207	Uppland, Enhälja	Sweden	66,09	15,81	*num*		17
203	Uppland, Halvbygda	Sweden	66,72	16,17	*num*		25
206	Uppland, Husby	Sweden	66,05	15,76	*num*	3 H3, 1 H5	17
205	Uppland, Knivsta	Sweden	66,26	16,1	*num*		25
209	Uppland, Knutby	Sweden	66,54	16,3	*num*		26
210	Uppland, Långalma	Sweden	66,95	16,43	*num*		25
202	Uppland, Svinnö	Sweden	66,77	16,43	*num*	2 H5, 1 H6	25
208	Uppland, Säva	Sweden	66,28	15,88	*num*		25
201	Uppland, Vigelsbo äng	Sweden	66,88	16,2	*num*		30
6	Östergötaland, Tryserum	Sweden	64,48	15,47	*num*	H6	24

Two opposite leaves from a shoot were rotated to make the apices pointing at the same direction and every leaf was horizontally directed after removal of the petioles. The leaves were fixed to sellotape (with the adaxial surface facing down), pressed and dried for subsequent image analysis. The outline of each leaf was digitized with a video camera connected to a computer via an analogue-to-digital converter. The shape of the leaf was described by moment invariants–seven parameters that describe the distribution of x and y coordinates of image points along the outline (Dudani *et al.* 1977, Rohlf & Archie 1984, White *et al.* 1988). The data obtained in the moment invariants analysis was subjected to canonical variates analyses (CVA, Dunn & Everitt 1982), where the variation in leaf shape was partitioned into different hierarchical components. By using the procedure CANDISC in SAS, we obtained estimates of Wilks' lambda (Λ), which quantify the within-group variation. Three different CVA analyses using the opposite leaves as replicate were carried out to give lambda-values for within region (Λ_{REG}), within population (Λ_{POP}) and within individual (Λ_{IND}). The proportion of diversity between regions was calculated as $1–\Lambda_{REG}$, the proportion of diversity between populations within regions as $\Lambda_{REG}–\Lambda_{POP}$, and between individuals within populations as $\Lambda_{POP}–\Lambda_{IND}$ (Runyeon & Prentice 1997, Rosquist & Prentice 2001). The pattern of variation in the leaf shape was displayed by plotting the population centroids on the first two canonical variates (CV1 and CV2) and also by plotting the population centroids on the first canonical variate (CV1) and latitude. To seek for relationships between leaf shape variation pattern and latitude, two linear regression tests (SPSS 15.0) were done between (i) latitude and CV1 across the overlapping distributions of the two subspecies and (ii) latitude and the CV1 including all populations.

DNA extraction and cpDNA microsatellite

Total genomic DNA was extracted from fresh, dried or frozen leaves by the CTAB method (Doyle & Doyle 1990) with adjustments according to Lodhi *et al.* (1994). Three noncoding cpDNA regions, (i) the

Table 2. List of the cpDNA microsatellite primers used in the study.

Primer pair	Primer sequence (5'-3')	TA (°C)	Type	Fragment length (bp)
mtrnSf-trnGr	F: CCATTTCGAAAATTGGAGAGA R: CGCATTAACAATACGAAACTATAGA	57	polyT	128/129
mtrnL2-trnF	F: AAT GGGCATCGGAATACCA R: CATTTTACGAGA GGGCTTGG	57	polyT	216/217
mtrnL5-trnL3	F: AAT GGGATTGAATGGCTTTG R: TCCTGGAGTGAAAGGGTTGA	57	polyA	241/242

trnL–trnF intergenic spacer, (ii) *trnL* intron (*trnL5-trnL3*) (Taberlet *et al.* 1991) and (iii) *trnS–trnG* intergenic spacer (Hamilton 1999) were selected for sequencing and later surveyed for polymorphic microsatellite loci. Three polymorphic mononucleotide (A/T) loci were detected and subsequently amplified by a set of three *Helianthemum* specific primer pairs designed in the Primer 3 program (Rozen 2000) (see Table 1). The PCR reactions were carried out in a total volume of 10 µl containing 6.8 µl distilled water, 1 µl (10x PCR) buffer, 0.8 µl (25 mM) $MgCl_2$, 0.2 µl (10mM of each) dNTPs, 0.08 µl (25 pmol/µl) each of the forward and reverse primers, 0.24 µl (1 unit/µl) *Taq* polymerase and 0.8 µl (14 ng/µl) DNA template. The PCR amplification was performed in a PTC-100 DNA thermal cycler. An initial 4 minutes of denaturation at 95°C was followed by 30 cycles at 92°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 1 minute. The PCR programs were ended with 10 minutes extension at 72°C. The PCR products were then mixed with appropriate size standards to determine the size of the amplified fragments. Dye-labelled fragments were separated on an ALF Express II DNA analyzer (GE Healthcare), and the size of fragments was determined by the ALFwin Fragment Analyzer 1.03.01 software (GE Healthcare).

Genetic analysis

All alleles of the three cpDNA loci in *trnL-trnF*, *trnL5-trnL3* and *trnS-trnG* were treated as ordered characters assuming that mutations primarily follow a stepwise mutation model (Ohta & Kimura 1973).

A hierarchical analysis of molecular variance (AMOVA) was performed to describe the partitioning of genetic diversity between populations within subspecies and within populations. Calculation of F_{ST} was based on haplotype frequency and significance level was tested with 10000 permutations. The sum of squared distances between pairs of haplotypes R_{ST} was calculated with 10000 permutations, producing a minimum spanning network (MSN) according to Slatkin (1995) using the Arlequin version 3.1 (Excoffier *et al.* 2005).

To test for the presence of a phylogeographic structure within species, G_{ST} and R_{ST} were calculated according to Pons & Petit (1996), and a test to determine if significantly $R_{ST}>G_{ST}$ was carried out with 10000 permutations according to Burban *et al.* (1999) with the software PERMUT 2 (http://www.pierroton.inra.fr/genetics/labo/software). If R_{ST}, which takes the genetic differences between haplotypes into account, is higher than G_{ST}, then it indicates the presence of a phylogeographic

structure; closely related haplotypes are more often found in the same geographic area (Pons & Petit 1996).

We estimated the completeness of haplotype sampling (i.e. total number of individuals analysed vs. number of different haplotypes they display), by using Stirling probability distribution and Bayes' theorem (Dixon 2006).

Results

Hair index

In the total material, there was a bimodal distribution of hair density with maxima at hair index 1 and 3 (Fig. 2). The frequency of plants with different hair indices differs significantly between the five regions (Kruskal-Wallis Test, $p<0.0001$). If we assign plants with a hair index 0–2 to subsp. *obscurum* (n = 587) and plants with hair index 2.5–3 to subsp. *nummularium* (n = 1127), our sampled populations will accordingly consist of 100% subsp. *obscurum* in Denmark and 100% subsp. *nummularium* in Uppland and along the coast of the Baltic Sea. Between these two regions (in southern Sweden) is a wide hybrid zone of populations with various proportions of the two subspecies (Fig. 3). Very few populations in this hybrid zone are monomorphic. For instance only three monomorphic populations of subsp. *obscurum* which are relatively small are found in Skåne and Blekinge and Småland, respectively (see Table 1). We found a significant correlation ($r = 0.638$, $p<0.0001$) between latitude and mean values for indumentum within populations.

Table 3. Hierarchical partitioning of leaf shape variation in *H. nummularium* in Scandinavia.

Source of variation	% of variation
Between regions	10.1
Between populations within regions	43.5
Within populations	46.4

Leaf shape

The hierarchical partitioning of the total variation in leaf shape was attributed to between the five regions (10.1%), between populations within regions (43.5%) and between individuals within populations (46.4%) (Table 3).

A CVA plot of the first two canonical variates (CV1 and CV2) of the 70 populations used in the leaf shape analysis is shown in Fig. 4. No clear difference between the five regions can be found. A significant correlation ($r = 0.459$, $p<0.001$) between latitude and population values along CV1 was found across the hybrid zone, but a non-significant correlation when all the populations (including Denmark and province of Uppland) were included ($r = 0.182$, $p = 0.141$) (Fig. 4).

Chloroplast DNA polymorphism

Each of the three cpDNA microsatellite loci yielded two alleles per locus among 82 individuals of subsp. *nummularium* and subsp. *obscurum.* The allelic combinations produced six haplotypes (see Table 4).

The MSN (Fig. 5a), summarizes the genetic relationships between the six cpDNA haplotypes. The connected haplotypes are separated by only one mutation length. H2 and H5 are centrally lo-

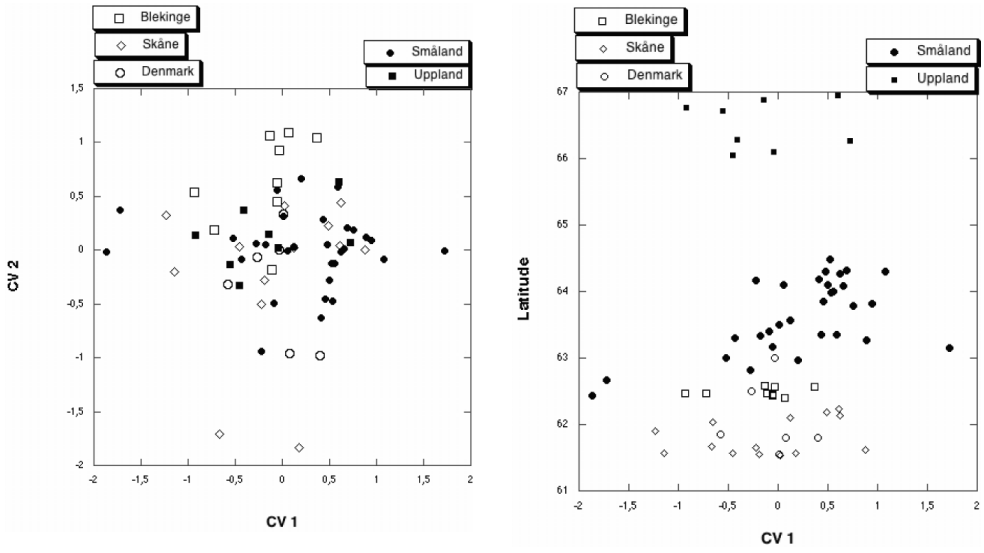

Figure 4. On the left panel, a canonical variates analysis of leaf shape-moment invariants of H. nummularium in five regions in Scandinavia based on the first two canonical variates (CV1 and CV2). On the right panel, a canonical variates analysis of leaf shape-moment invariants of H. nummularium in five regions in Scandinavia based on the first canonical variate (CV1) and latitude.

calized in the network and may represent basal haplotypes from which the other haplotypes are derived. H3 and H6 which only differ by one length mutation are distributed across the range of the species in Sweden and Denmark. H1 and H4 also differ by one length mutation and are confined to Denmark.

The distribution of cpDNA haplotypes overlapped but showed some geographic structuring in Scandinavia (Fig. 5b-d). The two most abundant haplotypes are H5 and H6, each found in 12 populations. Both are distributed across the species range in Denmark and Sweden. H2 (confined to Sweden) and H3 (found

Table 4. List of allelic composition, total frequency of plants (*N*) and frequency of subspecies per haplotype in *H. nummularium* in Scandinavia.

Haplotypes	*mtrnSf-trnGr*	*mtrnL2-trnF*	*mtrnL5-trnL3*	*N*	*nummularium*	*obscurum*
H1	128	216	241	3	0	3
H2	128	217	241	19	4	15
H3	128	217	242	14	6	8
H4	129	216	241	3	0	3
H5	129	217	241	22	9	13
H6	129	217	242	21	18	3
Total				82	37	45

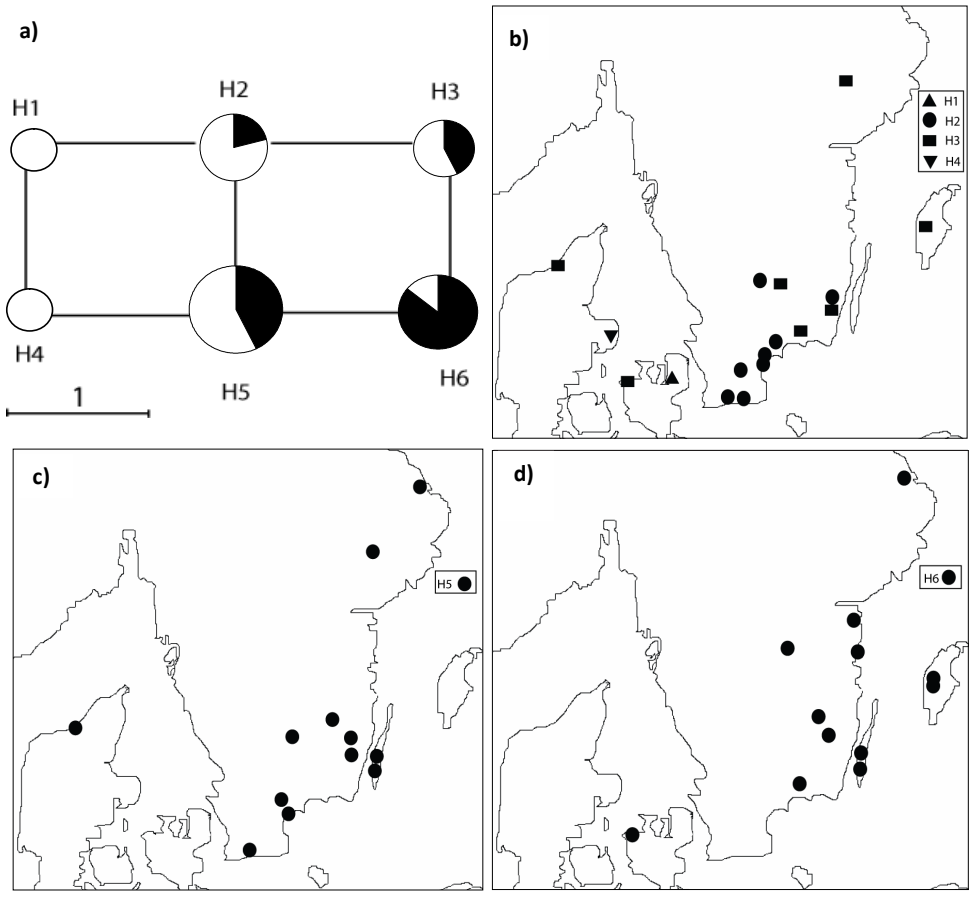

Figure 5. a) Minimum spanning network showing the genetic relationships between the six Scandinavian cpDNA haplotypes and taxon composition for each haplotype. The connection length (see scale bar) is proportional to the number of R_{ST} differences between a haplotype pair. The size of the pie diagrams is proportional to the frequency of haplotypes in the studied samples. White sectors denote the frequency of subsp. *obscurum* and black sectors denote the frequency of subsp. *nummularium*. (b-d) The geographic distribution of the six Scandinavian cpDNA haplotypes of *H. nummularium* in Denmark and southern Sweden.

elsewhere except in the province of Skåne, Sweden) are the second most abundant haplotypes found in eight and seven populations, respectively. The remaining two haplotypes, H1 and H4 were unique, found in one population, respectively and were restricted to Denmark.

Approximately, sixty-six percent of the total cpDNA haplotype diversity was distributed between populations ($F_{ST} = 0.657$) and the remaining was found within populations (see Table 5). The test for the presence of a phylogeographic structure within *H. nummularium* in Scandinavia was significant ($p = <0.05$); R_{ST} (0.667) was significantly higher than G_{ST} (0.589).

Using Dixon's (2006) method, the probability of cpDNA haplotype completeness was 1.0, suggesting that we have sampled all possible haplotypes in this study. Approximately half of the populations (52%) contained only one haploty-

pe, 44.4 % of the populations harboured two haplotypes while only one population harboured three haplotypes (see Table 1).

Haplotype vs. taxon

There was a weak cpDNA haplotype structuring across the two subspecies in Scandinavia. Only two haplotypes, H1 and H4 were peculiar to subsp. *obscurum* in Denmark. The remaining four haplotypes were shared among the two subspecies (Table 4, Fig. 5a).

Discussion

Characterization of indumentum and leaf shape variation pattern

The diagnostic character distinguishing the two subspecies of *H. nummularium* is the presence or absence of a grey felt of stellate hairs on the lower surface of the leaf. However, this trait is not a discrete character since a low frequency of large stellate hairs can be found in most plants without a dense grey felt. Some plants are actually intermediates with respect to stellate hairs (i.e. hair index 2 in Fig. 2, cf. also Azzouzi 1997). These intermediates are found in mixed populations in the hybrid zone in southern Sweden, but rarely also in Denmark. When seeds from individual plants in mixed populations in the hybrid zone are sown in cultivation, the progeny often segregates for the indumentum character (B. Widén, unpublished). Segregation in experimental crosses between the two subspecies suggests the presence of a few genes for indumentum characters and that the gene(s) for a dense felt of stellate hairs is (are) recessive (B. Widén, in prep.).

The distribution of the two subspecies as defined in the present study shows a distinct geographical pattern in Scandinavia (Fröberg 2006, Edqvist & Karlsson 2007, Tyler *et al.* 2007, Widén 2010).

Table 5. Analysis of molecular variance (AMOVA) based on cpDNA haplotype frequency of *H. nummularium* in Scandinavia.

Source of variation	d.f.	Sum of squares	Variance components	% of variation
Between populations	26	34.98	0.378	65.76*
Within populations	55	10.83	0.196	34.24
Total	81	45.81	0.575	

* ($p<0.0001$)

The most common one, subsp. *nummularium* has an eastern distribution in Scandinavia; predominantly in Sweden and Finland, but it has also been reported from Denmark (Widén 2010) though we did not find it in our sample. The subspecies *obscurum*, on the other hand, has a south-western distribution. In southern Sweden, a hybrid zone between the two subspecies with mixed populations occurs. Even in almost pure populations of one of the subspecies in the hybrid zone, the other subspecies may occur in a very low frequency and thus escape the detection in floristic inventories (B. Widén personal observation). Rare herbarium specimens of subsp. *obscurum* have been documented from areas north and east of the hybrid zone (i.e. Gotland and Östergötland) (Widén 2010, see also Proctor 1956 for the same phenomenon in areas outside Scandinavia). The records of herbarium specimens, together with the

segregation pattern in cultivation experiments (B. Widén in prep.) and the indumentum variation pattern, suggest that natural populations of *H. nummularium* in the hybrid zone differ in frequency for genes controlling the variation in indumentum.

Two scenarios are proposed to explain the geographical distribution of the two subspecies in Scandinavia. A postglacial colonization of a polymorphic lineage (i.e. in indumentum) may have been subjected to diversifying selection during range expansion. The distribution of subsp. *obscurum* suggests that natural selection may favour the genes coding for the absence of a felt of stellate hairs in the wetter and oceanic climate in south-western Scandinavia. However, a drier climate may favour the more pubescent subsp. *nummularium* in eastern and southern Sweden and further to the north. Other morphological characters, e.g. leaf shape, have not been subjected to diversifying selection. However, the fact that subsp. *obscurum* is absent from the more oceanic British Isles while subsp. *nummularium* is common, speaks against the ocean climatic influence on the gene frequencies coding for indumentum (Proctor 1956). Additionally, subsp. *obscurum* does not occur along the Swedish south-west coast, which has a more oceanic climate than further to the east in southern Sweden. A more plausible explanation for the observed geographical distribution is that the two subspecies represent two different postglacial migration lineages, one colonizing Scandinavia from the south and the other from the east (cf. Hedrén & Prentice 1996, Nordström & Hedrén 2008). The two lineages differed in frequency of genes for indumentum characters, the southern lineage without and the eastern lineage with genes coding for a dense felt of stellate hairs on the abaxial side of the leaf. Hybridization and introgression between the two lineages in the hybrid zone in southern Scandinavia gave rise to the present-day mixed populations with respect to indumentum. Possible differences in other morphological traits (i.e. leaf shape) may have disappeared by introgression between the two lineages.

Morphometric analysis of leaf shape variation pattern did not support the regional subdivision of populations in Scandinavia. Most of the variation was found between populations within regions and between individuals within populations. Also, the leaf shape did not support the subdivision of populations into the two subspecies, *nummularium* and *obscurum*. Since the leaf shape is considered a polygenic character, its taxonomical value is limited, especially at the intraspecific level where extensive overlapping of morphological characters occurs within species.

Chloroplast DNA distribution and diversity

In a preliminary wide-scale study (Soubani *et al.* in prep.) done on the *H. nummularium* complex in Europe, a total of 18 haplotypes were identified based on cpDNA microsatellite polymorphisms. Six of these haplotypes (H1-H6) were found in Scandinavia. H5 is the most frequent haplotype both in Scandinavia and the rest of the continent. It covers the species range in the central, eastern (i.e. Romania) and southern parts (i.e. Italy) of Europe, but is absent from the western parts. Apparently, this lineage survived the glacial cycles in potential refugia in southern or

south-eastern Europe and recolonized northern Europe via an eastern migration route after the last ice age. H6 is the second most frequent haplotype and found only in Scandinavia whereas H3 is found in Scandinavia and Estonia. Few studies have reported such cases where unique haplotypes were confined to Northern Europe e.g. *Alnus glutinosa* (King & Ferris 1998) and *Silene dioica* (Hathaway *et al.* 2009). H2 is the third frequent haplotype, found in Sweden, central, eastern and southern parts of Europe. The distribution of H2, H3 and H6 along with H5 supports an eastern postglacial immigration route into Scandinavia. In contrast, the absence of H1 and H4 from Sweden (although both are found in Denmark), supports a south-western immigration route into western Scandinavia. The immigration of H1 and H4 into Sweden might be hindered by pre-recolonization and establishment of other haplotypes such as H2, H3, H5 and H6.

The overall genetic diversity was significantly higher between populations than within populations and this may indicate restricted gene flow between distantly localized populations. The lower but substantially high genetic diversity within populations indicate occurrence of introgressive hybridization between populations in the hybrid zone (southern Sweden). According to the parameters R_{ST} and G_{ST}, there was a significant phylogeographic structure in haplotype distribution. For example, H3 and H6 are genetically closely related and have similar distributions in Sweden and Denmark. H1 and H4 are also closely related and are confined to Denmark. H2 and H5 were interpreted as basal haplotypes and have similar geographic distributions in southern Scandinavia.

Chloroplast DNA haplotype vs. taxon

The six cpDNA haplotypes do not support the subdivision *H. nummularium* into two subspecies, *nummularium* and *obscurum* in Scandinavia. For example, H1 and H4 are peculiar to regional populations of subsp. *obscurum* in Denmark. Haplotypes H2, H3, H5 and H6 are found in both subspecies but with different frequencies. Apparently, the distribution of cpDNA haplotypes is relatively geographically than taxonomically structured.

The lack of congruence between cpDNA haplotype and taxa has been demonstrated in several studies such as in the *Helianthemum oelandicum* complex (Widén *et al.* submitted) and *Hedera* spp. (Grivet & Petit 2002). This incongruence can be explained by introgression or pollen swamping between genetic lineages of a species (cf. Soubani *et al.* submitted) or by genetic drift as a result of dynamic shifts in species range; expansion and contraction during the Pleistocene period (Schaal 1998).

There is a long tradition to use indumentum traits as taxonomical key characters in *Helianthemum* (cf. Janchen 1909, Tutin *et al.* 1968, Widén 2010). However, our results question the validity of using stellate hairs for separating taxa (cf. Widén *et al.* submitted, Soubani *et al.* in prep.).

Conclusions

The geographical distribution of morphological variation and distribution of cpDNA haplotypes in Scandinavian populations of *H. nummularium* indicate two postglacial immigration lineages, one from west and one from east. The distri-

bution of four haplotypes (H2, H3, H5 and H6) supports an eastern postglacial immigration lineage whereas the distribution of H4 and H1 in Denmark supports a south-western postglacial immigration lineage into Scandinavia. The two lineages differ in the frequency of genes coding for a dense felt of stellate hairs on the lower side of the leaf. In a wide contact zone between the two lineages in south-east Scandinavia, hybridization and introgression have created a range of intermediate populations with high variability in indumentum density.

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Internal transcribed spacer and chloroplast DNA sequences reveal poor intraspecific phylogenetic resolution in the *Helianthemum oelandicum* complex (Cistaceae)

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Abstract

The rock rose *Helianthemum oelandicum* comprises a variety of morphs that display complex variation in both indumentum and flowering phenology. A previous study has shown that the traditional taxonomic subdivision of the complex is not supported by the geographical distribution of chloroplast haplotypes in Europe. Sequence data from the internal transcribed spacer region (ITS) and four chloroplast markers were used in the present study to examine relationships among the five European subspecies within the complex. Phylogenetic analysis of ITS and cpDNA resulted in poorly resolved trees; the subspecies seemed to be either poly- or paraphyletic. Thus, these molecular markers do not support the traditional subdivision of *H. oelandicum* into several subspecies. The most plausible explanation for the weak phylogenetic signals detected by the molecular markers is that *H. oelandicum* is of recent origin and does not show sufficient differentiation in genetic markers.

Introduction

Helianthemum oelandicum (Cistaceae) is a diploid (2n = 22), self-incompatible, insect and wind pollinated perennial dwarf shrub. The species is restricted to calcareous soils and limestones in open lowland to alpine grasslands, often with exposed outcrops (Janchen 1907, Proctor 1956). The species is distributed mainly in central and southern Europe, and it also extends into Turkey and the Caucasus. Marginal populations occur in the British Isles and in Scandinavia, where the complex is an important component in the alvar grasslands of the Baltic island of Öland (Sterner 1936a&b). Isolated populations are also found in the Pinega area in northern Russia and in the Atlas mountain range in North Africa (Proctor 1956).

The species comprises a variety of morphs that show complex variation in both indumentum and flowering phenolo-

gy and is therefore treated as a species complex (Janchen 1907, Sterner 1936a&b, Widén 1980, 2010). Three main types of trichomes (Grosser 1903) are found in different combinations in the *H. oelandicum* complex (Widén 1988): stellate hairs, bristles (or simple hairs) and glandular hairs. Indumentum may vary both within and between populations and shows considerable geographical variation even at the local scale (Widen 1988, 2010).

The species complex is characterized by two flowering phenologies; the concentrated flowering (CF) where the inflorescences are developed at the apex of the previous year's growth and the protracted flowering (PF) with inflorescences developed laterally on the current year's growth as well as at the apex of the previous year's growth. The taxonomy of the species complex in Europe has been treated in different ways. Janchen (1907) recognized five species, while Proctor in Flora Europaea (Tutin *et al.* 1968) divided the complex into two species based on the presence/absence of dense felt of stellate hairs on the abaxial surface of the leaves: *H. canum* with seven subspecies and *H. oelandicum* with five subspecies. Widén (2010) has studied the species complex on Öland and recognized one subspecies *H. oelandicum* subsp. *oelandicum* with two varieties; var. *oelandicum* (plants with a CF phenology and without stellate hairs) and var. *canescens* (plants with a PF phenology and variable with respect to stellate hairs on the abaxial surface of the leaf).

In the present study, the taxonomic subdivision of the complex is treated as one species *H. oelandicum* with five subspecies in Europe. Plants from Öland are regarded as the subspecies *oelandicum*. Following Widén (2010), I distinguish between var. *oelandicum* (with the CF phenology) and var. *canescens* (with the PF phenology) based on the flowering phenology. The subspecies *alpestre* refers to an alpine taxon, usually with large flowers, from higher altitudes in the Alps, Pyrenees, Tatra Mountains, Carpathians and other high mountains in south-eastern Europe. The subspecies *rupifragum* is restricted to lower and intermediate altitudes in south-eastern Europe. The subspecies *italicum* habituates low altitudes in the western and central Mediterranean areas. All morphs from continental Europe and the British Isles with a dense felt of stellate hairs on the lower side of the leaves were lumped together into the subsp. *incanum*.

A recent study of the phylogeography of the *Helianthemum oelandicum* complex (Widén *et al*. submitted) revealed a strong geographic structuring of chloroplast haplotypes within the complex in Europe. The three most common haplotypes are closely related. Two haplotype lineages dominate the geographical areas covered by the species; a western lineage (H1) and a eastern/south-eastern lineage (H2). The third common haplotype (H3) is confined to the Alps and adjacent areas in northern Italy. Six additional lineages have a more restricted distribution in south-western, central and southern Europe. These chloroplast haplotypes do not correlate with the taxonomic subdivision or the morphological variation within the complex; instead several chloroplast lineages cover most of the morphological variation within the complex. Widén *et al*. (submitted) suggested parallel evolution of morphs within allopatrically distributed chloroplast lineages in many mountain ranges belonging to the European Alpine System.

Phylogenetic studies performed at low taxonomic levels have become more attractive with the advent of molecular markers. Such studies can provide insights into species´ inter- and intrapopulation variation patterns as well as evolutionary processes governing divergence and speciation events (Mayer & Soltis 1999, Singh 2004). The internal transcribed spacer (ITS) of the 18S–5.8S–26S nuclear ribosomal DNA (nrDNA) combined with the chloroplast DNA (cpDNA) markers have proven valuable in investigating phylogeny at generic and specific level of several plants such as *Vasconcellea* spp. (Kyndt *et al.* 2005) and *Cotyledon* spp. (Mort *et al.* 2005). Chloroplast DNA is characterized by lower substitution rate compared to the nuclear DNA and thus may reveal limited variation (i.e. when used alone) among closely related taxa (Small *et al.* 2004). However, ITS is characterized by a higher substitution rate than organelle DNA. Although ITS is present in thousands of copies in the genome, it undergoes rapid concerted evolution driven by gene conversion and unequal crossing over, which tends to homogenize differing intragenomic nrDNA copies. Accordingly, ITS is considered as an informative marker for reconstruction of phylogenetic relationships between taxa (cf. Baldwin 1993, Baldwin *et al.* 1995, Alvares & Wendel 2003). However, there is no assurance that nuclear and/or cpDNA markers will provide sufficient phylogenetic resolution between closely related taxa, especially between subspecies or varieties within species of a recent origin (Pornpongrungrueng *et al.* 2009).

Table 1. List of material examined in European *Helianthemum oelandicum* and outgroup taxa. Under the heading "Haplotype" is given chloroplast haplotype belonging of the analyzed material according to (Widén *et al.* submitted). Under the headings "ITS" and "cpDNA" is indicated which material was successfully sequenced for either ITS or chloroplast DNA regions. In the cpDNA column is a further detail regarding how many cpDNA regions that were successfully sequenced for each accession (*trnL-trnF, trnL* intron, *trnS-trnT* and *trnT-psbD*). Further details regarding populations are given in Widén *et al.* (submitted) and Soubani *et al.* (submitted). Sequences for *H. scopulicolum* and *H. squamatum* were obtained from Genbank.

Taxon	Country	Haplotype	ITS	cpDNA	Collector
H. scopulicolum	Spain	-	X	-	GB: DQ092928
H. squamatum	Spain	-	X	-	GB: DQ092927
alpestre	Austria	H3	-	2X	Widén
incanum	Austria	H2	X	-	Widén
incanum	Austria	H2	X	4X	Widén
incanum	Austria	H2	X	2X	Widén
rupifragum	Bosnia & Herzegovina	H6	-	4X	Widén
incanum	Bulgaria	H2	-	4X	Widén
incanum	Czech Republic	H2	-	4X	Widén
incanum	England	H1	X	4X	Widén
incanum	France	H1	X	4X	Widén
incanum	France	H7	X	4X	Widén

italicum	France	H1	-	3X	Widén
italicum	France	H3	X	4X	Widén
incanum	Germany	H1	X	3X	Widén
incanum	Germany	H1	X	-	Widén
incanum	Greece	H2	-	4X	Engstrand
H. hymettium	Greece	H10	X	4X	Widén
incanum	Ireland	H1	X	-	Widén
alpestre	Italy	H3	X	4X	Widén
incanum	Italy	H2	X	-	Widén
italicum	Italy	H8	X	4X	Widén
incanum	Macedonia	H2	X	4X	Widén
rupifragum	Montenegro	H6	-	4X	Widén
incanum	Romania	H2	X	4X	Widén
rupifragum	Slovakia	H2	-	4X	Widén
rupifragum	Slovakia	H2	X	-	Widén
italicum	Spain	H4	X	4X	Widén
H. marifolium	Spain	H4	X	4X	Widén
canescens	Sweden	H1	X	-	Widén
canescens	Sweden	H1	X	-	Widén
canescens	Sweden	H1	-	4X	Widén
H. nummularium	Sweden	-	-	3X	Widén
oelandicum	Sweden	H1	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	-	Widén
oelandicum	Sweden	H2	X	4X	Widén
incanum	Switzerland	H1	X	2X	Widén
incanum	Turkey	H2	X	-	Soubani
incanum	Turkey	H2	X	4X	Soubani
incanum	Turkey	H2	-	4X	Soubani

Comparison between phylogenies derived from biparentally inherited nuclear regions and maternally inherited chloroplast DNA regions is advised, especially when reticulate evolution (e.g. introgression and hybridization) or incomplete lineage sorting is expected among taxa (Soltis *et al.* 1998, Linder & Rieseberg 2004, Small *et al.* 2004, Pornpongrungrueng *et al.* 2009).

Guzmán & Vargas (2005) investigated the systematics and molecular phylogeny of Cistaceae (based on cpDNA and ITS markers), including 20 species of *Cistus*, two *Halimium*, one *Tuberaria* and two *Helianthemum*. They found that *Halimium* and *Cistus* formed a monophyletic group which is a sister group to *Tuberaria*. *Helianthemum squamatum* and *H. scopulicolum* formed another monophyletic group which is a sister group to *Halimium*, *Cistus* and *Tuberaria*. The molecular phylogeny supports the circumscription of the species of *Cistus* based on morphological characters.

Recently, Guzmán and Vargas (2009) reinvestigated the cpDNA phylogeny of the same members of Cistaceae as mentioned above, but included several additional *Helianthemum* species, e.g. *H. oelandicum* and *H. marifolium.* They found that all accessions of *Helianthemum* formed a well supported monophyletic clade, and furthermore that *H. oelandicum* and *H. marifolium* were clustered in a well supported subclade.

Here I present a pilot study comprising a phylogenetic analysis of the *H. oelandicum* complex based on the nuclear and chloroplast genomes. I address the following questions: (1) Do the ITS and chloroplast DNA sequences give any phylogenetic signal within *H. oelandicum* and (2) Do these markers support the traditional subdivision of *H. oelandicum*? Also, (3) is there any congruence between the ITS and chloroplast DNA sequences?

Materials and Methods

Plant material

The phylogenetic analysis included 44 accessions covering the variation in taxonomy, chloroplast DNA haplotypes and geography, obtained from two recent studies; Widén *et al.* (submitted) and Soubani *et al.* (submitted). A total of 42 accessions belonged to the *H. oelandicum* complex (19 to subsp. *incanum*, four to subsp. *italicum*, four to subsp. *rupifragu*m, ten to subsp. *oelandicum* var. *oelandicum*, three to subsp. *oelandicum* var. *canescens* and two to subsp. *alpestre*). Two closely related taxa belonging to the same section (*Plectolobum*) as *H. oelandicum* were included: *H. hymettium* (2n = 22, Greece) and *H. marifolium* (2n = 22, Spain). Most of the accessions have been preserved as herbarium material for accurate identification. I also included three unrelated species as outgroups, *H. scopulicolum* (2n = 20, Spain), *H. squamatum* (2n = 10, Spain) and *H. nummularium* (2n = 20, Sweden) (Table 1).

DNA isolation, amplification and sequencing

Helianthemum DNA was isolated according to Widén *et al.* (submitted). For the amplification of the nuclear internal transcribed spacers (ITS), three published ITS primer pairs were tested; ITS4-ITS5 (White *et al.* 1990), 17SE-ITS4 (Sun *et al.* 1994 for 17SE and White *et al.* 1990 for ITS4) and P17S-P26S (Popp & Oxelman

2001). However, no or only weak amplification products were obtained. Therefore, a specific primer pair was designed based on ITS consensus sequence of *Helianthemum* and *Cistus* sequences obtained from Genbank. The forward primer *ITS1h* (`5-CGA TTC CTG CAC AGC AGA C-3´) and the reverse primer *ITS2h* (`5-GTT CGG GGT CCG AGA GAT-3´) successfully amplified the ITS region.

Four non-coding regions were sequenced in the cpDNA: the intergenic spacers *trnL-trnF*, *trnS-trnT* and *trnT-psbD* and the *trnL* intron. The *trnL* intron and the *trnL-trnF* spacer were amplified by the universal primer pairs c/d and e/f, respectively (Taberlet *et al.* 1991), and the *trnS-trnT* spacer by universal primers *trn*S/*trn*T (Demesure *et al.* 1995). The *trnT-psbD* spacer was amplified by *Helianthemum*-specific primers developed in Widén *et al.* (submitted). The same primers as for the amplifications were used in sequencing reactions.

The PCR reactions were carried out in a total volume of 10 µl containing 6.8 µl distilled water, 1 µl (10x PCR) buffer, 0.8 µl (25mM) $MgCl_2$, 0.2 µl (10 mM of each) dNTPs, 0.08 µl (25 pmol/µl) each of the forward and reverse primers, 0.24 µl (1 unit/µl) *Taq* polymerase and 0.8 µl (14 ng/µl) DNA template. The PCR amplification was performed in a PTC-100 DNA thermal cycler. An initial 4 minutes denaturation at 95ºC was followed by 39 cycles at 92ºC for 45 s, annealing at 57ºC for 1 minute and extension at 72ºC for 1 to 3 minutes depending on the fragment length. All the PCR programs were ended with 10 minutes extension at 72ºC.

PCR products of approximately 100 µl of each sample were purified with the QiaQuick PCR Purification kit (QIAGEN) and directly sequenced with BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). The reaction conditions for sequencing were 24 cycles at 96°C for 1 minute, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 minutes. The extension products were then purified and subsequently separated on an ABI PRISM 3100 automated sequencer (Applied Biosystems).

Sequence editing and Phylogentic analysis

Phylogenetic analyses were performed separately on sequence data obtained from the cpDNA (28 accessions) and ITS (36 accessions), respectively (see Table 1). The forward and reverse sequences for each sample were assembled and aligned with ClustalX (Thompson *et al.* 1997), with subsequent adjustment by eye in BioEdit 7.0.0 (Hall 1999). All regions of ambiguous alignment were excluded and gaps were treated as missing data. The resulting consensus sequences of the ITS and four cpDNA regions were then truncated so that all the accessions of the same genome had the same length. To increase the amount of informative characters for resolving the species phylogeny, the four cpDNA regions (*trnL* intron, *trnL-trnF*, *trnS-trnT* and *trnT-psbD*) were combined into one major consensus sequence for each accession. Separate phylogenetic analyses (Bayesian inference) were also performed on each of the four cpDNA regions (results not shown).

Phylogenetic trees were estimated using maximum parsimony (MP) implemented in PAUP 4.0 beta10 (Swofford 2002) and Bayesian inference (BI) by MrBAYES 3.1.2. (Huelsenbeck & Ron-

quist 2001, Ronquist & Huelsenbeck 2003). For the MP analysis, a heuristic search (1000 random addition replicates) was conducted using the optimality criterion of maximum parsimony in PAUP with Tree bisection-reconnection (TBR) branch-swapping and MulTrees options active. All characters were treated as unordered and equally weighted. Bootstrap support (BS, Felsenstein 1985) was estimated with 1000 replicates with the "collapse zero-length branches" option in effect. Both the consistency (CI) and retention indices (RI) were used to assess the amount of homoplasy present in the data set. A 50% majority–rule consensus tree was calculated for each data set.

For Bayesian inference (BI), a general model of DNA substitution (the GTR) with gamma-distributed rate variation across sites was applied and four Markov chain Monte Carlo (MCMC) chains, one cold and three heated, were performed. A 50% majority-rule consensus tree was calculated for each data set and posterior probability (PP) distributions of trees were produced as alternative estimate of robustness. Only PP ≥0.95 was considered statistically significant (Huelsenbeck & Ronquist 2001).

The Median Joining (MJ) network (Bandelt *et al.* 1999) implemented in Network 4.5.0.2 (available at: http://www.fluxus-engineering.com) was performed to obtain a better resolution among closely related taxa within *H. oelandicum* complex using the ITS and combined cpDNA sequences separately.

Results

The MP and the BI analyses gave rise to similar topologies and congruent statistical support for the clades identified in the ITS and the combined cpDNA trees (Figs. 1, 2, 3 & 4). A MJ network was constructed for the ITS data. However, it was not possible to construct a similar network for the combined cpDNA data due to extensive homoplasy.

ITS

A total of 34 ITS sequences was analyzed together with the two published outgroup sequences of *H. squamatum* and *H. scopulicolum* (see Table 1). The ITS (a partial sequence) had a total length of 427 characters (ITS1 = 72, 5.8S = 167 and ITS2 = 188). Three indels of 1-2 bp were required to align all the ingroup sequences with the two outgroup sequences.

The MP analysis resulted in 511 equally parsimonious trees of 134 steps (CI = 0.649, RI = 0.675). Forty-two (9.8%) characters were variable of which 35 (8%) were parsimony-informative.

Only four clades were supported in the resulting consensus tree (Fig. 1). The largest clade is Clade A (59% BS) contained 20 accessions and included representatives of subsp. *incanum*, var. *oelandicum*, var. *canescens*, subsp. *italicum* and subsp. *rupifragum*. This clade was dominated by samples with haplotypes H1 and H2, except for one accession from France with haplotype H3. It contained accessions from all over the sampled area except for the Iberian Peninsula. Two accessions of subsp. *incanum* from Macedonia and Austria formed a subclade with 76% BS. All other accessions were included in

a basal unresolved group Grade B composed of accessions of mixed origins. All taxa were represented in the group except for subsp. *rupifragum*, and all haplotypes were represented. Two accessions of subsp. *incanum* from France with haplotypes H1 and H7, one accession of subsp. *italicum* from Spain with haplotype H4 and one accession of *H. marifolium* from Spain with haplotype H4 formed a clade with 80% BS. The two samples from France formed a nested subclade within this clade with 78% BS.

The BI analysis resulted in a tree (Fig. 2) that largely reflected the consensus tree given by the MP analysis. However some further resolution was obtained by the BI, and within Clade A yet a subclade was identified that was composed of 2 accessions of var. *oelandicum* from Sweden with H2 and four accessions of subsp. *incanum* of mixed origins containing haplotypes H1 and H2.

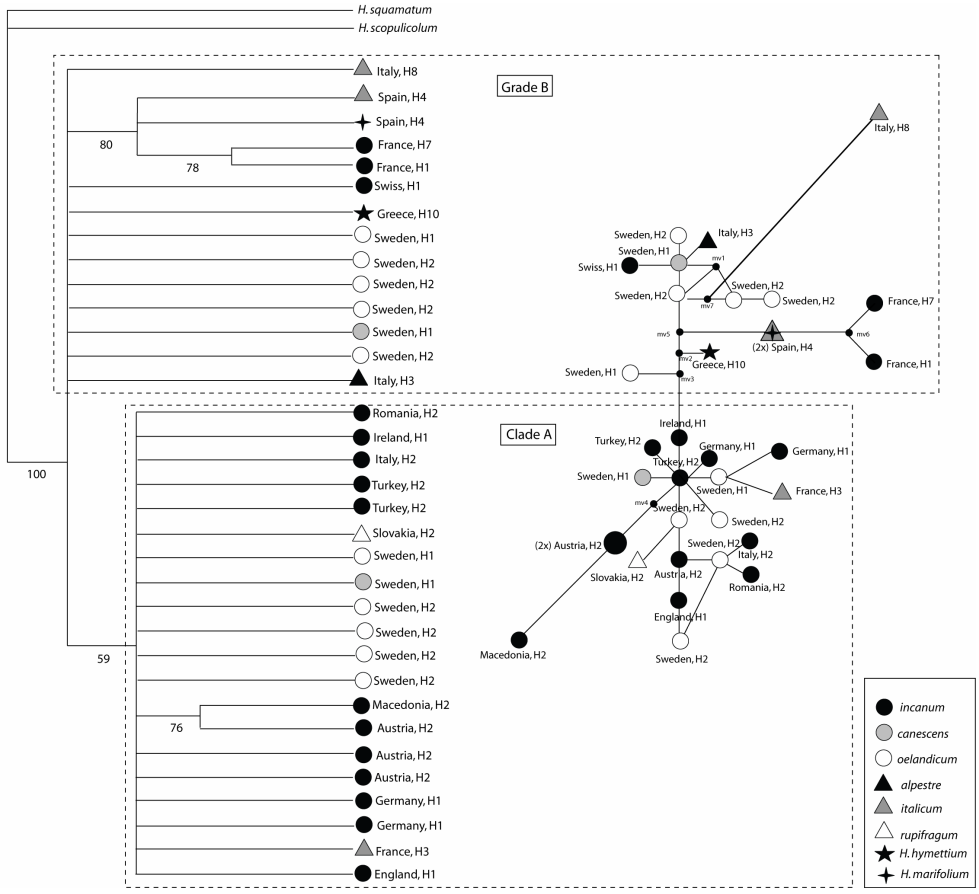

Figure 1. Left: The 50% majority–rule consensus tree of 511 equally parsimonious trees of the *H. oelandicum* complex, *H. hymettium* and *H. marifolium* based on ITS sequences (total length 134 steps; CI = 0.649, RI = 0.675). The tree is rooted with two outgroups, *H. squamatum* and *H. scopulicolum.* Right: The median-joining network based on ITS data. Distances correspond roughly to the number of differences between the accessions. Seven-median joining vectors (mv) are included in the network denoted by small black dots.

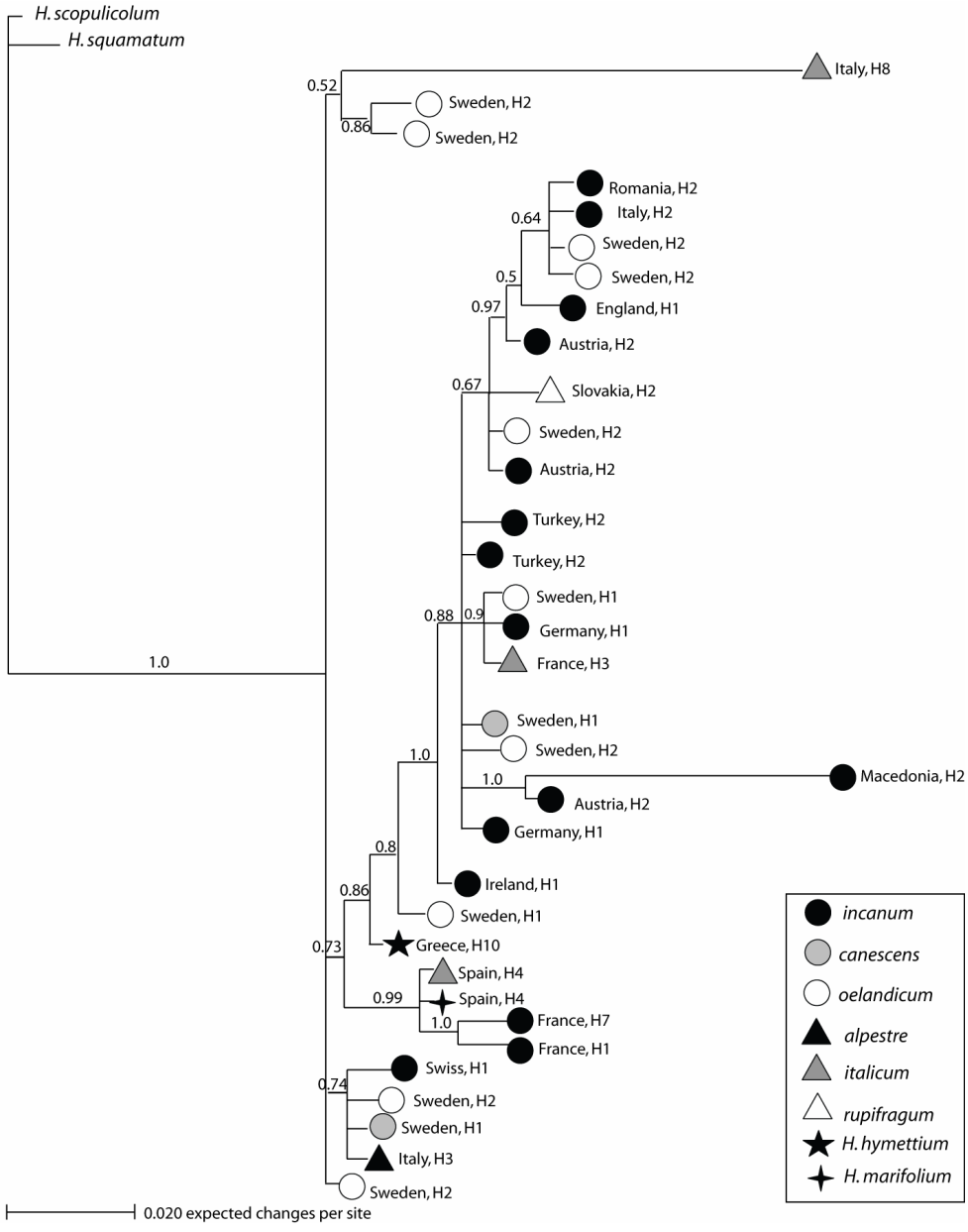

Figure 2. Bayesian phylogram based on ITS sequences. Values above the branches indicate posterior probabilities (PP). Only PP ≥0.95 are considered as significant. The tree is rooted with two outgroups, *H. squamatum* and *H. scopulicolum.*

In the MJ network, all the ingroup accessions (without outgroups) were assembled into two major groups; Grade B and Clade A which were already identified in MP in Fig. 1. However, the MJ network (similar to BI) provides better resolutions concerning the relationships between the internal unresolved branches in MP. For example, subsp. *incanum* with H2 from Turkey in Clade A, represents a central haplotype with highest number of connection lengths; suggesting it to represent an ancestral taxon. The seven median joining vectors (*mv*) distributed in the network, indicate missing intermediates probably due to unsampled data, extinct accessions or as a consequence of reticulate evolution (i.e. hybridization and introgression (Bandelt *et al.* 1999).

Chloroplast DNA regions

The total alignment length of the 28 sequences (including one outgroup, *H. nummularium*) in the present analysis was 3435 characters (complete sequences of *trnL intron* = 558, *trnL-trnF* = 395 and partial sequences of *trnS-trnT* = 1327, *trnT-psbD* = 1155).

The MP analysis resulted in 1341 equally parsimonious trees of 1510 steps (CI = 0.547, RI = 0.200). Four hundred and sixty-three characters (13.5%) were variable of which 282 (8.2%) were parsimony-informative. Seven polymorphic microsatellite loci were detected ranging from 13-22 bp. Forty indels were required to align all sequences (see Fig. 3).

Both the MP (Fig. 3) and BI (Fig. 4) analyses resulted in similar tree topologies where successively one accession of subsp. *alpestre* (H3, Austria) and one accession of subsp. *italicum* (H1, France) were placed as sister to the remaining accessions. The remaining accessions formed a large polytomy with no substructure except for three small clades of two accession each: one clade composed of var. *canescens* (H1) and var. *oelandicum* from Sweden (H2) with 85% BS, one clade composed of two accessions of subsp. *incanum* from Turkey with 100% BS, and one clade composed of subsp. *rupifragum* from Slovakia (H2) and *H. marifolium* from Spain with 62% BS.

Again, the BI analysis (Fig. 4) resulted in a tree that largely reflected the consensus tree given by the MP analysis, but some additional clades could be identified. Several isolated accessions were split off as successive sisters from the base of the tree; one accession of subsp. *alpestre* (Austria, H3), followed by an accession of subsp. *italicum* (France, H1), an accession of *H. hymettium* (Greece, H10), and an accession of subsp. *incanum* (Bulgaria, H2).

The separate analysis performed on each of the four cpDNA regions resulted in trees with lower support and less resolution than the combined tree. The Bayesian analysis of *trnS-trnT* and *trnT-psbD* regions resulted in relatively similar trees in which identical haplotypes were more or less clustered together, in contrast to trees resulting from analyses of the *trnL-trnF* and *trnL intron* regions.

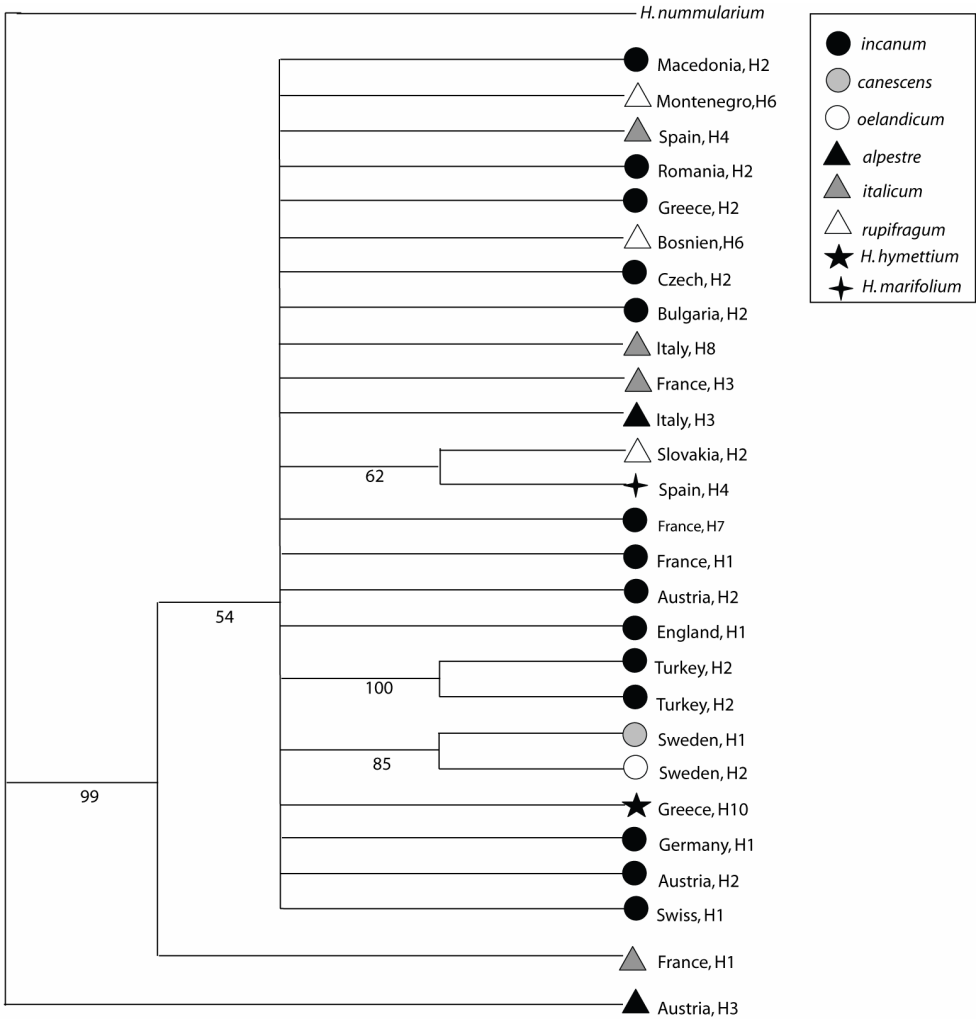

Figure 3. The 50% majority–rule consensus tree of 1342 equally parsimonious trees of *H. oelandicum*, *H. hymettium* and *H. marifolium* based on combined cpDNA regions (total length 1510 steps, CI = 0.547, RI = 0.200). The tree is rooted with one outgroup, *H. nummularium.*

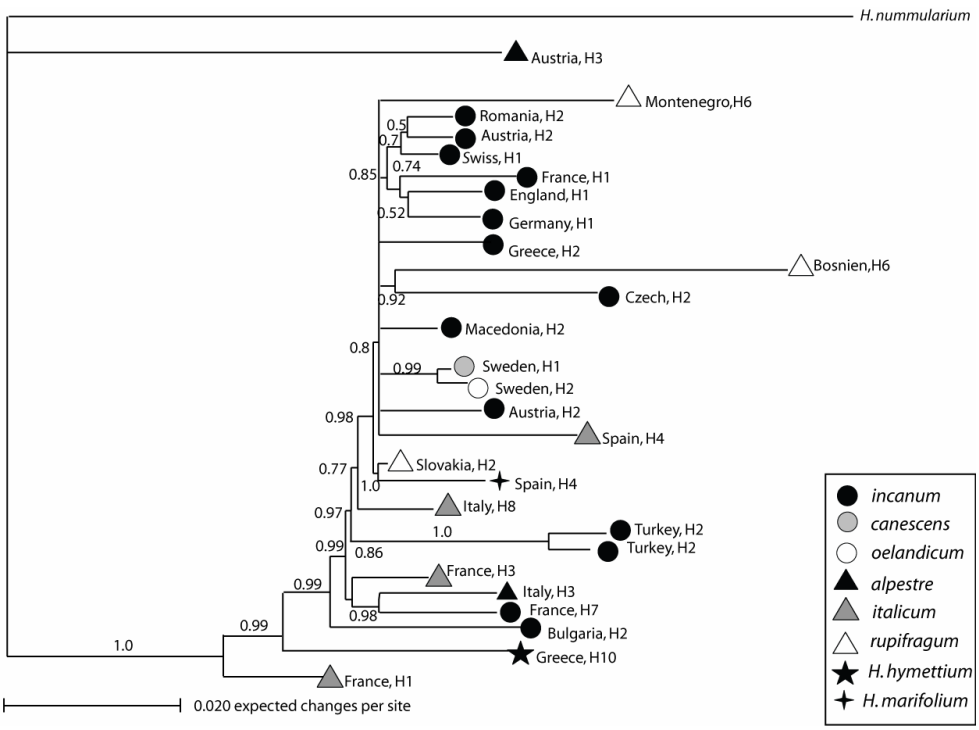

Figure 4. Bayesian phylogram based on the combined cpDNA regions. Values above the branches give posterior probabilities (PP). Only PP ≥0.95 are considered as significant. The tree is rooted with one outgroup, *H. nummularium.*

Discussion

Both the ITS and the four combined cpDNA regions provided weak phylogenetic substructures within the *H. oelandicum* complex. Despite the broad morphological and ecological variation within *H. oelandicum,* these regions performed poorly in resolving the relationships within the species. The difficulty of finding an appropriate marker system for intraspecific phylogenetic inference is a common problem in phylogeny (Wendel & Doyle 1998).

Polyphyletic relationships of subspecies within H. oelandicum

The phylogenetic analysis based on the ITS sequences and combined sequence data of the four cpDNA regions revealed that all subspecies are para- or polyphyletic. The observed polyphyly in *H. oelandicum* may reflect differences in phylogenetic history of the nuclear and chloroplast regions (Funk & Olmland 2003). Polyphyly can arise as an effect of homoplasy (i.e. recurrent mutation), incomplete lineage sorting from a polymorphic ancestor, or reticulate evolution (introgression and hybridization). Furthermore, the observed polyphyly may reflect a weak phylogenetic

signal due to inadequate information obtained from the gene trees (Funk & Olmland 2003). However, a reason for the polyphyly of the *Helianthemum* taxa could be that some very appealing and easily scored characters (i.e. indumentum) have been emphasized in the circumscription of taxa, although it has become increasingly clear that these characters do not correlate well with the overall morphology of the plants (Widén, in preparation).

Incongruence between the combined cpDNA sequences and cpDNA haplotypes

The recognized cpDNA haplotypes of *H. oelandicum* (Widén *et al.* submitted) were found to be polyphyletic in both the MP and BS analyses. Two of the partially sequenced cpDNA regions, *trnS-trnT* and *trnT-psbD* were also analysed in Widén *et al.* (submitted) for haplotype designation. When I performed phylogenetic analysis separately on each cpDNA region, I found that the *trnS-trnT* and *trnT-psbD* gave rise to relatively well structured tree topologies and that some of the similar haplotypes were clustered together. However, the phylogenetic analyses based on the *trnL-trnF* and *trnL* intron regions gave rise to less well structured trees with an assemblage of divergent cpDNA haplotypes in one clade, probably due to low sequence variation. Accordingly, the phylogenetic analyses of the various regions in the chloroplast genome may give rise to different gene trees and thus the inference about the chloroplast history may be difficult. The difference in phylogenetic signal between cpDNA regions can be explained by difference in mutation rate, as some regions evolve faster than other regions.

Congruence between ITS and chloroplast DNA sequences?

Sequence divergence and potentially informative characters varied slightly between the ITS and combined cpDNA regions. The proportion of parsimony informative characters (8%) in the ITS was almost the same as that of the cpDNA (8.2%). The ITS sequences displayed less homoplasy compared to cpDNA sequences, despite the fact that the evolution rate for the ITS is higher than cpDNA (Baldwin *et al.* 1995).

There are some conflicts between the ITS and cpDNA phylogenetic trees but the high number of collapsed branches and the polyphyletic grouping of the five subspecies and the two closely related species is predominant in both data sets. For example, the two well supported subclades of subsp. *incanum* (one clade with 76% BS/1.0 PP and one clade with 78% BS/1.0 PP) in the ITS are not supported in the cpDNA trees. There is a strong support for the two Turkish subsp. *incanum* and for the two Swedish varieties (var. *oelandicum* and var. *canescens*) in the cpDNA but not in the ITS phylogeny. Several studies have reported incongruence or conflicts between plastid and nuclear based phylogenies in several plants including *Helianthus* (Rieseberg 1991), *Gossypium* (Wendel *et al.* 1991), *Populus* (Smith & Sytsma 1990) and *Heuchera* (Soltis *et al.* 1991). Such incongruence is probably due to the occurrence of homoplasy as repeated mutations evolve independently in different gene regions.

Taxonomic implications

Morphological characters traditionally used to distinguish the five subspecies within *H. oelandicum* complex have focused primarily on the presence/absence of indumentum (i.e. stellate hairs or bristles), size and shape of leaves and flowers, and growth form. Our molecular data do not support the taxonomic designation (i.e. monophyly) of the subspecies as in traditional treatments where certain morphological characters based on indumentum have been overstated. These morphological characters are suggested to have simple genetic backgrounds (B. Widén, in preparation), and with genetic heterozygosity maintained all over the distribution area of the complex, certain morphs recognized as subspecies may have evolved recurrently, and continue to do so, in different regions (cf. Widén *et al.* submitted).

Remarkably, the ITS and cpDNA phylogeny do not support the treatment of *H. hymettium* and *H. marifolium* as closely related species to *H. oelandicum* but rather as members of the complex. *H. marifolium* has recently been shown to be closely related to *H. oelandicum* (Guzmán & Vargas 2009) and it shares the same chloroplast haplotype as *H. oelandicum* from the same geographical area (Widén *et al.* submitted). However, there are no indications that *H. hymettium* should be a member of the same species complex as *H. oelandicum*. The clustering of *H. hymettium* and *H. marifolium* with *H. oelandicum* is a strong indication that the phylogenetic signals obtained from the sequences used in this study is too weak to be used in any reconstruction of intraspecific phylogeny of *H. oelandicum*.

Concluding remarks

The high number of unresolved internal nodes, and the level of incongruence between the ITS and cpDNA trees do not support the subdivision of *H. oelandicum* into clear subspecies. Instead, the species is probably of recent origin and therefore the genetic differentiation within the species has not yet reached the level that gives a clear phylogenetic signal.

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