Plastid DNA microsatellite data do not support recognition of subspecies in *Coeloglossum viride* (L.) Hartm. (Orchidaceae) in northern Europe

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The orchid *Coeloglossum viride* (L.) Hartm. has a circumboreal distribution and is widespread in the Nordic countries, especially in the boreal and montane regions. Whereas plants from the lowlands are generally slender and have greenish-yellowish flowers, plants from mountain areas tend to be low-growing with fewer, reddish-brown flowers, leading some authors to recognize the latter as a separate subspecies, *C. viride* subsp. *islandica* (Lindl.) Kreutz. In course of collecting data for the treatment of the Orchidaceae in the Flora Nordica, we have analysed material of *C. viride* for plastid microsatellite variation in order to assess the taxonomic justification of subsp. *islandica*. We found two major groups of haplotypes in *C. viride* in the Flora Nordica area. The two groups were widespread in both mountain and lowland regions in Scandinavia, but only one of the groups was present in material from Iceland. Accordingly, plastid microsatellite data give no support for the recognition of mountain/Icelandic plants as a separate taxon. Based on the distribution of haplotypes and their relationships, we suggest that the present-day population of *C. viride* in the Nordic countries originated from populations in refugia close to the Weichselian ice sheet

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Introduction

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Coeloglossum Hartm. is mostly regarded as a monotypic genus with the single species *C. viride* (L.) Hartm. However, the species has a nearly continuous circumboreal distribution, extending southwards into montane regions in Europe, Asia and North America (Hultén

Table 1. Sites sampled for *Coeloglossum*. The column "N" reports the number of individuals sampled at each site, "DNA Bank accession number" give the accession numbers to DNA extracts stored with the first author at Dept. of Biology, Lund University, and "Locality abbreviation" is the locality abbreviation used in Table 4.

Country	Region	Locality	Lat.	Long.	N	DNA Bank acc. #	Locality abbreviation
Georgia	Kazbegi	Gudauri peace monument	42°30′N	44°27′E	1	6512	Gudauri
Italy	Südtirol	Val Gardena, Santa Christina	45°34′N	11°40′E	1	8968	Val Gardena
Italy	Südtirol	Sellajoch	46°31′N	11°45′E	3	8969–8971	Sellajoch
France	Hautes-Alpes/Savoie	Col du Galibier	45°03′N	06°24′E	4	8959–8962	Col du Galibier
France	Savoie	Mt Cenis	45°17′N	06°54′E	3	8963–8965	Mt Cenis
Switzerland	Graubünden	Furka Pass	46°35′N	08°26′E	2	8966–8967	Furka
Austria	Salzburg	Schafberg	47°47′N	13°26′E	2	8972–8973	Schafberg
England	Hampshire	Noar Hill	51°04′N	00°56′W	15	6873–6887	Noar Hill
Sweden	Gotland	Djaupdy	57°15′N	18°42′E	10	8447–8456	Djaupdy
Sweden	Ångermanland	Svedbergsviken	63°37′N	15°58′E	1	3237	Svedbergsviken
Sweden	Jämtland	Odensala, V Lillsjön	63°09′N	14°41′E	1	284	Odensala
Sweden	Jämtland	Skalberget	62°34′N	14°27′E	2	497, 8958	Skalberget
Sweden	Jämtland	Surmyran	63°28′N	15°20′E	4	3438–3440	Vackermyran
Sweden	Lycksele lappmark	Formliden, S Västansjö	65°42′N	15°07′E	10	6767–6776	Formliden
Sweden	Lycksele lappmark	Rödingsnäset, NV Tängvattnet	65°52′N	14°43′E	10	6777–6786	Rödingsnäset
Sweden	Torne lappmark	Njulla, NV Abisko	68°22′N	18°41′E	10	6853–6862	Njulla
Norway	Rogaland	Jæren	58°47′N	05°33′E	1	8974	Jæren
Norway	Møre og Romsdal	Tågdalen	63°03′N	09°05′E	5	6762–6766	Tågdalen
Norway	Sør-Trøndelag	Sølendet, Kjerrstokenget	62°41′N	11°50′E	10	6863–6872	Sølendet
Iceland	Mi-Ísland	Kalmanstunga	64°44′N	20°49′W	8	8431–8438	Kalmanstunga
Iceland	Norur-Ísland	Björg	65°57′N	17°36′W	8	8439–8446	Björg
Iceland	Norur-Ísland	Kolugafjall	65°48′N	19°55′W	8	8407–8414	Kolugafjall
Iceland	Norur-Ísland	Ljosavatn	65°42′N	17°42′W	8	8423–8430	Ljosavatn
Iceland	Norur-Ísland	Reykjarhóll	66°00′N	19°00′W	8	8415–8422	Reykjarhóll

1950; Hultén & Fries 1986), and geographic segregates have sometimes been recognized at infraspecific levels. For example, the North American population is often treated as subsp. *bracteatum* (Muehlenbeck ex Willd.) Hultén (e.g. in Hultén & Fries 1986) and material from Iceland has been separated as subsp. *islandicum* (Lindl.) Kreutz (occasionally treated as species, variety or form).

Coeloglossum viride is widespread in most parts of the Nordic countries (hereafter Norden) (Hultén 1950, 1971), although it is rare and decreasing in the southern nemoral and nemo-boreal regions. In Denmark it has not been observed since 1950 (Pedersen & Faurholdt 2010; Hartvig 2015), and it is probably extinct from several provinces in southern Sweden, including Skåne (Tyler et al. 2007); Blekinge (Fröberg 2006); Småland (Edqvist & Karlsson 2007), and Västergötland (Bertilsson et al. 2002). It is rare and scattered elsewhere in Götaland, including Öland (Sterner 1986), Gotland (Ingmansson & Johansson 2005), Dalsland (Andersson 1981) and Östergötland (Genberg 1992), and it is absent from southern Norway (Hylander 1966; Lid 1985). Elsewhere in Norden it is regionally common, both in boreal lowland areas and in the mountain regions of Scandinavia and Iceland (Hultén 1950, 1971; Hylander 1966; Mossberg & Nilsson 1977; Kristinsson 2010).

Whereas plants in the Nordic lowlands are often found in shaded or semi-shaded locations, and are typically slender with greenish-yellowish flowers (and hence often difficult to spot), plants in mountain regions are mostly found at exposed sites, and tend to be low-grown with reddish-brown flowers. The mountain form was grouped with the Icelandic subspecies by Selander (1950). Although this taxon has mostly not been formally accepted in the botanical literature, it is often still mentioned and discussed (e.g. in Hylander 1966; Luer 1975; Lid 1985; Mossberg & Nilsson 1977; Løjtnant 1977; Landwehr 1977; Nilsson 1991; Delforge 2001; Baumann et al. 2006). Yet another form of *C. viride* is a low-growing and stout form with rounded leaves confined to dune slacks in Jæren, SW Norway (Elven, undated; S. Imsland, pers. comm.) – see also Vik-Mo (2008) who referred the Jæren population to subsp. *islandicum*. Similar plants are known from dune slacks in Britain and Ireland (see e.g. Baumann et al. 2006).

As we are in the process of revising the Orchidaceae for a forthcoming treatment of the family for the *Flora Nordica* project (Jonsell 2004), we decided to perform a molecular study to describe the differentiation pattern within *C. viride*. On the basis of the views of previous authors describing the variation within *Coeloglossum* in Norden, we examined the two hypotheses that either (i) the Icelandic population is separated from the entire Scandinavian population, or (ii) that the combined Icelandic / Scandinavian mountain population is separated from the Scandinavian lowland population. We also wanted to consider the suggestion that the dune slack form known from SW Norway is divergent from other Scandinavian forms of the species. To test these hypotheses, we collected data from hypervariable regions in the plastid genome, combined the observed variation patterns into haplotypes and analysed the relationships of these haplotypes.

Materials and methods

Plant material and collecting sites

Material for this study was collected in connection with studies for *Flora Nordica* and during excursions to Central Europe or the Caucasus. Altogether 135 samples from 24 sites were collected (Table 1). Voucher material in the form of dried flowers (with the first author), or ethanol-preserved flowers (with the second author) was prepared from the sampled plants. The voucher material will be deposited in the botanical museums at Lund (LD) and Copenhagen (C), respectively. Accession numbers for DNA extracts stored at the DNA bank of the first author at Department of Biology, Lund University, are given in Table 1.

No	Type	General region and universal primers	Location	Specific primers for fragment	Sequence 5′--3′	Ann	Length of repeat	No of size var.
1	polyA (Soliva & Widmer 1999)	*trn*L exon 1 – *trn*L exon 2; c/d (Taberlet et al.1991)	*trn*T – *trn*L intergenic spacer	Cy5trnL5 (= c, Taberlet et al. 1991) trnLR5 (Hedrén et al. 2008)	**F:** CGAAATCGGT-AGACGCTACGC **R:** CGTTAGAAC-AGCTTCCATTG	57	11−13 (187)	3
7c	polyA	*trn*H – *trn*K; *trn*H/*trn*K (Demesure et al. 1995)	*trn*H – *rps*19 intergenic spacer	Cy5HK1F HK2R	**F:** GATTCTTAC-CCTCATACTTC **R:** CGTGTCACG-GCTGATTACTC	54	10−12 (98)	3
10b	polyA-TA-T	*trn*H – *trn*K; *trn*H/*trn*K (Demesure et al. 1995)	*psb*A – *trn*K exon 1 intergenic spacer	Cy5trnK1A (= *trn*K, Demesure et al. 1995) HK10F (Hedrén et al. 2008)	**F:** CCGACTAGT-TCCGGGTTCGA **R:** GAAAGGCTT-GTTATTTCACAG	56	34−39 (138)	6
11b	polyA	*rpl*16 intron (Wallace 2003); F71 (Jordan et al. 1996)/ R622 (Les et al. 2002)	*rpl*16 intron	Cy5F71 (= F71, Jordan et al. 1996) F71R2 (Hedrén et al. 2008)	**F:** GCTATGCTTAGT-GTGTGACTCGTTG **R:** AGTTTATAG-TGGGGTCAGCC	53	7−9 (87)	3
12	polyA	*rpl*16 intron (Wallace 2003); F71 (Jordan et al. 1996)/ R622 (Les et al. 2002)	*rpl*16 intron	Cy5F71F2 R622R2	**F:** CGAGATCCC-AAGAAACAGTC **R:** TCCGCCTT-TTCTACATCCC	56	11 (122)	1
14	polyA	*trn*C – *trn*D; *trn*C/*trn*D (Demesure et al. 1995)		Cy5trnC4F trnC2R	**F:** GATTCAAAA-GGGGACGTTGC **R:** GGGTTCAGA-TGAAACCACAC	58	9 (192)	1
16	polyT (Weising & Gardner 1999, Cozzolino et al. 2003)	*rpl*2 – *rps*19; ccmp10 (Weising & Gardner 1999)	*rpl*2 – *rps*19 intergenic spacer	Cy5ccmp10F (= Weising & Gardner 1999) ccmp10R2 (Hedrén et al. 2008)	**F:** TTTTTTTTTA-GTGAACGTGTCA **R:** CAGATTAAGA-TACGAGATATTGG	50	11−13 (90)	3
18	polyTA	*trn*S – *trn*G; *trn*S (GCU)/ *trn*G (UCC) (Hamilton 1999)	*trn*S – *trn*G intergenic spacer	Cy5trnSGf2 (Hedrén et al. 2008) trnSGr2 (Hedrén et al. 2008)	**F:** CCTAATTCTTA-GAAAGAATATGAG **R:** GAATAGATAT-AGAATCTTACTC	54	13−21 (87)	2
19B	polyT (Pillon et al. 2005)	*trn*S – *trn*G; *trn*S (GCU)/ *trn*G (UCC) (Hamilton 1999)	*trn*S – *trn*G intergenic spacer	Cy5trnSGf3 (Hedrén et al. 2008) trnSGr3 (Hedrén et al. 2008)	**F:** GAGTAATAGT-GTTCTAATAAGAG **R:** CAGACGCAG TCAAGATAGCA	58	10−11 (146)	2

LEFT. Table 2. Description of the nine plastid microsatellite regions examined in *Coeloglossum*. "Ann": annealing temperature. In the column "Length of repeat" the approximate size of the shortest amplified fragment is given within paranthesis.

Haplotype	Locus								
	1	7	10b	11b	12	14	16	18	19
A (1)	11	12	36	8	11	9	13	13	10
B (1)	11	12	37	8	11	9	13	13	10
C (1)	11	12	38	8	11	9	13	13	10
D (1)	11	12	39	8	11	9	13	13	10
E	12	10	36	8	11	9	11	13	10
F (2)	12	11	35	8	11	9	12	13	11
G (2)	12	11	36	8	11	9	12	13	11
H (2)	12	11	39	8	11	9	12	13	11
I (2)	12	11	35	8	11	9	12	13	10
J (2)	12	11	35	9	11	9	12	13	11
K (2)	12	12	36	8	11	9	13	13	11
L	12	12	37	8	11	9	13	13	11
M	12	12	34	8	11	9	13	13	10
N	13	12	35	8	11	9	13	13	10
O (2)	12	11	34	8	11	9	12	13	11
P	12	12	35	8	11	9	13	13	10
Q	11	12	35	7	11	9	13	21	10

Table 3. Characterization of the 17 different plastid haplotypes found in *Coeloglossum*. Numbers within parentheses denote the major group to which haplotypes identified in the Nordic material is referenced in the text. The numbers reported under each locus are the size of the repeats.

Molecular methods

A few flowers with bracts, or the upper parts of leaves, were collected in the field and rapidly desiccated in plastic bags with dry silica gel (Chase & Hills 1991). One flower from each sample, or about 1 cm^2 of leaf area was used to extract DNA according to the 2× CTAB procedure (Doyle & Doyle 1990).

Nine putatively size-variable marker sites were studied in the plastid genome. Most of these markers and PCR conditions have been reported elsewhere (Hedrén et al. 2008), but the marker loci 7, 12 and 14 are added here (Table 2). The markers included seven mononucleotide microsatellites, one dinucleotide microsatellite, and one combined mononucleotide/dinucleotide repeat. The combined variation patterns at all marker sites were recognized as haplotypes and denoted by capital letters (Table 3).

Data analysis

The relationships between plastid haplotypes were described by means of a median-joining network (MJ; Bandelt et al. 1999) in which variants recognized at the nine investigated loci were treated as ordered characters according to fragment size and the estimated numbers of repeats. MJ identifies unrooted trees using parsimony, and presents multiple trees in the form of a network in which alternative, equally parsimonious, pathways are visible as cycles.

MJ networks may link derived haplotypes directly to ancestral ones and may also include median vectors, which are hypothetical character combinations introduced to reduce the network length. The MJ network was calculated using the computer program NETWORK 4.5.1.6 (Fluxus Technology 2007).

Results

Of the nine plastid loci analysed, seven were found to be variable within the sampled material, with between two and six size variants each (Table 3). In the material sampled from Norden, six loci were variable. Seventeen haplotypes were identified based on the combined variation pattern at all variable loci. Eleven of these haplotypes were identified in the material sampled from Norden (Table 4). A few haplotypes were more common than the rest. Haplotype C was identified in 43 samples out of 135 samples, but was confined to mainland Scandinavia and Britain. Haplotype G dominated at the Icelandic sites, but was also identified in one sample from northern Scandinavia. The third most common haplotype, F, was widespread and identified in plants from England, Gotland, mainland Scandinavia and Iceland. Two moderately common haplotypes, D and J, found in nine plants each, were confined to plants from Norway and Iceland, respectively. The remaining twelve haplotypes were identified in between one and five samples each. Five of these haplotypes were restricted to material from the Alps and one to the sample from Georgia.

The haplotypes were linked to each other in a median-joining network (Fig. 1). Haplotypes identified in Nordic material were clustered in two sections of the network. Haplotypes A to D (hereafter group 1) were separated only by differences at the most variable locus 10b, and were confined to material from Britain, Gotland and mainland Scandinavia. Haplotypes F to K and O formed a more lose group (2), and were separated by differences at various loci. All haplotypes identified in material from Iceland belonged to group 2, along with haplotypes in material from Britain, Gotland, mainland Scandinavia and the Alps. The two groups were linked by haplotypes identified in material from the Alps. The haplotype from the single plant from Georgia was clearly divergent, mostly due to a difference of four repeats at locus 18.

Discussion

Based on the results obtained in this study, we reject the hypothesis that the Icelandic population is genetically separated from the Scandinavian population, since group 2 haplotypes characteristic of *C. viride* on Iceland are also present in Scandinavia and Britain. Moreover, we also reject the hypothesis that plants from high elevations in the Scandinavian mountains are genetically separated from plants in the lowlands, since haplotypes from both major groups are encountered in both of these areas. It should be noted that our findings are consistent with the results from a previous assessment of patterns of allozyme variation among a subset of the populations sampled for the present study (H. Æ. Pedersen, unpublished data).

The distribution of haplotypes and their relationships are still of interest for understanding the recolonization history of *Coeloglossum* in Norden. The haplotypes found in Nordic material formed two relatively distinct groups in the plastid network. Haplotypes from the Alps material formed an intermediate group, linking the two Nordic groups together, and in fact very few haplotypes were shared between Norden and the Alps. The clear pattern of divergence in haplotype composition between the two regions gives little support to the idea that the present-day population of *Coeloglossum* in Norden was established by dispersal of seeds from the Alps or a Central European refugium after the last glaciation. It seems more likely that the Nordic population was re-established from populations that were able to survive at closer distance from the Weichselian ice sheet; and the fact that the Scandinavian haplotypes form two quite distinct groups linked to each other by haplotypes from the Alps suggests that the source population has a long evolutionary history, dating back well before the last ice age. Some authors have observed that plants from high elevation in the Alps may approach the Scandinavian mountain form in being low-growing and having reddish flowers (Buttler

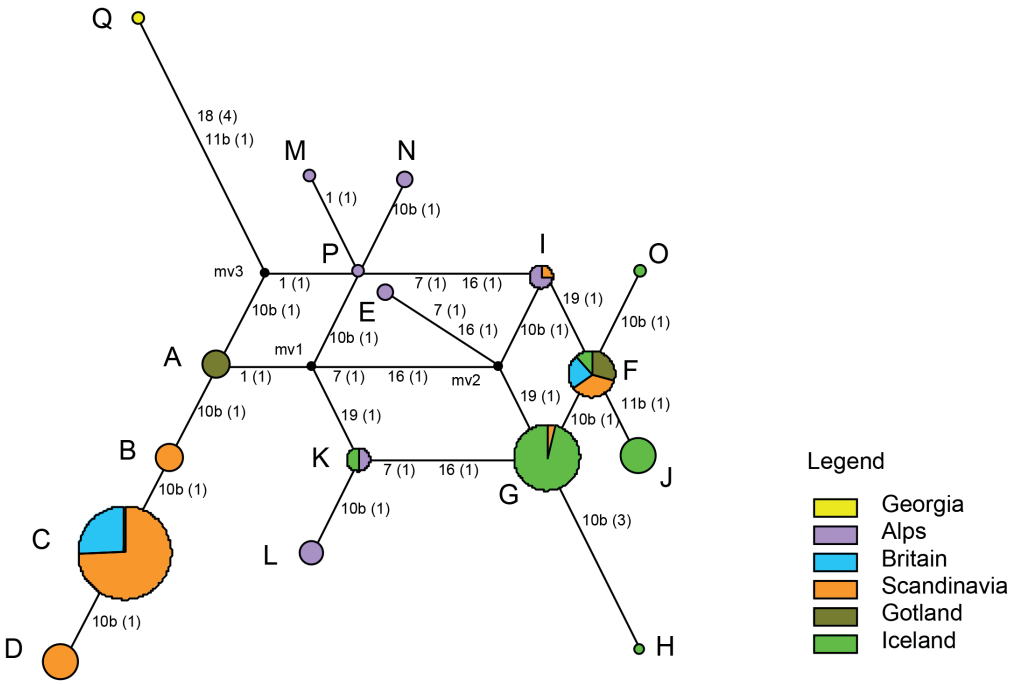

Fig. 1. Median-joining network of the plastid haplotypes identified in *Coeloglossum viride*. The network is composed of three equally parsimonious unrooted trees of 27 steps each. Observed microsatellite fragments were arranged according to size at each marker site and treated as ordered characters. The size of each symbol is proportional to the number of individuals carrying the particular haplotype, and the sectors are proportional to the different regions in which the haplotype has been found (Table 4). Marker sites and mutational steps (within parentheses) separating haplotypes are indicated. Branch lengths are approximately proportional to the numbers of mutational steps. Small black diamonds denote median vectors. Haplotypes are annotated by capital letters (see Table 3).

1991), but on the basis of our data plants of this type must have been formed in parallel in the two areas.

The Icelandic population may have been established by seed dispersal from north-western Europe but, since we have not had access to any material from North America, we cannot rule out the possibility that the Icelandic population has been established following long distance dispersal from the west; and perhaps even that material of western origin contributed to the colonization of Scandinavia and mixed in with plants carrying group 1 haplotypes. Similarly, we have not had access to material from Russia, so we can also not speculate on the eastward extension of the haplotypes occurring in Scandinavia today.

The single specimen from Jæren, representing the dune slack form of the species, carried haplotype I, which was otherwise present in material from the Alps. Based on this finding, it may be speculated that the dune slack form represents an independent dispersal event to Scandinavia, but it may also be observed that this haplotype differs only by a single mutational step from the widespread and relatively common haplotype F. Thus, the haplotype I on Jæren may well represent an independent formation of this haplotype.

Overall, it may be observed that the haplotype network given as Fig. 1 contains several cycles with alternative mutational pathways between haplotypes, and at least three equally short trees were inferred by the MJ analysis. On the

Haplotype Haplotype group	A 1	B 1	C 1	D 1	E	F 2	G 2	H 2	I 2	J 2	K 2	L	M	N	O 2	P	Q	Row sum
Caucasus																	1	1
Gudauri																		
Alps																		
Val Gardena													1					1
Sellajoch									2		1							3
Col du Galibier					2							1				1		4
Mt Cenis												1		2				3
Furka									1		1							2
Schafberg												2						2
Britain																		
Noar Hill			11			4												15
Gotland																		
Djaupdy	5					5												10
Scandinavia																		
Svedbergsviken			1															1
Odensala			1															1
Skalberget			2															2
Vackermyran			4															4
Formliden		2	8															10
Rödingsnäset			4			5	1											10
Njulla			9			1												10
Jæren									1									1
Tågdalen				5														5
Sølendet		3	3	4														10
Iceland																		
Kalmanstunga							6			2								8
Björg							4			3					1			8
Kolugafjall						2	4				2							8
Ljosavatn							3	1		4								8
Reykjarhóll							8											8
Column sum	5	5	43	9	2	17	26	1	4	9	4	4	1	2	1	1	1	135

Table 4. Distribution of plastid haplotypes within and between population samples of *Coeloglossum* (figures denote numbers of individuals). "Haplotype group" denotes the major group to which haplotypes identified in the Nordic material is referenced in the text.

one hand, we need loci with high mutation rates, such as microsatellite loci, to analyze differentiation patterns and relationships among haplotypes within species. On the other hand, with several such loci, there is a distinct possibility that parallel mutations may occur, such that a particular haplotype may evolve from several parental haplotypes. While there is a small probability that common haplotypes have originated repeatedly from rare ones, rare haplotypes are more likely to have evolved repeatedly from common ones (Lowe et al. 2004). Accordingly, the differentiation patterns given by the haplotypes identified in a high number of samples should be more trustworthy than differentiation patterns given by the haplotypes found in only a few samples.

It should also be noted that the sampling for this study is quite uneven. While about ten individuals were sampled from most of the Nordic sites, only a few individuals per site were sampled from the sites in the Alps and in the Caucasus. We would probably have identified additional haplotypes from these areas if we had included more samples from each site, but it is also possible that some of the haplotypes from these areas would have turned out to be as dominant in these regions as the most common haplotypes identified in Nordic material; and if so, the discussion on the origins of particular haplotypes may have been different. It may also be commented that sampling over the Flora Nordica area is uneven and, for instance, no material from Finland was available for this study. For a better understanding of the origin and position of the dune slack population in Jæren, we would obviously also need additional samples from the same region as well as reference samples of the dune slack form from Britain and Ireland.

Authors who have discussed the status of subsp. *islandica* seem to agree that the taxon should be dismissed on the basis that plants of intermediate appearance connect subsp. *islandica* with subsp. *viride* (Hylander 1966; Mossberg & Nilsson 1977; Løjtnant 1977; Nilsson 1991). However, according to the criteria given in *Flora Nordica* (Jonsell 2004) the subspecies category may still be adopted to recognize geographical, ecogeographical, or even ecological variants within a species. Therefore, on basis of morphology and distribution alone, we are still left with the possibility keeping subsp. *islandica* as a separate subspecies, and we need to evaluate this standpoint on the basis of patterns shown by molecular data.

Given the criteria listed in *Flora Nordica*, any species with geographically correlated morphological variation and a reasonably large distribution may be subdivided into subspecies. As there is no objective way to decide on the number and circumscription of such subspecies, additional criteria are needed to achieve taxonomic stability at the subspecies level. First, we emphasize that subspecies need to agree with some other apparent type of differentiation pattern that is not just clinal variation resulting from isolation by distance. Apart from morphology, such patterns may be manifested in habitat requirements or phenology. Subspecies do not need to be completely separated in any character or trait, but their boundaries should correlate with the steepest zones of transition between forms. Second, we propose that patterns of genetic differentiation should be taken into account in the recognition of subspecies. Preferably, individuals of one subspecies should be more closely related to each other than to individuals of another subspecies, and vice versa (i.e. evolutionarily significant units, ESUs sensu Moritz 1996). Such subspecies may arise due to historical processes, such as lineage splitting or migration history, or to more recent and ongoing processes, such as high unifying gene flow within subspecies, geographic, genetic or ecological separation of different subspecies, or a combination of these processes. Subspecies integrity can be tested by means of molecular markers that are effectively "neutral", i.e. not directly linked to genes responsible for morphological or ecological differentiation. Subspecies proposed on the basis of morphological and geographic data may be accepted if they also agree with differentiation patterns at molecular markers, and if not then other arguments must be evaluated very carefully. Previous authors have already questioned the distinctness of infraspecific taxa proposed within *C. viride* in Norden. Here we also show that these taxa do not constitute genetically coherent subgroups, and hence that they do not merit taxonomic recognition at the subspecies level.

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