

Genetic and morphological differentiation across a contact zone between two postglacial immigration lineages of *Helianthemum nummularium* (Cistaceae) in southern Scandinavia

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Abstract *Helianthemum nummularium* (L.) Mill. has two subspecies in southern Scandinavia, which differ in an indumentum character that is inherited as a Mendelian gene with two alleles. The dominant allele is the genetic basis for the key character of subsp. *obscurum* (absence of a dense felt of stellate hairs on the abaxial surface of the leaf), whereas the recessive allele is the genetic basis for the key character of subsp. *nummularium* (presence of a dense felt of stellate hairs). Subsp. *obscurum* occurs in southwest Scandinavia, whereas subsp. *nummularium* occurs in southeast Scandinavia. The distributions of the two subspecies overlap in a wide zone in south Sweden, where natural populations consist of a mixture of the two subspecies in various proportions. Eighty-five percent of the investigated populations in this zone are polymorphic. A significant positive correlation exists between latitude/longitude across the zone and the frequency of the recessive indumentum allele. There is no significant difference in the leaf shape between the two subspecies, but there is a geographical trend across the zone. Twenty-seven populations displayed six plastid 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 postglacial immigration lineage from southwest.

Keywords *Helianthemum* · Hybrid zone · Plastid DNA · Indumentum · Allele frequency · Shape analysis

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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, 2001).

Patterns and levels of genetic diversity in plants allow for detection and estimation of past and present evolutionary processes, shaping the present genetic structure. Historical events (i.e. during the Pleistocene), the species' ecological requirements (i.e. selection pressure) and life history traits are responsible for shaping the genetic variation in species. Interaction between different evolutionary processes such as genetic drift and gene flow also accounts 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 populations and differentiation between populations (Slatkin 1987; Hamrick and Godt 1990).

Traditionally, taxonomy has been based on morphological characters supported by ecological factors of the species. Gottlieb (1984) pointed out that simple morphological differences 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. Westerberg 1992; Kärkkäinen and Ågren 2002; Kivmäki 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 and 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 dog roses (*Rosa* sect. *Caninae*). Lönn and Prentice (1995) could separate between regional populations of *Hippocrepis emerus* (Leguminosae) based on leaf shape variation pattern.

The objective of this paper was to use a polymorphic species for studies of genetic and morphological differentiation across a contact zone between two postglacial lineages in southern Scandinavia. *Helianthemum nummularium* shows great morphological variation in leaf shape (length and width) and in indumentum characters (Janchen 1909; Soubani et al. 2014; Widén 2010; Widén unpubl. data). The species reached southern Scandinavia via two migration routes, one from southwest and one from east (Soubani et al. 2014; Volkova P. A. unpubl. data).

Materials and methods

The species

Helianthemum nummularium (L.) Mill. is an evergreen, dwarf shrub that belongs to Cistaceae. The species is outbreeding (Widén unpubl. data), insect pollinated (Proctor 1956) and produces racemes of yellow (rarely pink) flowers in early summer. It favours dry, sunny sites, open grasslands and meadows. It is decreasing in Scandinavia due to changes in 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* (Celak.) Holub. The subspecies *obscurum* has a southwestern distribution in Scandinavia (Denmark, the Swedish provinces: Skåne, western Blekinge and central Småland), while subsp. *nummularium* has a more south eastern distribution in Scandinavia and is distributed as far north as the Swedish province of Uppland and southern Finland. The two subspecies often occur in mixed populations in a wide zone in southern Sweden (Edqvist and Karlsson 2007; Widén 2010; Widén unpubl. data).

Using microsatellites, Soubani et al. (2014) identified 18 plastid haplotypes that show geographical structuring across the west and south European range of the distribution area of *H. nummularium*. The mosaic distribution of plastid haplotypes in south and central Europe indicates a complex glacial and post-glacial migration history of the species. Some unique haplotypes restricted to the Baltic area indicated an eastern postglacial migration route to Scandinavia, whereas other haplotypes indicated another migration route reaching Denmark from southwest. Based on geographically wider samples of the species, this general view of the phylogeography of *H. nummularium* has recently been confirmed by Volkova P. A. (unpubl. data), using sequences of the *rsp 16-trnk* chloroplast intergenic spacer.

The only diagnostic character distinguishing between subsp. *obscurum* and subsp. *nummularium* is the absence/presence of a dense cover of stellate hairs on the abaxial surface of the leaf. However, there are no correlation between taxonomic subdivision and distribution of plastid haplotypes within *H. nummularium* (Soubani et al. 2014; Volkova P. A. unpubl. data). Widén (unpubl. data) has recently shown that the key character distinguishing subsp. *obscurum* and subsp. *nummularium* is inherited as a Mendelian gene with a dominant allele for subsp. *obscurum* (absence of a dense cover of stellate hairs) and a recessive allele for the key character of subsp. *nummularium* (presence of a dense cover of stellate hairs). Subspecies *obscurum* usually has a variable number of single, large stellate hairs on the abaxial surface of the leaves. The most hairy of these plants, which also have a dense cover of bristles on leaves and other parts, are sometimes reported as intermediates between the two subspecies (cf. Azzouzi et al. 1997; Widén 2010). However, common garden experiments have shown them to be extreme morphs of subsp. *obscurum* (Widén unpubl. data).

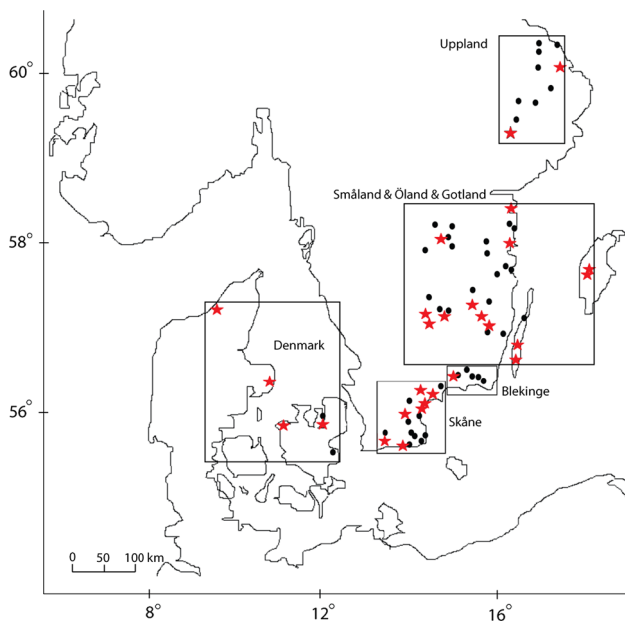


Fig. 1 Distribution of the 72 sampled populations of *H. nummularium* from Denmark and southern Sweden included in the study. Stars denote populations sampled for plastid DNA microsatellite analysis

Plant material

The material for the present study was collected from five regions in southern Scandinavia: Denmark (the provinces of Jylland and Sjælland), Skåne, Blekinge, Småland (including Öland, Gotland, Östergötland) and Uppland in Sweden. Based on the data obtained from recent floristic surveys (Fröberg 2006; Edqvist and Karlsson 2007; Tyler et al. 2007), a representative collection of 72 sites with subsp. *nummularium* and/or subsp. *obscurum* was visited (Fig. 1). A random sample of individuals was collected from each site (for details see Widén unpubl. data), the distance between samples was usually between 1 and 10 m, giving rise to sample sizes ranging from 5 to 39 individuals per site (see Table 1). Each individual 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 was sown in a separate pot for each sample in the experimental garden at Lund University. A total of 82 plants representing 27 sites (average $n = 3$ individuals/site) were surveyed for plastid DNA polymorphism.

Allele frequencies for the key character

Based on the frequency of plants of subsp. *nummularium* in natural populations, we calculated the frequency of the

recessive indumentum allele in each population. To test for significant differences in allele frequencies within populations between regions, a Kruskal–Wallis test was performed in SPSS 20.0. Also, a linear regression test in SPSS 20.0 was performed between latitude/longitude and the frequency of the recessive allele for subsp. *nummularium*.

Leaf shape

Two opposite leaves from a shoot were rotated to make the apices point in 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 of the leaf (Dudani et al. 1977; Rohlf and Archie 1984; White et al. 1988). The data obtained in the moment invariants analysis were subjected to canonical variate analyses (CVA, Dunn and Everitt 1982), where the variation in leaf shape was partitioned into different hierarchical components. 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 $1 - \Lambda_{\text{REG}}$, the proportion of diversity between populations within regions as $\Lambda_{\text{REG}} - \Lambda_{\text{POP}}$ and between individuals within populations as $\Lambda_{\text{POP}} - \Lambda_{\text{IND}}$ (Runyeon and Prentice 1997; Rosquist and 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 20.0) were done between (1) latitude and CV1 across the overlapping distributions of the two subspecies and (2) latitude and the CV1 including all populations.

Plastid microsatellites and genetic analysis

DNA extraction and PCR procedure followed Soubani et al. (2014). Alleles of three plastid DNA loci in *trnL-trnF*, *trnL5-trnL3* and *trnS-trnG* were detected and all were treated as ordered characters assuming that mutations primarily follow a stepwise mutation model (Ohta and Kimura 1973).

Table 1 Population code (pop. code), locality, country, coordinates [Swedish grid (SG) x , y], frequency of the *num*-allele (the recessive allele determining the presence of a dense felt of stellate hairs on the lower side of the leaves) per population, plastid DNA haplotype (the number of individuals is indicated before the haplotype code) and number of individuals (N) per population used for morphometric analysis

Pop. code	Locality	Country	SG- x	SG- y	Frequency of <i>num</i> -allele	Haplotype	N
19	Jylland, Glatved Strand	Denmark	11.88	62.50	0	3 H4	30
12	Jylland, Sebbesund	Denmark	11.08	63.00	0	2 H3, 1 H5	6
20	Sjælland, Køge	Denmark	12.68	61.55	0		17
17	Sjælland, Skibby	Denmark	12.60	61.80	0	3 H1	35
18	Sjælland, Skuldelev	Denmark	12.60	61.85	0		14
26	Sjælland, Vesterlynge	Denmark	12.16	61.80	0	2 H3, 1 H6	31
105	Skåne, Benestad	Sweden	13.79	61.56	0.894	3 H2	30
125	Skåne, Brantevik	Sweden	14.08	61.56	0.267		14
107	Skåne, Everöd	Sweden	13.99	61.90	0.717		37
109	Skåne, Genarp	Sweden	13.46	61.62	0.707		12
102	Skåne, Gislöv	Sweden	14.03	61.52	0.640		22
111	Skåne, Grödbby	Sweden	14.19	62.18	0.577	4 H2	39
104	Skåne, Kverrestad	Sweden	13.86	61.56	0.609		35
128	Skåne, Lyngsjö	Sweden	13.91	62.03	0.986	3 H2	36
110	Skåne, Lämmeströ	Sweden	13.49	61.54	0.688	1 H2, 2 H5	19
114	Skåne, Oderberga	Sweden	13.98	62.23	0		22
127	Skåne, Onslunda	Sweden	13.89	61.66	0.480		13
126	Skåne, Snoggedal	Sweden	13.92	61.64	0		6
103	Skåne, Tosteberga	Sweden	14.16	62.10	0.461		33
106	Skåne, Åby	Sweden	14.18	62.14	0.707	1 H2, 2 H5	16
134	Blekinge, Askaremåla	Sweden	14.46	62.44	0.535		7
132	Blekinge, Eringsboda	Sweden	14.72	62.56	0	2 H3, 1 H6	9
121	Blekinge, Flyerud	Sweden	15.04	62.43	1		26
101	Blekinge, Flymen	Sweden	14.96	62.46	0.825		25
133	Blekinge, Makrilla	Sweden	14.68	62.57	0.707		8
120	Blekinge, Rödeby	Sweden	14.90	62.47	0.796		30
113	Blekinge, Vilshult	Sweden	14.17	62.46	0.183	3 H5	30
131	Blekinge, Åbyholm	Sweden	14.87	62.57	0.707		6
112	Blekinge, Åkeholm	Sweden	14.34	62.40	0.180	3 H2	31
118	Öland, Alburna	Sweden	15.39	62.44	1	1 H5, 2 H6	31
211	Öland, Djupvik	Sweden	15.60	63.20	1		30
117	Öland, Resmo	Sweden	15.43	62.67	1	2 H5, 1 H6	30
1087	Gothem, Ekstakusten	Sweden	16.55	63.48	1	3 H6	0
2078	Gothem, Ekstakusten	Sweden	16.55	63.48	1	1 H3, 2 H6	0
119	Småland, Adelöv	Sweden	14.32	64.32	0.968		32
10	Småland, Boanäs	Sweden	14.29	63.34	0.569	3 H2	34
9	Småland, Braås	Sweden	14.55	63.27	0.586		32
23	Småland, Bredestad	Sweden	14.47	64.10	0.983		30
4	Småland, Fagerhult	Sweden	14.87	63.34	0.935	2 H5, 1 H6	24
25	Småland, Forserum	Sweden	14.22	63.98	0.983		30
11	Småland, Hågeryd	Sweden	14.32	63.14	0	3 H5	5
2	Småland, Högsby	Sweden	15.12	63.4	1		27
8	Småland, Klavreström	Sweden	14.58	63.33	0.658	3 H3	30
116	Småland, Kulla	Sweden	15.03	63.00	0.933	1 H2, 1H3, 1 H5	31
115	Småland, Köpstad	Sweden	15.03	62.97	0.894		30

Table 1 continued

Pop. code	Locality	Country	SG-x	SG-y	Frequency of <i>num</i> -allele	Haplotype	<i>N</i>
14	Småland, Loftahammar	Sweden	15.5	64.26	1		12
123	Småland, Mada	Sweden	14.52	64.29	0.861		31
24	Småland, Marbäck I	Sweden	14.45	64.18	0.913	3 H6	30
22	Småland, Marbäck II	Sweden	14.43	64.16	0.730		30
1	Småland, Nottebäck	Sweden	14.64	63.29	0.858		19
13	Småland, Nässja	Sweden	14.37	63.49	0.913		12
21	Småland, Smedby	Sweden	15.23	62.82	1		26
129	Småland, Spångenäs	Sweden	15.16	63.85	1		32
122	Småland, Södra Vi	Sweden	15.00	64.10	1		31
108	Småland, Vena	Sweden	15.07	63.78	1		31
124	Småland, Vimmerby	Sweden	15.00	64.00	1		22
7	Småland, Virserum	Sweden	14.87	63.57	1		27
130	Småland, Vånghult	Sweden	15.23	63.82	1		30
16	Småland, Västerik	Sweden	15.45	64.08	1	2 H6	30
15	Småland, Västra Ed	Sweden	15.44	64.30	1		30
3	Småland, Älghult	Sweden	14.97	63.17	0.816	2 H5, 1 H6	30
6	Östergötaland, Tryserum	Sweden	15.47	64.48	1	3 H6	24
204	Uppland, Berkinge	Sweden	16.22	66.95	1		5
207	Uppland, Enhälja	Sweden	15.81	66.09	1		17
203	Uppland, Halvbygdä	Sweden	16.17	66.72	1		25
206	Uppland, Husby	Sweden	15.76	66.05	1	3 H3, 1 H5	17
205	Uppland, Knivsta	Sweden	16.1	66.26	1		25
209	Uppland, Knutby	Sweden	16.3	66.54	1		26
210	Uppland, Långalma	Sweden	16.43	66.95	1		25
202	Uppland, Svinnö	Sweden	16.43	66.77	1	2 H5, 1 H6	25
208	Uppland, Säva	Sweden	15.88	66.28	1		25
201	Uppland, Vigelsbo äng	Sweden	16.2	66.88	1		30

A hierarchical analysis of molecular variance (AMOVA) was performed to describe the partitioning of genetic diversity between and within populations. The calculation of F_{ST} was based on sum of squared distances between pairs of haplotypes (R_{ST} -like comparisons; Slatkin 1995), and significance levels were tested with 10,000 permutations. Calculations were performed in Arlequin version 3.1 (Excoffier et al. 2005). The Arlequin programme was also used to calculate a minimum spanning network (MSN) between haplotypes.

To test for the presence of a phylogeographic structure within species, G_{ST} (which does not take numbers of mutations between haplotypes into account) and N_{ST} (which includes numbers of steps between haplotypes) were calculated according to Pons and Petit (1996), assuming that closely related haplotypes are more often found in the same geographic area (Pons and Petit 1996). To determine whether N_{ST} was significantly larger than G_{ST} , 10,000 permutations according to Burban et al. (1999)

were carried out in the software PERMUT 2 (<http://www.pierroton.inra.fr/genetics/labo/software>).

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

Results

Frequency of the recessive indumentum allele

The frequency of the recessive allele for presence of a dense felt of stellate hairs on the abaxial side of leaves in natural populations varied from 0.0 to 1.0 (Table 1), the mean allele frequency being 0.0 in Denmark, 0.552 in Skåne, 0.548 in Blekinge, 0.897 in Småland and 1.0 in Uppland. The frequency differs significantly between the five regions (Kruskal–Wallis test, $p < 0.001$). Accordingly,

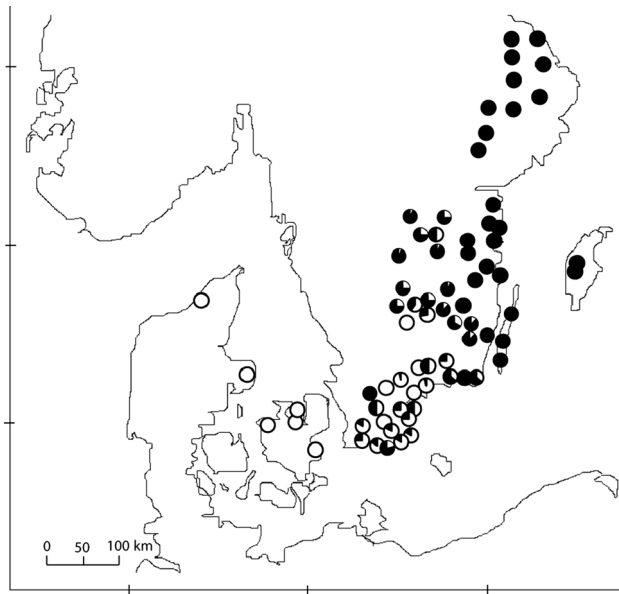


Fig. 2 Distribution of the two morphological subspecies recognized in *H. nummularium* in southern Scandinavia. Each population is represented by a pie diagram in which the relative frequency of subsp. *nummularium* is given by the black sector and subsp. *obscurum* by the white sector

our sampled populations consist of 100 % subsp. *obscurum* in Denmark and 100 % subsp. *nummularium* in Uppland, whereas the proportion of the two subspecies varies in Skåne, Blekinge and Småland. A narrow zone in Småland along the coast of the Baltic Sea is occupied exclusively by subsp. *nummularium* (Fig. 2). Between this coastal area and Denmark is a wide zone of populations with various proportions of the two subspecies (Fig. 2). Very few populations in this zone are monomorphic (<15 % of the investigated populations in the zone). For instance only four, relatively small monomorphic populations of the *obscurum* subspecies are found in Skåne, Blekinge and Småland (see Table 1). We found a significant correlation ($p < 0.001$) between the frequency of the recessive allele and latitude ($r = 0.56$) and longitude ($r = 0.75$) based on all populations. The correlations are still significant if only populations in the zone in mainland Sweden (Småland, Blekinge and Skåne) are compared.

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 %).

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. 3. No clear difference between the five regions can be found. A significant correlation ($r = 0.459$,

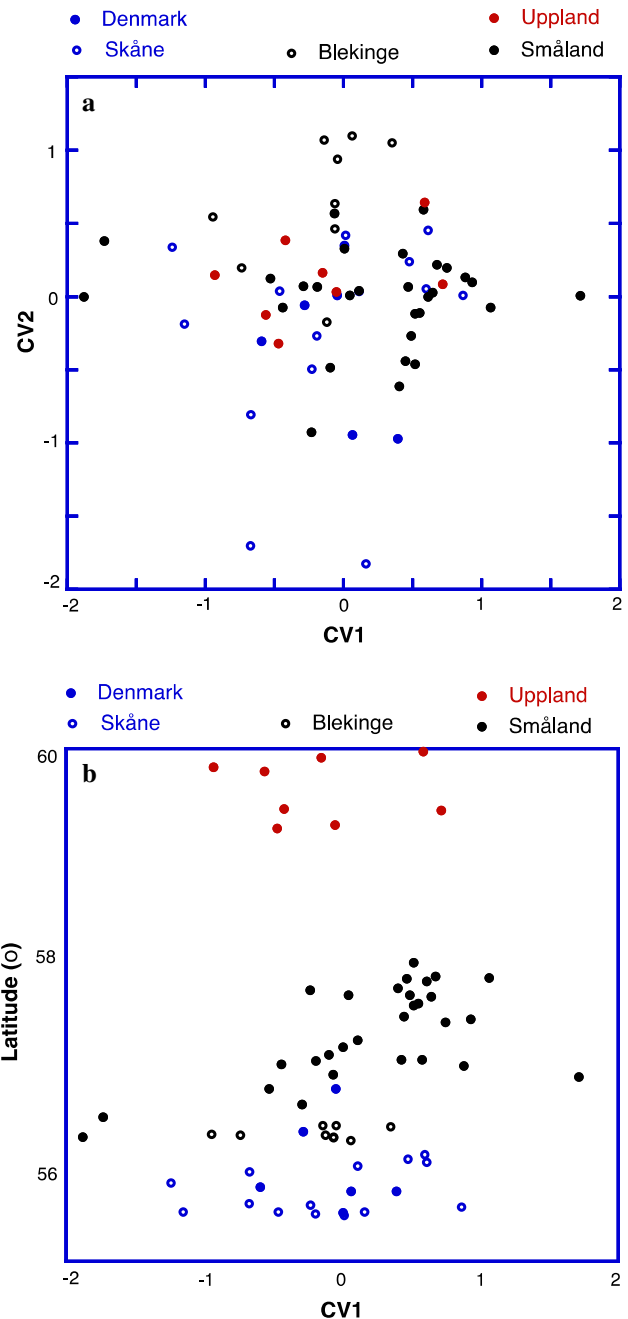


Fig. 3 **a** Plot of the first two canonical variates of a CVA of leaf shape parameters (moment invariants) in *H. nummularium* in southern Scandinavia. Leaf shape data were grouped by population in the analysis. Regional origins of the samples are indicated by different symbols. **b** Plot of the first canonical variate from the CVA vs. latitude (°)

$p < 0.001$) 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 the province of Uppland) were included ($r = 0.182$, $p = 0.141$) (Fig. 3).

Table 2 Plastid haplotypes recorded in *H. nummularium* in Scandinavia

Haplotypes	<i>mtrnSf-trnGr</i>	<i>mtrnL2-trnF</i>	<i>mtrnL5-trnL3</i>	<i>N</i>	<i>nummularium</i>	<i>obscurum</i>
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

Allelic composition, total numbers of plants (*N*) and numbers of plants per subspecies is given for each haplotype

Plastid DNA polymorphism

Each of the three plastid DNA microsatellite loci yielded two alleles per locus among 82 individuals of subsp. *nummularium* and *obscurum*. The allelic combinations produced six haplotypes (see Table 2).

The minimum spanning network (MSN) (Fig. 4a) summarizes the genetic relationships between the six plastid DNA haplotypes. The connected haplotypes are separated by only one mutation length. H2 and H5 are centrally localized 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 plastid DNA haplotypes overlapped, but showed some geographic structuring in Scandinavia (Fig. 4b, c). 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 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 each and were restricted to Denmark.

Approximately, sixty-six percent of the total plastid DNA haplotype diversity was distributed between populations ($F_{ST} = 0.657$) and the remaining was found within populations (see Table 3). The test for the presence of a phylogeographic structure within *H. nummularium* in Scandinavia was significant ($p = < 0.05$); N_{ST} (0.667) was significantly higher than G_{ST} (0.589).

Using Dixon's (2006) method, the probability of plastid DNA 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 haplotype, 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 plastid DNA 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 between the two subspecies (Table 2; Fig. 4a).

Discussion

Plastid DNA distribution and diversity

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 indicates occurrence of gene flow between populations in the contact zone (southern Sweden). According to the parameters N_{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 are also closely related and have similar geographic distributions in southern Scandinavia.

In a wide-scale study (Soubani et al. 2014), done on the *H. nummularium* complex in Europe, a total of 18 haplotypes were identified based on plastid DNA 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 southeastern 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

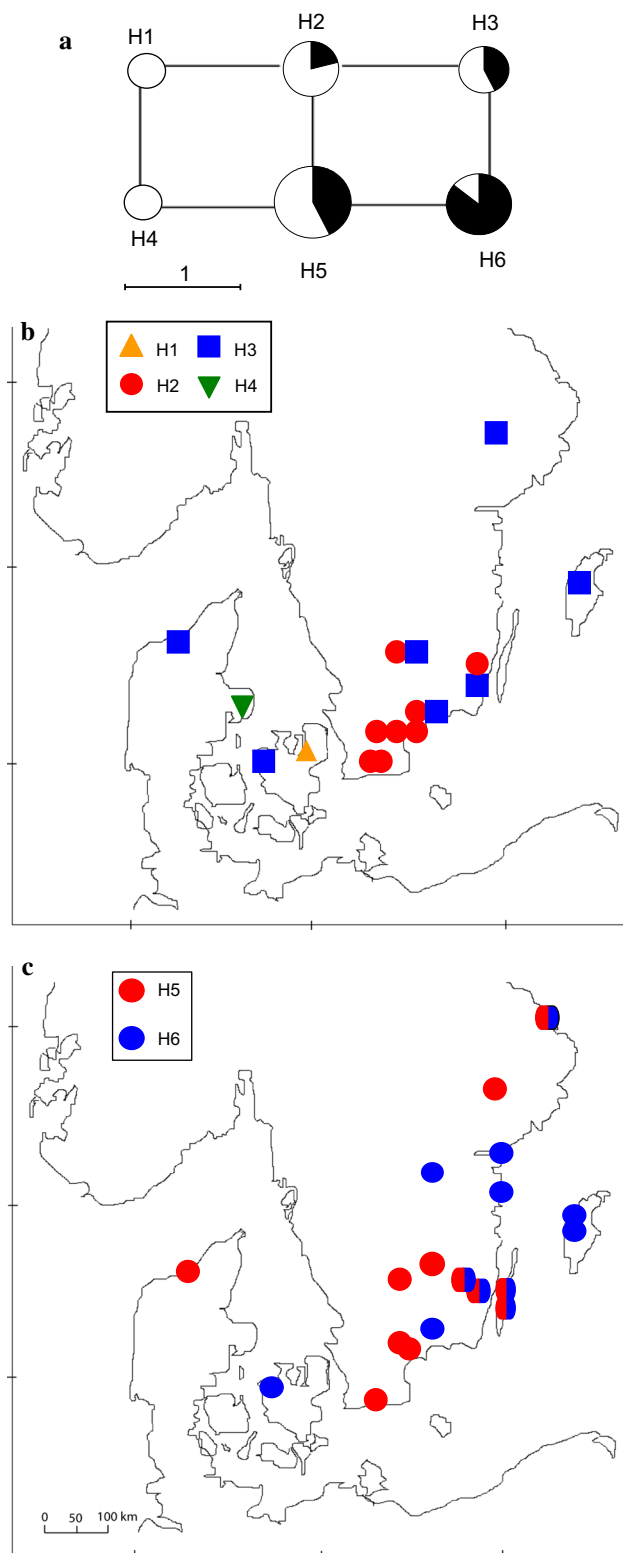


Fig. 4 a Minimum spanning network showing the genetic relationships between the six Scandinavian plastid DNA haplotypes and taxon composition for each haplotype. Each linked pair of haplotypes differs by a single mutation. The size of the pie diagrams is proportional to the frequency of haplotypes in the studied samples. White sectors denote the frequency of subsp. *obscurum* and black sectors denote the frequency of subsp. *nummularium*. b, c The geographic distribution of the six plastid DNA haplotypes of *H. nummularium* present in Denmark and southern Sweden

Table 3 Partitioning of genetic diversity as given by plastid haplotype variation (AMOVA; R_{ST} -like comparisons) in *H. nummularium* in Scandinavia

Source of variation	df	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	

* $p < 0.0001$

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 southwestern immigration route into western Scandinavia.

In a phylogeographic study based on analysis of *rps16-trnK* chloroplast intergenic spacer sequences comprising the whole geographical range of *H. nummularium*, Volkova P. A. (unpubl. data) found one haplotype C with a wide distribution in eastern Europe and reaching southern Scandinavia and central Europe. The geographical distribution in Continental Europe of our H2 and H5 coincided with the distribution of haplotype C (cf. Soubani et al. 2014). Haplotype D (Volkova P. A. unpubl. data), with a central European distribution reaching Denmark, overlapped the distribution of our H1 and H4.

Characterization of indumentum and leaf shape variation pattern

The distribution of the two subspecies shows a distinct geographical pattern in Scandinavia (Fröberg 2006; Edqvist and Karlsson 2007; Tyler et al. 2007; Widén 2010). 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. Subsp. *obscurum*; on the other hand, has a southwestern distribution. In southern Sweden, a wide zone exists with mixed populations consisting of both subspecies. Thus,

reported such cases where unique haplotypes were confined to northern Europe, e.g. *Alnus glutinosa* (King and Ferris 2000) and *Silene dioica* (Hathaway et al. 2009). H2 is the third most frequent haplotype, found in Sweden, and in

natural populations of *H. nummularium* in this zone differ in frequency of alleles controlling the character absence/presence of a dense felt of stellate hairs on the abaxial surface of the leaves. Even in almost pure populations of one of the subspecies in the 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 zone (i.e. Gotland and Östergötland) (Widén 2010; see also Proctor 1956 for the same phenomenon in areas outside Scandinavia).

Two scenarios may explain the geographical distribution of the two subspecies in Scandinavia. The least plausible of these scenarios may involve a postglacial colonization of a polymorphic lineage (in indumentum) that was subjected to diversifying selection during range expansion. The distribution of subsp. *obscurum* suggests that natural selection may favour the allele coding for the absence of a felt of stellate hairs in the wetter and oceanic climate in southwest Scandinavia. 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 allele frequencies coding for indumentum (Proctor 1956). Additionally, subsp. *obscurum* does not occur along the Swedish southwest coast, which has a more oceanic climate than further to the east in southern Sweden. The only study known to us that tested adaptation to different substrates in *H. nummularium* did not find any correlation between subspecies and calcicolous or siliceous substrates (Azzouzi et al. 1997).

The scenario of a polymorphic postglacial migration lineage does not explain the geographical structuring of plastid haplotypes. In our view, 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 and Prentice 1996; Nordström and Hedrén 2008). The two lineages differed in allele frequency for the indumentum character, the southern lineage without, and the eastern lineage with, the allele coding for a dense felt of stellate hairs on the abaxial side of the leaf. Gene flow between the two lineages in the zone in southern Scandinavia (cf. Widén unpubl. data) 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. The existence of a correlation between leaf shape and latitude across the contact

zone may indicate that introgression between the two migration lineages is still going on. However, 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* (cf. Soubani et al. 2014).

The distribution of the two subspecies and plastid haplotypes in the contact zone in southern Scandinavia does not coincide with each other. Migration of H1 and H4 into the 'suture zone' in southern Scandinavia was probably prevented by pre-colonization and establishment by other haplotypes (H2, H3, H5 and H6). Early gene flow between the two migration lineages and 'chloroplast capture' may have created the lack of correlation between morphology and plastid haplotypes. However, the mechanisms behind this 'chloroplast capture' are unknown.

Conclusions

The geographical distribution of morphological variation and distribution of plastid DNA haplotypes in Scandinavian populations of *H. nummularium* indicate two postglacial immigration lineages, one of subsp. *obscurum* from west and one of subsp. *nummularium* from east. The distribution 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 southwestern postglacial immigration lineage into Scandinavia. In a wide contact or hybrid zone between the two lineages in south Sweden, gene flow have created a range of populations with a variable frequency of the two subspecies.

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