

Phylogeography of the European rock rose *Helianthemum nummularium* s.l. (Cistaceae): western richness and eastern poverty

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Abstract *Helianthemum nummularium* s.l. is a young, morphologically diverse species distributed from western Europe to the Caucasus and the Southern Urals in the east. We analysed the *rps16-trnK* plastid intergenic spacer sequences from 85 localities covering most of the range of *H. nummularium*. Thirteen haplotypes were very unevenly distributed throughout the range of the species, and exhibited a strong phylogeographic signal. The results confirm range expansions of *H. nummularium* from Mediterranean refugia northwards, but also show the major role of eastern European (the Caucasus and the Southern Urals) refugia in rapid postglacial colonization of east, north and central Europe. The plastid haplotypes form distinct clades, one representing an eastern European lineage with few haplotypes and the other representing a western European lineage with many haplotypes. Parallel

to this split in haplotype diversity is the pronounced differentiation in morphological variation displayed by the taxa found in west and east Europe. We discuss the role of topography in generating differences in morphological and genetic diversity between these two groups. We also discuss the taxonomical status of *Helianthemum arcticum*, which is regarded as an endangered local endemic of the Kola Peninsula. *Helianthemum arcticum* appears to represent an outlying peripheral population of *H. nummularium* preserved since the last postglacial major range expansion of this species, and bears the same plastid haplotype as the bulk of east and north European populations.

Keywords Endangered species · *Helianthemum* · Phylogeography · Peripheral populations · *rps16-trnK*

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Introduction

Local relief is one of the main factors shaping biodiversity. Mountain ranges are important for diversity and speciation in temperate regions in two ways; they can act as a barrier to species migration during Quaternary climate fluctuations (Taberlet et al. 1998), and they can serve as refugia due to their complex topography and varied habitat types (Tribsch and Schönswetter 2003). The mountain ranges in western Europe (the Alps and the Pyrenees) form so-called ‘suture zones’, where different genetic lineages come into contact and possibly hybridize during periods of favourable climate conditions (Hewitt 2000). The altitudinal distribution of mountain and alpine species fluctuated with the Quaternary climate oscillations, which caused repeated contraction and expansion that could enhance differentiation among mountains (Kropf et al. 2003; Ronikier et al. 2008a). Many studies have documented high genetic

differentiation of alpine species in western European mountain ranges (the Pyrenees, the Alps and the Carpathians) and surrounding areas (e.g. Stehlik et al. 2002; Tribsch and Schönswetter 2003; Ronikier et al. 2008a). The origin of this differentiation is often attributed to glacial refugia in ice-free areas in southern Europe (e.g. Iberian Peninsula, Italy and the Balkans), and to periglacial refugia north of the glaciated mountain areas of southern Europe (the Pyrenees, the Alps and the Carpathians) (Petit et al. 2003; Stewart and Lister 2001).

In contrast to western Europe, the relief of eastern Europe is more homogenous, with no prominent mountain ranges in its central part (the Caucasus and the Urals form the border of this region). The southern part of the East European plain was never glaciated during the late Quaternary climate fluctuations (Svendsen et al. 2004). Physical obstacles had less influence on range contractions and expansions of species during the climate oscillations than in western Europe. The risk of local refugia being locked in by mountain ranges during glacial maxima was low, so we would expect lower genetic differentiation between populations in eastern Europe than in western Europe. However, as far as we know, this has never been tested with appropriate sampling of plants from the entire distribution area (Hewitt 2004). Inclusion of samples from eastern Europe in phylogeographic studies is especially important, as this region was regarded as being an important source of genetic variation for plants in the western part of the continent (Taberlet et al. 1998). There have been few pan-European phylogeographic studies of plants, but all of them support the general idea of westward postglacial migrations (e.g. Palmé et al. 2003; Pyhäjärvi et al. 2008; Tollefsrud et al. 2009; Treier and Müller-Schärer 2011; Eidesen et al. 2013).

Helianthemum nummularium s.l. (Cistaceae) is a species aggregate that shows great morphological differentiation in Europe. Many taxa have been described on the basis of flower colour, petal and leaf size, and indumentum (Janchen 1909; Proctor and Heywood 1968). Most morphological variation is found in western Europe, displayed by a greater mosaic of taxa distributed in this area than in eastern Europe. This is in accordance with the greater impact by glaciation in western Europe during the Quaternary climate fluctuations (Svendsen et al. 2004). The phylogeography of the species in western Europe based on studies of plastid microsatellites has recently revealed genetic data that did not support the current taxonomical subdivision of the *H. nummularium* s.l. (Soubani et al. 2014). The most important key character (presence/absence of a dense cover of stellate hairs on the abaxial surface of the leaves) in the species complex turned out to be governed by a single Mendelian gene with two alleles (Widén 2015).

In this study, we extend the sampling of *H. nummularium* s.l. to comprise almost the entire species range of the yellow-flowered morphs in the species complex. We explore the phylogeography of the species based on plastid DNA sequences. We focus on the contrasts in genetic differentiation between the two regions, western and eastern Europe. We compared our results to the previous taxonomy of *H. nummularium* s.l. based on morphology and plastid microsatellites (Janchen 1909; Soubani et al. 2014). Based on our analysis of plastid DNA sequences in *H. nummularium* s.l., we can suggest scenarios of the biogeographical history of the species.

Materials and methods

Study model

Helianthemum Mill. is a young genus (Guzman and Vargas 2009), comprising several species complexes (Janchen 1907, 1909) that have not reached reproductive isolation (Widén 1986, 2015). *Helianthemum nummularium* s.l. is a European dwarf shrub characterized by $2n = 20$. The morphological variation in this species is complex, where the indumentum characters in particular have been considered to be taxonomically important, and the species has been divided into several taxa, most of them in western Europe (Janchen 1909; Proctor and Heywood 1968). According to the ‘Flora Europaea’ (Proctor and Heywood 1968), the *H. nummularium* s.l. consists of eight subspecies (sometimes regarded as separate species (e.g. *H. grandiflorum* (Scop.) Lam. et DC., *H. tomentosum* (Scop.) Spreng in Yuzepchuk 1974, see also “Appendix”), five of which belong to a yellow-flowered group. Additional taxa have been regarded as important local endemic morphs, e.g. *H. arcticum* (Grosser) Janch. in the Kola Peninsula (Belousova et al. 2008).

Helianthemum nummularium s.l. is distributed from the Iberian Peninsula, through central and southern Europe, northward to Scotland, Denmark, central Sweden and southern Finland; scattered populations are found in the Caucasus and central Russia, extending to the Urals in the east and to Lake Ladoga in the north (Proctor 1956; Vasari and Vasari 1999). The species is listed as rare or decreasing in a number in the Red Data Books of many regions of central Russia (Prisyazhnyuk 2009), East Fennoscandia (Kotiranta et al. 1998) and Sweden (Artdatabanken 2015). In addition, one highly isolated population of *H. nummularium* s.l. is located on the southern coast of the Kola Peninsula, more than 500 km away from the nearest conspecific locality. This large (hundreds of individuals along several kilometres of coastline) and stable (definitely existing for more than a century; Yuzepchuk 1974)

population is sometimes regarded as a separate and endangered endemic species (*H. arcticum*). It is included in the Red Data Book of the Russian Federation (Kostina 2008) and has been protected in the Kandalaksha State Nature Reserve since 1977.

The evolutionary history of this population is closely connected with the glacial history of the area, as the Kola Peninsula was ice-covered during the last (Weichselian) glacial maximum; the ice retreated from its southern shore as recently as approximately 13,000 years ago (Svendsen et al. 2004). The fossil pollen spectra suggest that *H. nummularium* s.l. was abundant in late-glacial European grasslands and virtually disappeared from the areas where post-glacial forests spread (e.g. Hirsch et al. 2015). Today, it is restricted to relatively small treeless refugia on base-rich rocks with thin soils (Proctor 1956, 1958; Vasari and Vasari 1999 and references therein).

According to Proctor and Heywood (1968), the two main subspecies of the yellow-flowered *H. nummularium* (L.) Mill. are subsp. *nummularium* and subsp. *obscurum* (Čelak.) Holub, with overlapping distributions; the latter is mainly found in western Europe, while the former covers the main distribution area of the species. *H. nummularium* subsp. *nummularium* is characterized by a dense cover of stellate hairs on the abaxial surface of the leaves, and subsp. *obscurum* has abaxial surfaces of the leaves without this key character. The two subspecies may occur together and, when they do, they cross freely; the diagnostic character—a dense cover of stellate hairs on the abaxial surface—is inherited as a Mendelian gene with a recessive allele for subsp. *nummularium* and a dominant allele for subsp. *obscurum* (Widén 2015). Besides the two main subspecies, the yellow-flowered part of the complex comprises three altitudinal subspecies mainly distributed in mountains of southern and central Europe (Proctor and Heywood 1968)—subsp. *tomentosum* (Scop.) Schinz & Thell., with large flowers and a dense cover of stellate hairs on the abaxial surface of the leaves, and two subspecies without the dense cover of stellate hairs—subsp. *glabrum* (Koch) Wilczek with more or less glabrous leaves and subsp. *grandiflorum* (Scop.) Schinz & Thell., with large flowers.

Recent studies (Soubani et al. 2014) have shown that the variation patterns of indumentum, along with the leaf and petal shape and plastid haplotype variability do not correlate with the subdivision of *H. nummularium* into subspecies. Morphological variation of *H. nummularium* s.l. has been shaped by hybridization, selection and by historical processes, such as range fluctuations during Pleistocene glaciations (Soubani et al. 2014). The species origin was attributed to the Mediterranean. The Alps and the surrounding areas constituted the centre of genetic diversity, and northern areas displayed lower diversity. The

main refugia were assumed to be located in central and southern Europe (around the Alps and the Balkans). There was a refugium in eastern Europe, and an assumed partial colonization of Scandinavia from it, but this hypothesis could not be tested in that study because of the absence of samples from the eastern part of the continent (Soubani et al. 2014).

In the present study, we analysed five taxa of *H. nummularium* from western Europe (all yellow-flowered morphs in Proctor and Heywood 1968) and four taxa from eastern Europe [subsp. *nummularium*, subsp. *obscurum*, subsp. *glabrum* (= *H. nitidum* Clem.) and *H. arcticum*]. We added the endemic morph *H. arcticum*, even though the species status of *H. arcticum* seems to be doubtful, since it differs from *H. nummularium* s.s. only by reduced indumentum of leaves, peduncles and sepals (Yuzepchuk 1974).

Sampling

We used plant material of *H. nummularium* s.l. collected in the field or cultivated in a greenhouse at Lund University from seeds collected in the field. All the populations sampled are listed in Table 1. Plants were determined to subspecies level, according to Proctor and Heywood (1968) (Table 1 in Soubani et al. 2014). We analysed one plant per population except for *H. arcticum*, where we sampled three plants from different parts of its population (indicated as 6, 11 and 12 in Table 1; the taxonomical status and morphological variation of this morph were one of the original foci of the study). Leaf samples from all the plants were dried in silica gel prior to DNA analysis, and one plant per population was pressed as a voucher. Voucher specimens are deposited at the Herbaria of Moscow State University (MW), Russia and Lund University (LD), Sweden. In this study, we also included additional specimens of *H. nummularium* from the Herbarium of the Main Botanical Garden in Moscow (MHA), Russia. Altogether we sampled 85 localities (Table 1), covering almost the entire range of *H. nummularium* (Proctor 1956), and including six taxa (*H. nummularium* subspp. *nummularium*, *tomentosum*, *obscurum*, *grandiflorum*, *glabrum* and the putative species *H. arcticum*). One-third of the studied populations (Table 1) were also included in the study of plastid microsatellites in Soubani et al. (2014). Two herbarium samples of *H. ledifolium* (L.) Mill. from Azerbaijan were included in the study as an outgroup (following Guzman and Vargas 2009 and Parejo-Farnés et al. 2013).

DNA isolation and sequencing

DNA was extracted from dehydrated leaf material using the CTAB method (Doyle and Doyle 1987). We sequenced the chloroplast intergenic spacer *rps16-trnK* (Takahashi

Table 1 Geographic origin, plastid DNA haplotypes and subspecies treatment for the investigated populations of *Helianthemum nummularium* s.l.

Pop. no.	Subspecies ^a	Region	Latitude (deg)	Longitude (deg)	Haplotype	Soubani
1	<i>glabrum</i>	Russia, Krasnodarsky kraj	43.85	39.73	A	
2	<i>nummularium</i>	Georgia	41.43	44.58	A	
3	<i>nummularium</i>	Azerbajdzhan	39.82	46.75	A	
6	<i>arcticum</i>	Russia, Murmansk region	66.53	34.50	C	
7	<i>nummularium</i>	Russia, Bryansk region	52.83	32.67	C	
9	<i>nummularium</i>	Russia, Bashkiria	53.87	58.27	A	
11	<i>arcticum</i>	Russia, Murmansk region	66.53	34.50	C	
12	<i>arcticum</i>	Russia, Murmansk region	66.53	34.50	C	
15	<i>nummularium</i>	Georgia	41.63	42.98	A	
17	<i>nummularium</i>	Daghestan	42.78	46.15	A	
19	<i>nummularium</i>	Southern Ossetiya	42.47	43.75	A	
20	<i>nummularium</i>	Russia, Stavropolskij kraj	43.48	43.08	A	
22	<i>nummularium</i>	Russia, Krasnodarsky kraj	44.78	37.73	A	
24	<i>nummularium</i>	Russia, Moscow region	55.03	38.88	C	
25	<i>nummularium</i>	Russia, Belgorod region	51.12	37.25	C	
26	<i>nummularium</i>	Russia, Belgorod region	50.99	37.59	C	
27	<i>nummularium</i>	Russia, Belgorod region	51.30	37.83	C	
28	<i>nummularium</i>	Russia, Lipetsk region	52.63	38.93	C	
31	<i>nummularium</i>	Russia, Bryansk region	52.42	34.65	C	
32	<i>nummularium</i>	Russia, Bryansk region	52.50	31.78	C	
33	<i>nummularium</i>	Russia, Bryansk region	52.92	33.40	C	
50	<i>obscurum</i>	Austria	47.92	15.78	C	
108	<i>nummularium</i>	Sweden	57.53	15.93	C	
112	<i>obscurum</i>	Sweden	56.28	14.73	C	H2
124	<i>nummularium</i>	Sweden	57.77	15.86	C	
128	<i>nummularium</i>	Sweden	55.94	14.06	C	H2
211	<i>nummularium</i>	Sweden	57.00	16.80	C	
665	<i>nummularium</i>	Greece	41.30	24.05	G	
667	<i>glabrum</i>	Greece	40.08	22.37	I	
669	<i>nummularium</i>	Greece	40.08	22.38	G	
670	<i>nummularium</i>	Greece	40.08	22.40	G	
1031	<i>grandiflorum</i>	Switzerland	46.68	7.83	B	H4, H12
1087	<i>nummularium</i>	Sweden, Gotland	57.15	18.00	C	H6
1091	<i>obscurum</i>	France	44.08	6.27	B	H14
1107	<i>obscurum</i>	Italy	44.50	8.07	B	H14
1108	<i>obscurum</i>	Italy	45.75	7.67	C	H5
2001	<i>nummularium</i>	Estonia	58.37	22.18	C	
2006	<i>glabrum</i>	Italy	46.45	10.40	B	H14
2011	<i>grandiflorum</i>	Italy	45.32	11.57	E	H4
2013	<i>obscurum</i>	France	48.10	3.77	B	H14
2014	<i>obscurum</i>	France	46.17	6.57	B	H14
2034	<i>nummularium</i>	Romania	46.15	23.55	C	H4
2045	<i>glabrum</i>	Slovakia	48.80	20.20	E	H4
2051	<i>obscurum</i>	Slovakia	48.67	19.67	D	H5
2052	<i>obscurum</i>	Germany	47.90	7.93	C	H5
2058	<i>nummularium</i>	Belgium	50.17	4.83	E	H13
2067	<i>obscurum</i>	Polen	54.08	23.07	C	H5
2070	<i>obscurum</i>	Austria	46.72	13.28	C	H5

Table 1 continued

Pop. no.	Subspecies ^a	Region	Latitude (deg)	Longitude (deg)	Haplotype	Soubani
2075	<i>obscurum</i>	Germany	51.63	11.55	D	H1
2087	<i>nummularium</i>	Bulgaria	42.00	25.00	F	H8
2088	<i>nummularium</i>	Bulgaria	42.95	23.10	G	H4
2093	<i>nummularium</i>	Estonia	58.75	22.50	C	
3020	<i>obscurum</i>	France	45.62	6.76	C	
3021	<i>tomentosum</i>	France	42.96	0.33	E	
3029	<i>obscurum</i>	Italy	42.11	14.06	H	
3040	<i>obscurum</i>	Austria	47.48	10.71	B	
3042	<i>nummularium</i>	Estonia	59.18	23.78	C	
3045	<i>obscurum</i>	France	48.73	6.19	D	
12sw	<i>obscurum</i>	Denmark	56.97	9.55	C	
16sw	<i>nummularium</i>	Sweden	57.77	16.56	C	
17sw	<i>obscurum</i>	Denmark	55.73	11.95	D	H1
19sw	<i>obscurum</i>	Denmark	56.30	10.82	D	H4
20sw	<i>obscurum</i>	Denmark	55.45	12.17	C	
26sw	<i>obscurum</i>	Denmark	55.72	11.27	C	H3, H6
AL	–	Austria	40.95	13.18	C	
IS	<i>nummularium</i>	Spain	42.75	-0.47	J	
it19	<i>obscurum</i>	Italy	42.04	14.08	H	
it22	<i>obscurum</i>	Italy	42.80	13.30	C	
it26	<i>obscurum</i>	Italy	42.53	13.07	C	
it32	<i>obscurum</i>	Italy	42.41	13.55	C	
it41	<i>obscurum</i>	Italy	43.70	12.66	C	
it53	<i>obscurum</i>	Italy	42.76	12.83	C	
it61	<i>obscurum</i>	Italy	45.78	10.86	K	
it63	<i>obscurum</i>	Italy	45.76	10.87	C	
it70	<i>nummularium</i>	Italy	46.28	11.80	E	
K1	<i>grandiflorum</i>	Croatia	44.98	14.96	C	
KOR	<i>nummularium</i>	Russia, Karelia	62.07	30.84	C	
KR	<i>glabrum</i>	Crimea	44.60	33.72	C	
NU1	<i>nummularium</i>	Russia, Bashkiriya	53.26	57.06	A	
RAD1	<i>nummularium</i>	Russia, Karelia	61.93	35.12	C	
REV	<i>nummularium</i>	Spain	42.60	0.13	E	
SHK	<i>nummularium</i>	Russia, Krasnodarsky kraj	44.32	38.71	A	
SV	<i>nummularium</i>	Spain	42.45	0.18	L	
UBT	–	Germany	49.95	11.65	M	
UL2	<i>nummularium</i>	Russia, Ulyanovsk region	53.05	47.37	A	

Soubani refers to plastid DNA microsatellites found in the same populations according to Soubani et al. (2014)

^a Two samples remained undetermined as to subspecies due to the absence of herbarium vouchers

et al. 2005). Polymerase chain reactions (PCR) were conducted in 20 µl reaction volumes containing 4 µl of Ready-to-Use PCR MaGMix (200 µM of each dNTP, 1.5 mM MgCl₂, 1.5 U SmarTaqDNA Polymerase and reaction buffer; Dialat Ltd., Moscow, Russia), 15 µl deionised water, 3.4 pmol of each primer and 1 µl of template DNA of unknown concentration. PCR cycling was performed

with a MJ Research PTC-220 DNA Engine Dyad Thermal Cycler (BioRad Laboratories, USA) with the following parameters: initial denaturation for 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 2 min at 72 °C, ending with 10 min extension at 72 °C. Double-stranded PCR products were checked on agarose gel and purified using centrifugation with a solution of

ammonium acetate in ethanol. Sequencing was performed in both directions using ABI PRISM BigDye[®] Terminator v 3.1 Kit (Applied Biosystems) according to the manufacturer's manual, except that we used 10 µl reaction volumes, and further analysis was carried out using 3130 and 3500 Genetic Analyzers (Applied Biosystems). The GenBank accession numbers of the sequences are JQ927012–JQ927212 (<http://www.ncbi.nlm.nih.gov/genbank/>) (cf. Online Resources 1, 2).

Data analyses

The sequences were aligned manually using BioEdit 7.0.5.3 (Hall 1999). Conventional phylogenetic methods may perform poorly in revealing intraspecific relationships (Jakob and Blattner 2006), so we carried out statistical parsimony analysis using the network algorithm described in Templeton et al. (1992), and implemented in the TCS v. 1.21 program (Clement et al. 2000). This method estimates an unrooted haplotype network and a 95 % plausible set of all haplotype lineages in that network. Since the alignment contained multiple gaps of various lengths, we performed separate analyses with and without gap data included. In the latter case, gaps were treated as missing data, otherwise they were coded using the simple method of indel coding (Simmons et al. 2001) as implemented in the GapCoder software (Young and Healy 2003).

Rooting of the network was performed using sequences of *H. ledifolium* as an outgroup. *H. ledifolium* has been shown to belong to a sister clade of the *H. nummularium* clade in two independent phylogenetic surveys of the genus *Helianthemum* and other Cistaceae (Guzman and Vargas 2009; Parejo-Farnés et al. 2013). We performed two separate analyses using the NJ algorithm as implemented in the SplitsTree4 ver. 4.9.1 program (Huson and Bryant 2006), and independently using the ML analysis as implemented in the Treefinder program (Jobb 2011). In the latter case, a substitution model was assessed by means of the same program.

AMOVA analyses were calculated in Arlequin 3.1 (Excoffier et al. 2005). Geographic groups were defined in terms of three major haplotype lineages revealed by the statistical parsimony analysis.

Correspondence between subspecies identity of *H. nummularium* s.l. and plastid DNA haplotype was tested with Pearson's Chi-square test with simulated *p* value, appropriate for correspondence tables with many zeroes, as in our case, *p* value was calculated by Monte Carlo simulation based on 2000 replicates. The test was performed in statistical environment R 2.9.2 (R Development Core Team 2011).

Results

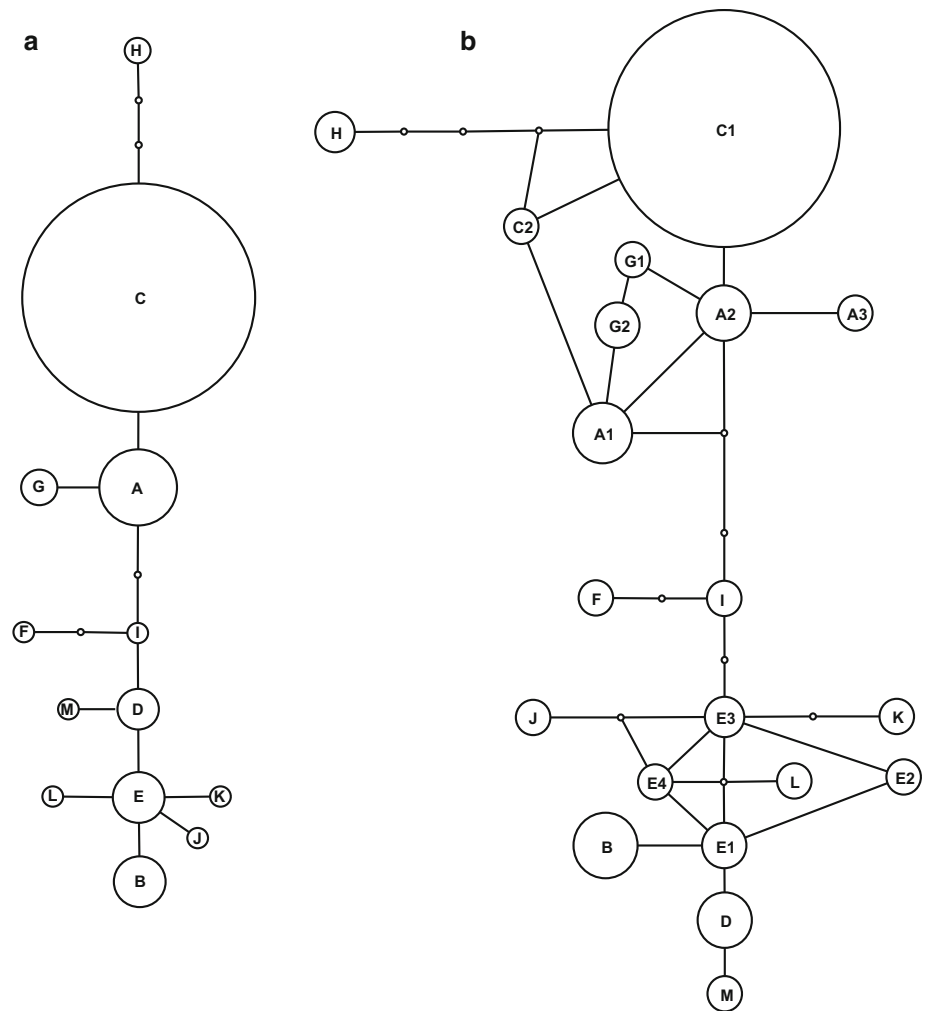
The *rps16-trnK* sequences were 570–699 positions long. The alignment was 809 positions long and contained seven parsimony informative sites and six singletons within the ingroup. The outgroup differed from the ingroup by mutations in 13 sites. The alignment included ten gaps 1–217 positions long, two of them in poly-A and poly-T sequences. For the purpose of the analysis they were coded using simple indel coding method in the GapCoder program as 17 indels.

Analysis of the *rps16-trnK* alignment in TCS without outgroup, with indels treated as missing data, revealed 17 haplotypes, four of which were not in the data and represented hypothetical extinct haplotypes. The TCS program calculated the 95 % limit of parsimony of 13 mutational steps, and connected all the haplotypes into a single network (Fig. 1a). The outgroup was too distant genetically to be included in the network (not shown). When indels were included in the analysis, the program revealed 30 haplotypes, ten of which were not in the data and represented hypothetical extinct haplotypes. In this case, the haplotypes A, C, E, and G revealed in the previous analysis were split into 2–4 closely related haplotypes. However, the analysis introduced eight loops into the network caused by homoplasy (Crandall and Templeton 1993), obscuring the relationships of these additional haplotypes (Fig. 1b).

Geographic distribution of these split haplotypes did not add much to the analysis either. Within haplotype A, haplotype A1 is restricted to the Caucasus, but A2 occurs both in the Caucasus, the Urals, and in Ulyanovsk Province on the East European Plain. The A3 haplotype is represented by a single specimen from the Urals and may constitute a product of local diversification. Within the major haplotype C, C2 is represented by a single specimen from southern Germany, while all the other specimens belong to the haplotype C1. The haplotype E1 occurs in Slovakia, northern Italy, and Belgium; E2 is represented by a single specimen from the Pyrenees; E3 is represented by two specimens from southern slopes of the Alps in northern Italy; E4 also belongs to a single specimen from the Pyrenees. Consequently, no clear geographic pattern can be seen in these split haplotypes, and the relationships between them cannot be easily interpreted.

Among the haplotypes derived from the haplotype G, the G1 is known from a single specimen from Bulgaria, while all the other specimens bear the haplotype G2 and occur in Greece. This may reflect some geographical diversification at a local scale. However, relationships between haplotypes G1 and G2, and their relations to haplotypes A1 and A2, are unresolved.

Fig. 1 Statistical parsimony networks of *Helianthemum nummularium* *rps16-trnK* haplotypes: **a** indels treated as missing data. **b** Indels included in the analysis. Circles are proportional to the number of sequences representing each haplotype



We assume that variation in indel length and composition in *H. nummularium* is strongly homoplastic. For this reason, the initial network based on the analysis with all the gaps treated as missing data was used for all further analyses and calculation of genetic indices. However, one major incongruence between the trees is worth mentioning and should be kept in mind, i.e. the position of the haplotype D and its derivative haplotype M. In the tree based on nucleotide substitution data only, the clade is internal, i.e. positioned deep inside the network. The inclusion of indel data in the analysis changes position of this clade to tip, though relations between haplotypes D and E are retained.

To root the network, we performed NJ and ML analyses on a matrix comprising 13 ingroup haplotypes and the outgroup. The Treefinder program assessed the most likely substitutional model as TVM+G. In both cases the resulting trees were topologically identical. The analysis connected the outgroup with the internal haplotype I (Fig. 2), which appears to be basal to the rest of the

haplotypes. The root position of the haplotype I is also supported by its deep internal position in the network. With its only derivative tip haplotype F, haplotype I forms a basal clade from which two major evolutionary lineages originate. The eastern lineage 1 includes the clade AGCH distributed mostly in eastern Europe, the Caucasus and the Balkan Peninsula, but also penetrating into central Europe. The western lineage 2 includes two other clades (DM and ELBJK) distributed in central and western Europe.

The results of AMOVA are given in Table 2. Most of the variation is due to differences between clades, i.e. evolutionary lineages, which indicate their rather wide divergence.

We found no correspondence between subspecies identity of *H. nummularium* plants and their haplotypes [Table 3, Chi-square test: $N = 85$, $p = 0.17$, (Chi square) = 76.63]: plants referred to one subspecies were characterized by different haplotypes (usually quite distant) and each non-single haplotype was associated with different subspecies. Different subspecies in a given area tended

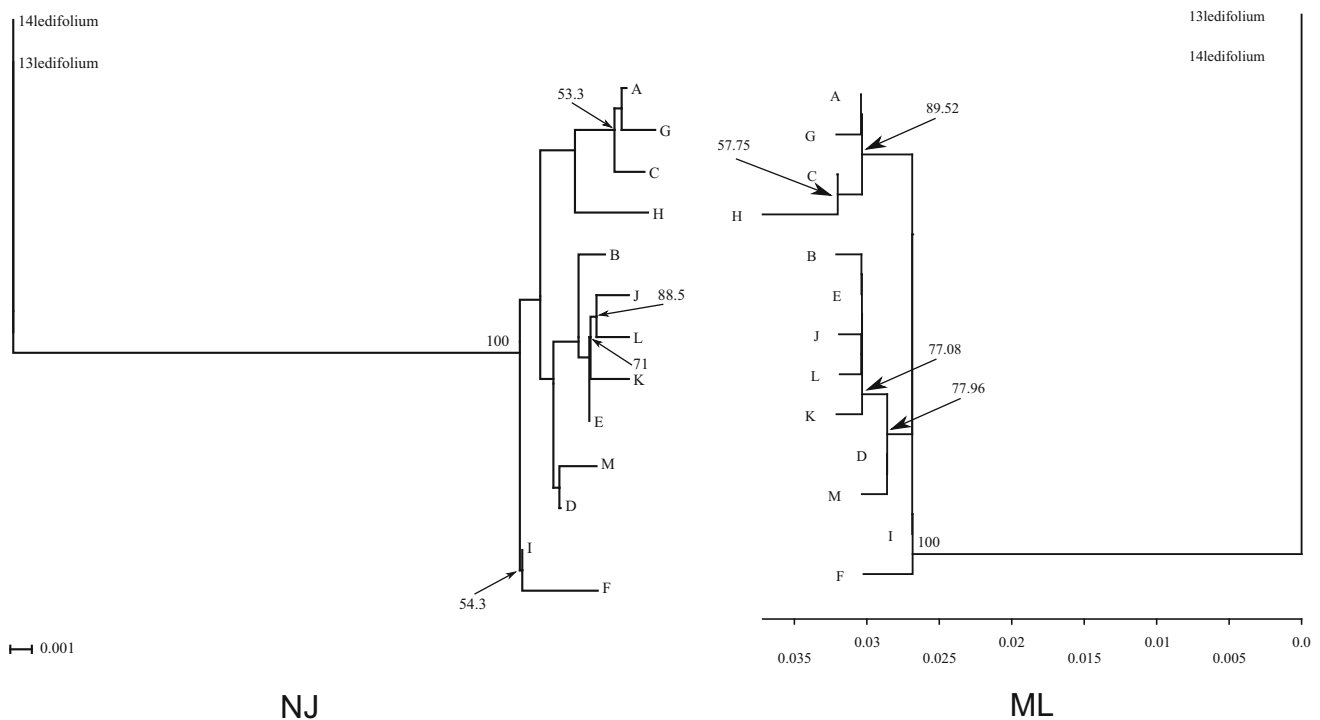


Fig. 2 Neighbour Joining (NJ) and Maximum Likelihood (ML) trees of *Helianthemum nummularium* *rps16-trnK* haplotypes rooted on *H. ledifolium*. Numbers above branches indicate bootstrap support above 70 %

Table 2 Partitioning of genetic diversity in *Helianthemum nummularium* *rps16-trnK* haplotypes (AMOVA) throughout Europe

Source of variation	df	Sum of squares	Percentage of variation
Among clades	2	55.991	77.83
Within clades	82	35.397	22.17
Total	84	91.388	100

The clades are indicated with different colours in Fig. 3

to share the same haplotype (e.g. populations 112 and 128 in southern Sweden and populations 3021 and REV in the Pyrenees: Table 1). The three *H. arcticum* plants sampled

in different parts of a population from the Kola Peninsula were characterized by haplotype C. This haplotype is found in many plants of subsp. *nummularium* and *obscurum*, but only in a few plants of subsp. *glabrum* and *grandiflorum* (Table 3).

The contrast in haplotype diversity between western and eastern Europe is striking. Dividing Europe in two halves with different glacial histories along an arbitrary line from the southeastern Baltic to the western Black Sea gives twelve haplotypes in western Europe but only two in eastern Europe. This means 2.4 haplotypes per taxon and 0.5 (or 0.75 if *H. arcticum* is included in *H. nummularium* subsp. *nummularium*) for western and eastern Europe, respectively.

Table 3 Correspondence of plastid DNA haplotypes to subspecies of *Helianthemum nummularium* s.l.

Taxon	N	Plastid DNA haplotype											
		A	B	C	D	E	F	G	H	I	J	K	L
<i>arcticum</i>	3	0	0	1.00	0	0	0	0	0	0	0	0	0
<i>glabrum</i>	5	0.20	0.20	0.20	0	0.20	0	0	0	0.20	0	0	0
<i>grandiflorum</i>	3	0	0.33	0.33	0	0.33	0	0	0	0	0	0	0
<i>nummularium</i>	42	0.24	0	0.52	0	0.07	0.02	0.1	0	0	0.02	0	0.02
<i>obscurum</i>	29	0	0.17	0.55	0.17	0	0	0	0.07	0	0	0.03	0
<i>tomentosum</i>	1	0	0	0	0	1.00	0	0	0	0	0	0	0

Proportion of plants with each combination of haplotype (A–L) and taxon is given
N number of plants per taxon

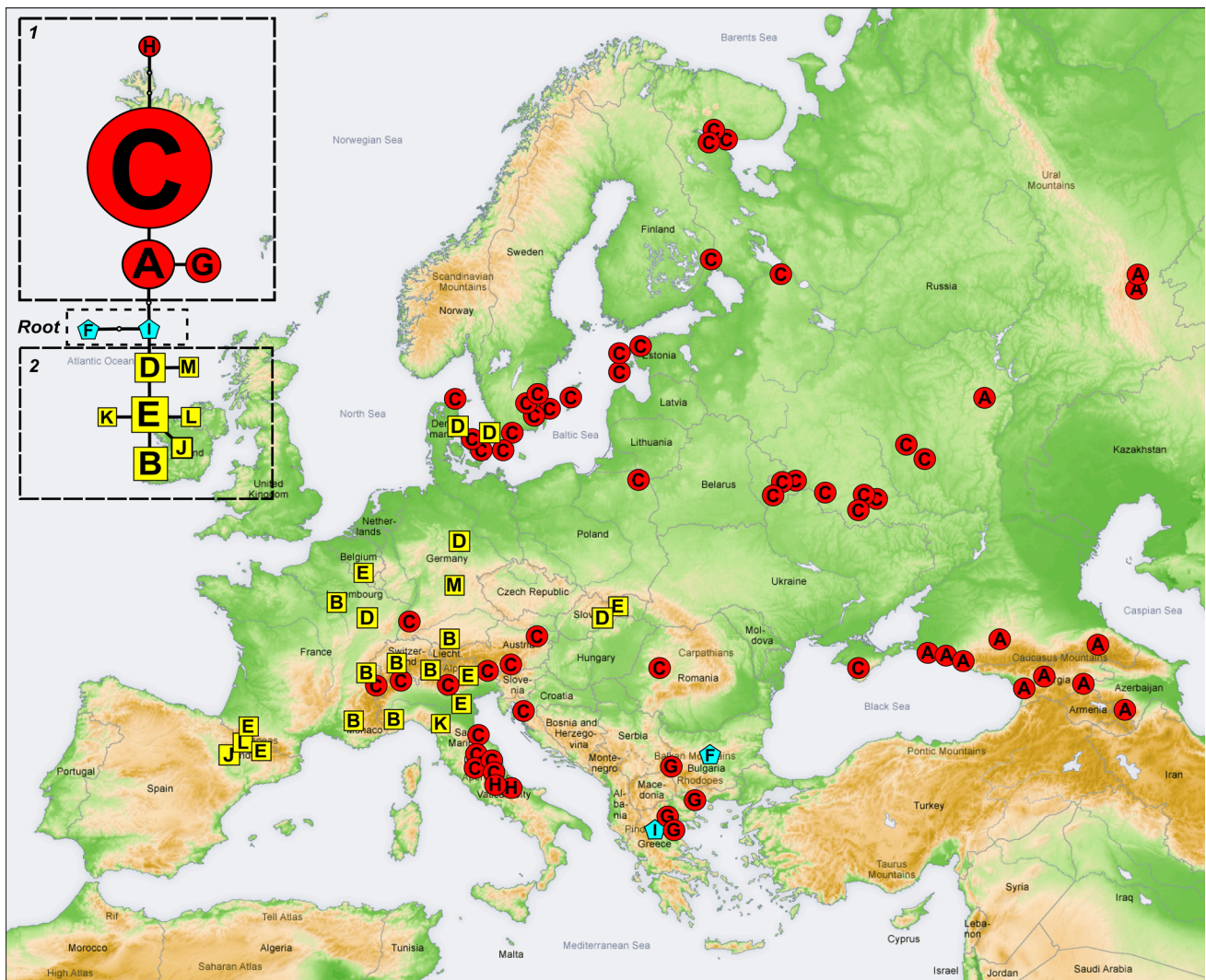


Fig. 3 Geographical distribution of *Helianthemum nummularium* rps16-trnK haplotypes. Haplotypes designated with letters, clades with colours and symbols (encircled with dashed lines). Symbol size in the network is proportional to the number of sequences representing each haplotype

Discussion

We revealed a clear spatial pattern of plastid DNA haplotypes in *H. nummularium* s.l. in Europe, which is largely consistent with the earlier independent phylogeographic analyses of plastid microsatellites of western European samples (Soubani et al. 2014). As discussed further below, plant populations from the plains of eastern Europe are characterized by low morphological and genetic diversity compared with mountainous western Europe. Based on the analysis of variation in one plastid marker, we discuss alternative scenarios of refuge areas and migration routes of *H. nummularium*—hypothesizes that will have to be tested in the future using independent nuclear markers (cf. Després et al. 2003; Petit et al. 2005).

Taxonomic implications

We revealed up to seven not closely related plastid haplotypes in a single subspecies of *H. nummularium* s.l., and several haplotypes shared among up to five taxa (cf. Soubani et al. 2014). The sharing of haplotypes among taxa may be due to introgression or incomplete lineage sorting from a polymorphic ancestor (Wolfe and Elisens 1995; Comes and Abbott 2001). Both of these processes occur mostly between young and reproductively not completely isolated taxa (such as representatives of subgen. *Helianthemum* Willk., radiating in Pleistocene; Guzman and Vargas 2009; Parejo-Farnés et al. 2013), and distinguishing them in phylogenetic analyses is often impossible (Jakob and Blattner 2006). The maintenance of shared

ancient polymorphisms along with constantly growing effective population size is suggested by the small number of hypothetically extinct haplotypes in the network, resulting in the fixation of nearly all newly arising haplotypes. Local introgression may be indicated by the tendency of different morphological subspecies to share the same haplotype in a given region.

Although sharing of several plastid haplotypes among a multitude of species has been shown (e.g. Jakob and Blattner 2006; Hathaway et al. 2009; Christe et al. 2014), we believe that the current splitting of *H. nummularium* into taxonomic units of any rank, especially of specific level (Yuzepchuk 1974), seems unjustified, as morphological characters that were treated as diagnostic are not stable but correlated with ecological conditions (Kupatadze 1978; Soubani et al. 2014). Furthermore, the most important key character in the species, a dense cover of stellate hairs on the abaxial surface of the leaves, has turned out to be governed by a single Mendelian gene (Widén 2015). The conclusion of low taxonomical rank of morphs in *H. nummularium* also applies to '*H. arcticum*', whose morphology lies within the variation range of subsp. *nummularium* (Widen, unpubl.) and which is not genetically distinct in terms of plastid DNA haplotype variation. The isolated occurrence of *H. arcticum* on the Kola Peninsula can be explained by the postglacial history of *H. nummularium* (Jalas 1980).

Phylogeography

Helianthemum nummularium is a young species (Guzman and Vargas 2009), which was split into a western and an eastern lineage early in its evolution during the Pleistocene. We could not date the split with our methods, but this probably occurred before the LGM.

The internal root haplotype I, together with its nearest derivative F, is found in two populations in the Balkan Peninsula, suggesting a Mediterranean origin for the species (cf. Soubani et al. 2014), a pattern typical for members of the Cistaceae family (Guzman and Vargas 2009). This also supports the idea of the Balkans as one of the main sources of genetic diversity in Europe (Hewitt 1999, 2000).

The eastern lineage covers a vast area of east, north and south-central Europe. This lineage was split into two clades; haplotype H (now confined to southern Italy) and the more widespread clade ACG. It may be speculated that the earliest *H. nummularium* migration was directed from the Balkans eastwards (haplotype G and/or haplotype A), colonizing the Caucasus, and further to the north of the Southern Urals and Ulyanovsk region. This migration was rapid, as no mutations accumulated apart from a few deletions. Unfortunately, we do not have samples from Asia Minor, but it seems likely that the Caucasus may have

been colonized via this region. The time of this eastward expansion may be estimated as one of the glacial maxima, since it encompassed mostly territory only partially covered with ice in high mountains.

The origin and the migration routes of haplotype C are less obvious. This haplotype could have originated in the Caucasus or in the eastern part of the Russian Plain region, and then expanded northward and westward to the late- and post-glacial landscapes of the Russian Plain and onward to central Europe, including southern slopes of the Alps and central Italy. An alternative hypothesis to explain the current distribution of haplotype C could be an origin in the Balkans or in the Italian peninsula and a successive expansion into central Europe and northward and eastward to the post-glacial landscape of the Russian Plain.

Irrespective of origin and direction of migration, the expansion of haplotype C must have occurred very recently, probably after the last glacial maximum (LGM), and rapidly, since no mutations have accumulated apart from a few locally occurring deletions. This eastern lineage of *H. nummularium* should also have colonized northern Europe, including southern Sweden and eastern Denmark, as was suggested earlier for *H. nummularium* (Soubani et al. 2014, 2015) and for several other plant species (e.g. Van Rossum and Prentice 2004 and references therein).

Although palaeobotanical data for eastern Europe are very scanty, fossil pollen of *Helianthemum* spp. was reported for periglacial shrub tundra expanded in the north of central and eastern Europe (Hirsch et al. 2015). This includes the Kola Peninsula (Kultina and Spiridonova 1972) and southernmost Sweden in the Bølling-Allerød interstadial complex approximately 11–12,000 years BP (van Kolfschoten et al. 2008).

Scattered distribution of modern northeasterly populations of *H. nummularium* is consistent with their proposed relict status, as they were virtually displaced by post-glacial forests (Proctor 1958); this is proved by fossil pollen findings in that area (Vasari and Vasari 1999). Relict character of stony slopes where *H. nummularium* grows in Southern Karelia was postulated more than a century ago (Litvinov 1902), and the unique edaphic properties of Cape Turij (the Kola Peninsula) are also favourable for a number of relict species (Bubenets et al. 1993). The occurrence of several relict species in Cape Turij and the fact that *H. nummularium* lacks mechanisms for current long-distance dispersal (the seeds drop to the ground close to the parent and lack any morphological adaptation to long-distance dispersal; Widén, personal observation) makes a recent long-distance dispersal unlikely. Consequently, we found no genetic differentiation of isolated peripheral populations of *H. nummularium*—this is difficult to explain without knowledge of their evolutionary history (cf. Lesica and Allendorf 1995; Eckert et al. 2008).

The western lineage of *H. nummularium* covers a more restricted area than the eastern lineage. The distribution of haplotype D and its descendant M covers areas north of the central European mountains that have been suggested as periglacial refugia for several temperate plant species (Stewart and Lister 2001; Ronikier et al. 2008b). This lineage (haplotype D) colonized western Denmark, a region that was partly ice-free during the glacial maximum (Tzedakis et al. 2013). The other haplotypes in the western lineage are distributed across western Europe (haplotype E and its descendants B, L, K and J). Haplotype B is distributed along the basin of the River Rhone and along the northern slopes of the Alps, as well as south of the Alps. The distribution of haplotype B coincides with that of haplotype H14, identified on the basis of plastid microsatellites in Soubani et al. (2014), a haplotype that surrounds the Alps.

The observed high genetic differentiation in western Europe is partly explained by the existence of the well-known ‘suture zone’ located in the Alps, which was covered by ice during the LGM (Hewitt 2000). The distribution of the two haplotypes of *H. nummularium* on the northern (haplotype B) and southern (haplotype C) slopes of the Alps, i.e. along the Alpine arc, is consistent with distributions of other calcicolous species, explained by availability of calcareous bedrock in these mountains (Alvarez et al. 2009).

A secondary contact zone detected in Denmark is probably explained by the uneven melting of the LGM ice cap, which allowed eastern and western lineages to meet. This contact zone can be traced in the morphology of present-day populations of *H. nummularium* in southern Scandinavia. The distributions of the almost exclusive morph in Denmark (subsp. *obscurum*) and the most common morph in southern Sweden (subsp. *nummularium*) constitute a wide hybrid zone in southwestern Sweden, where plants of the two taxa cross freely (Soubani et al. 2015; Widén 2015). The western haplotype D, represented by subsp. *obscurum*, reached the ice-free areas in western Denmark, while the eastern haplotype C in the form of subsp. *nummularium*, arrived via more easterly routes. The distributions of taxa and haplotypes in the hybrid zone today do not coincide with each other (cf. Soubani et al. 2015), probably due to “chloroplast capture” (Jakob and Blattner 2006; Hathaway et al. 2009). Similar contact zones between western and eastern postglacial migration routes have previously been described for several species in Scandinavia (Malm and Prentice 2002; Tyler et al. 2002; Van Rossum and Prentice 2004; Nordström and Hedrén 2008; Hathaway et al. 2009; Prentice et al. 2011), outside of the current distribution range of *H. nummularium*.

The morphological variation of plants represented by the haplotypes A and C in the east is nearly homogenous, and

the plants in this area are mostly recognized as one subspecies (subsp. *nummularium*). This is in sharp contrast to the haplotypes in western and southern Europe, where variation in morphology has been divided into five subspecies. The altitudinal fluctuations of the ice cap, for instance in the Alps, probably created a lot of local reductions and expansions in population size and diverse opportunities for adaptation to new habitats. Morphs with large flowers and leaves within different haplotype lineages may have been adapted repeatedly to mountain and alpine habitats. It is significant that three of the five western European subspecies are found in the Alpine belt (subsp. *grandiflorum*, *glabrum* and *tomentosum*). A single dominant mutation in the nuclear genome that changed the phenotype from plants with a dense cover of stellate hairs on the abaxial surface of the leaves to plants without this cover (or vice versa) may have been fixed in small populations once or repeatedly (a mutation from subsp. *nummularium* to subsp. *obscurum* probably occurs today, see Soubani et al. 2015).

Genetic and morphological diversity of *H. nummularium* in western Europe are shaped not only by older dispersal from southern refugia as was shown for many other species (Hewitt 2000, 2004) but also by more recent colonization from eastern Europe—the area that is still ignored in most phylogeographic reconstructions. In contrast, eastern and northern Europe virtually lacks both morphological and genetic variation among populations of *H. nummularium*. This may be regarded as a signature of rapid and recent colonization of these territories following the melting of glaciers, with subsequent extinction of many of these populations after forest vegetation became established and suitable habitats disappeared in most of the area.

Conclusions and future perspectives

We found a very distinct pattern in molecular and morphological variation in *H. nummularium* throughout Europe. Our results clearly show the importance of phylogeographic analyses of the entire species range for recovering species history. Questions remain about glacial refuges and postglacial migration routes, so areas not covered in the present study (e.g. southernmost Italy, western parts of the Balkans, Turkey and Sierra Nevada) should be included to give a deeper understanding of the evolution of *H. nummularium*. Moreover, to further understand the complex pattern of genetic and morphological differentiation of the *H. nummularium* s.l., closer examinations of introgression between different haplotypes of the western lineage and between western and eastern lineages are needed, using other molecular methods (Després et al. 2003; Petit et al. 2005) as well as morphometric analysis.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Appendix

Some names used for the yellow-flowered morphs in the *Helianthemum nummularium* s.l. according to the International Plant Names Index (IPNI), Janchen (1909), Proctor and Heywood (1968) and Yuzepchuk (1974).

IPNI	Janchen (1909)	Yuzepchuk (1974)	Proctor and Heywood (1968)
<i>H. nummularium</i> Mill.	<i>H. nummularium</i> (L.) Dunal.	<i>H. nummularium</i> (L.) Dunal.	<i>H. nummularium</i> (L.) Mill. subsp. <i>nummularium</i>
<i>H. obscurum</i> Pers.	<i>H. hirsutum</i> (Thuill.) Mérat.	<i>H. hirsutum</i> (Thuill.) Mérat.	<i>H. nummularium</i> subsp. <i>obscurum</i> (Čelak.) Holub
<i>H. grandiflorum</i> DC	<i>H. grandiflorum</i> (Scop.) Lam. et DC.	<i>H. grandiflorum</i> (Scop.) Lam. et DC	<i>H. nummularium</i> subsp. <i>grandiflorum</i> (Scop.) Schinz & Thell.
<i>H. glabrum</i> Kerner	<i>H. nitidum</i> Clem.	<i>H. nitidum</i> Clem. included in <i>H. grandiflorum</i>	<i>H. nummularium</i> subsp. <i>glabrum</i> (Koch) Wilczek
<i>H. tomentosum</i> Gray	<i>H. tomentosum</i> (Scop.) Spreng.	<i>H. tomentosum</i> (Scop.) Spreng	<i>H. nummularium</i> subsp. <i>tomentosum</i> (Scop.) Schinz & Thell.
<i>H. arcticum</i> Janch.		<i>H. arcticum</i> (Grosser) Janch. included in <i>H. nummularium</i>	<i>H. arcticum</i> (Guss.) Juz. included in <i>H. nummularium</i> subsp. <i>nummularium</i>

Information on Electronic Supplementary Material

Online resource 1. Matrix of sequence data for haplotypes.

Online resource 2. Matrix of sequence data for individual samples.

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