Master of Science thesis

Phylogeography and population structure in *Dryas octopetala* L. analysed by microsatellite markers

Unni Vik

Centre for Ecological and Evolutionary Synthesis

Department of Biology,University of Oslo

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Abstract

Dryas octopetala L. is an arctic-alpine plant that belongs to a genus where the number and delimitation of species are not fully resolved. The plant is unusual, compared with most other arctic-alpine plants in its diploid, dwarf-shrub habit with slow generation turnover. In this study, ten newly developed microsatellite markers have been tested and used to infer both phylogeography and population structure in 471 individuals of *D. octopetala* sampled from seven main localities in the North Atlantic area. In addition, the microsatellite markers were used to investigate whether there is genetic support for assigning three morphotypes of *D. octopetala* on Svalbard to taxa, as previously proposed by Russian scientists.

Nine of the ten microsatellites were analysed in this study, and provided a resolution high enough to distinguish between individuals. At a phylogeographic scale, the analysed material grouped into three main groups, a southern group, an intermediate group and a northernmost group. It is likely that there have been two main colonization routes following the glacial retreat in the North Atlantic region, one southern and one eastern. This study supports a colonization route from the south for the southern Norwegian populations, while an eastern immigration route probably has colonized Greenland, northern Norway and Svalbard.

At a smaller, population scale, the microsatellites separated between different genets of *D. octopetala*. The local *D. octopetala* populations were mainly in Hardy-Weinberg equilibrium and no latitudinal trend was found regarding the proportion of ramets in the sampled populations or for heterozygote deficiency.

No evidence for the assigning the three morphotypes occurring on Svalbard to taxa was found, and thus this study concludes that there is only one *Dryas* species on Svalbard, i.e., *D. octopetala*.

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Appendices as CD in the back-cover

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"Hvorledes er nu denne merkelige koloni av fjeldplanter kommet til Langesundsfjorden?"

Joh. Dyring - 1911

Introduction

The genetic variation of plant species is shaped in time and space and is influenced by both present and past migration histories, life history traits, mutation, selection and drift (Loveless and Hamrick, 1984; Hewitt and Butlin, 1997; Thiel-Egenter et al., 2009). Present and past migration histories are important first of all at the larger (phylogeographic) scale (Hewitt and Butlin, 1997), whilst life history traits and ecological factors are important first of all at the smaller (population) scale (Loveless and Hamrick, 1984).

An increasing amplitude in the Earth's climatic oscillations throughout the Tertiary lead to the series of major ice ages of the Quaternary (2.4 million years to the present; Hewitt, 2000). In Northern Europe, the severe fluctuations between the long glacial and the relatively short interglacial episodes produced great changes in species distributions by habitat fragmentation and expansion, and have had a huge impact on the genetic structure of current species (Hewitt, 1999; Hewitt, 2000). Since the introduction of phylogeography as a discipline (Avise et al., 1987), a large amount of phylogeographic studies on various organisms have revealed some general trends concerning the glacial history of species. High level of genetic diversity is typically found in refugia that have been inhabited for the longest periods, whereas populations that have been subjected to repeated bottlenecks during leading edge phases of colonization following the glaciations are characterised by a loss of genetic diversity. Contact zones where migrants from different refugia meet, on the other hand, tend to have a higher diversity (Hewitt, 1996; Ibrahim et al., 1996; Comes and Kadereit, 1998; Hewitt, 1999; Widmer and Lexer, 2001).

At the population scale, life history traits and ecological factors affecting reproduction and dispersal in plants are likely to be of particular importance in determining genetic structure (Loveless and Hamrick, 1984). In the study by Loveless and Hamrick (1984), life form, geographic range, breeding system and taxonomic status were found to have significant effects on the partitioning of genetic diversity within and among plant populations. In a recent comparative study by Thiel-Egenter

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et al. (2009), wind pollination and wind dispersal were found to be associated with high genetic diversity in high-mountain plants.

Figure 1. *Dryas octopetala* populations sampled for this study. The distribution of *Dryas* (dotted line and hatched) is redrawn from Hultén and Fries (1986)

Dryas L. (Rosaceae) is one of the hardiest of all woody plants and, in comparison to most other arctic-alpine plants, unusual in its diploid, dwarf-shrub habit with slow generation turnover within populations (Max et al., 1999). Evolution in *Dryas* might therefore be slower than in herbaceous arctic-alpine plants, which to a high degree display allopolyploidy (Brochmann et al., 2004). *Dryas octopetala* is a mainly outcrossing species (Elkington, 1971; McGraw, 1987; Molau, 1993). The flower is insect pollinated, the seeds are wind dispersed and the plant has the potential to spread lateral by vegetative ramets (Wookey et al., 1995).

Dryas octopetala L. is circumpolar and distributed from the boreal to the arctic zone where it is an important component of the vegetation carpet of tundra and calcareous alpine heaths (Fig. 1; Walker et al., 1994). The species belongs to a circumpolar genus where the number and delimitation of species have not been fully resolved (Elkington, 1965; McGraw, 1987; Yurtsev, 1997; Nordal et al., 1999; Siegismund and Philipp, 1999; Philipp and Siegismund, 2003). *Dryas* has a

geographically structured morphological variation that can be divided into several species, and the species frequently hybridize (Hultén, 1968). Historically, there are two main approaches to the taxonomy of the genus, the Russian approach; with fifteen species recognized globally (Yurtsev, 1997) and the Hultén approach, accepting only three species and some subspecies worldwide (Hultén, 1968; Elven et al., 2008). The major difference between the Hultén and the Russian approaches lies in the circumscription of *D. octopetala*. Hultén's *D. octopetala* s. lat. with six sub-species was considered as three subsections with nine species and seven additional subspecies by Yurtsev (1997). Russian botanists and a few North American botanists have mainly followed the Russian approach, whilst most botanists in North America, Greenland and non-Russian Europe have followed the Hultén approach for the last 30 years (Elven et al., 2008). The Hultén approach recognises only one species (*D. octopetala*) on Svalbard (Elven and Elvebakk, 1996; Siegismund and Philipp, 1999) whereas the Russian botanists, represented by Yurtsev (1997), have recognized three taxa: *D. octopetala* subsp. *subincisa* Jurtz., *D. punctata* Juz. subsp. *punctata* and their hybrid, *D.* x *vagans* Juz. *Dryas punctata* is distinguished morphologically from *D. octopetala* by having stipitate, yellowish, brown or purple glands, and the hybrid *D.* x *vagans* in having glands but at a much lower density (Yurtsev, 1997). Nordal et al. (1999) considered the gland character as an intraspecific polymorphism within *D. octopetala*.

Skrede et al. (2006) investigated the phylogeography of *D. octopetala* in Eurasia using amplified fragment length polymorphisms (AFLPs; Vos et al., 1995). They found that the Eurasian plants were separated into two main groups that probably reflect isolation in and expansion from two major glacial refugia, located south and east of the North European ice sheets. Their results indicated that virtually all of northwestern Europe as well as East Greenland have been colonized by the Southern lineage, while northwestern Russia, the Tatra Mountains and Svalbard have been colonized by the Eastern linage. Plants from East Greenland clustered together with Beringian populations of *Dryas*.

AFLPs have frequently been used in plant phylogeography (Eriksen and Topel, 2006; Alsos et al., 2007; Eidesen et al., 2007). However, AFLPs are dominant and anonymous, and co-migrating fragments are not necessarily homologous (Skrede et al., 2008), and to this date, no ideal marker has been developed for phylogeographic inference in plants. Microsatellite markers have in the later years

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emerged as one of the most popular choices for studies at population level, including migration rates, population size, bottlenecks and kinships (Selkoe and Toonen, 2006). Microsatellites are co-dominant, simple sequence repeats of one to six nucleotides that are present in the genome of most taxa. They mutate frequently by slippage and proofreading errors during DNA replication, causing a change in the number of repeats and thus the length of the repeat string (Eisen, 1999). Hence, the polymorphisms of microsatellites derive from variability in length rather than in point mutations (Ellegren, 2004). However, the rapid evolution of microsatellites may cause homoplasy, which again may blur genetic structure at a larger phylogeographic scale.

For this study, ten microsatellite primer pairs have been developed to infer phylogeographic patterns and genetic structure of *Dryas octopetala* populations within the North Atlantic region. The microsatellite results will be compared to the results from the AFLP study by Skrede et al. (2006) on the phylogeography of *D. octopetala*. The population structure at different spatial scale will be related to reproductive biology, growth habit and other life history traits of *D. octopetala*. In addition, the molecular data will be used to discuss the recognition of one or more *Dryas* taxa on Svalbard.

Material and Methods

Sampling

Leaves from 461 plants of *Dryas octopetala* were collected from five main localities situated in mainland Norway and Svalbard (Fig. 1; Table 1). At each main locality plants were sampled from three 6 x 6 m sub-localities separated by approximately one kilometre. At each sub-locality one plant was sampled in each square of the 6 x 6-meter grid, when possible. If fewer than 30 individuals were found inside the grid, the grid was extended by additional squares to meet this requirement. At the geographically restricted Langesund locality, plants were collected only from one sub-locality. Green leaves were collected on silica to quickly dry the organic material to preserve the DNA for the later extraction. On Svalbard the presence or absence and the density of glands on the leaves were recorded for all collected plants. In addition, five plants from each sub-locality were pressed as voucher specimens, and deposited at the Natural History Museum in Oslo (O). DNA material previously obtained from three Greenlandic and five Russian locations (Skrede et al., 2006) were also included (Fig. 1; Table 1). Ten to eleven individuals were sampled from each of these localities.

DNA extraction

DNA was isolated from silica-dried leaves from between 24 and 40 individuals from each sub-locality (Table 1) following a slightly modified version of the acidic DNA extraction protocol by Ziegenhagen (1993). The modification included an extra purification step; before the pellet was air-dried, 1 mL 70% ethanol was added, and the samples were centrifuged for 2 min at 13,000 rpm. The DNA concentrations were measured with a spectrophotometer (NanoDrop, ND-1000, Thermo Fisher Scientific, Wilmington) and diluted 100 times to obtain an approximate concentration of 5ng/μL.

Microsatellite analysis

For this study, ten *D. octopetala* microsatellite markers (Table 2) were developed by the firm ecogenics GmbH (Zürich) using the following protocol. An enriched library was made from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour et al., 1994) and enriched by magnetic bead selection with biotinlabelled $(CT)_{13}$, $(GT)_{13}$, $(GTAT)_7$, and $(GATA)_7$ oligonucleotide repeats (Gautschi et al.,

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2000a; Gautschi et al., 2000b). Of 378 recombinant colonies screened, 81 gave a positive signal after hybridization. Plasmids from 63 positive clones were sequenced and primers were designed for 21 microsatellite inserts, 20 of which were tested for polymorphism. Out of these, ten microsatellite markers were found to have a suitable amount of polymorphism.

The sampled plant material was screened for microsatellite variation using the M13 tailing approach by Schuelke (2000). Each microsatellite was amplified separately in 20 µL reaction volumes including 2.0 µL 10x CoralLoad PCR Buffer (Qiagen, Hilden), 2.0 µL 10 mM dNTP, 0.4 µL 10 µM forward primer, 1.6 µL 10 µM reverse primer, 1.6 µL 10 µM fluorescent-labelled M13 primers (Table 2), 0.1 µL HotstarTaq Plus DNA polymerase (Qiagen), 8.3 µL mqH$_2$O and 4 µL diluted DNA. The following PCR conditions were used: an initial denaturation step at 95°C for 5 min, followed by 30 cycles consisting of a denaturation step at 95°C for 30 s, an annealing step with temperatures specified for each marker (Table 2) for 45 s and an extension step at 72°C for 45 s. This was again followed by 8 cycles with a denaturation step at 95°C for 30 s, an annealing step at 53°C for 45 s and an extension step at 72°C for 45 s. The PCR program ended with an extension step at 72°C for 30 min.

Electrophoresis of the microsatellites was done on an ABI 3730 (Applied Biosystems, Foster City) sequencer. Five non-overlapping microsatellites labelled with different colours (4.0 µL of 6-FAM, and 2.0 µL of each of PET, NED and VIC, see Table 2) were prepared to be co-loaded in each electrophoresis run by mixing and diluting ten times before 1 µL of this mix was applied together with 0.2 µL GeneScan 500 (-250) LIZ (Applied Biosystems) size standard and 8.8 µL HiDi formamide (Applied Biosystems). During PCR and electrophoresis, each 96 well plate included two positive internal controls and one negative control. Four samples were included on all plates, as a control between the different runs.

Table 1. Overview of the collected material of *Dryas octopetala*. N is the number of plant individuals collected from each population. Latitude and longitude are given in degrees, positive and negative values indicate west and east respectively. Voucher specimens are deposited at the Natural History Museum, Oslo (O). Percentage of linked loci were only calculated for the populations that were sampled in the standardized way (see text).

Population name (abbreviation)	Country	Locality	Sublocality	N	Year	Collector*	Latitude	Longitude	% linked loci
Langesund (L-1)	Norway	Langesund	Langøya	39	2006	AKB, UV, IN	59.0023	9.7563	14.81 %
Finse (F-1)	Norway	Finse	Kvannjolsnut	31	2006	AKB, UV	60.6076	7.5499	4.94 %
Finse (F-2)	Norway	Finse	Sanddalsnut	34	2006	AKB, UV	60.6149	7.5201	13.58 %
Finse (F-3)	Norway	Finse	Jomfrunut	31	2006	AKB, UV	60.6045	7.5189	9.88 %
Troms (T-1)	Norway	Troms	Fløifjellet	36	2007	UV, MFMB	69.6222	19.0038	6.17 %
Troms (T-2)	Norway	Troms	Lyngsalpene	30	2007	UV, MFMB	69.6941	20.7775	13.58 %
Troms (T-3)	Norway	Troms	Lyngsalpene	30	2007	UV, MFMB	69.7516	20.7386	11.11 %
Longyearbyen (S-1)	Norway	Longyearbyen	Endalen	30	2006	AKB, UV, IN	78.1897	15.7812	3.70 %
Longyearbyen (S-2)	Norway	Longyearbyen	Longyeardalen	24	2006	UV, IN	78.2005	15.5861	12.35 %
Longyearbyen (S-3)	Norway	Longyearbyen	Bjørndalen	31	2006	UV, IN	78.2313	15.3333	8.64 %
Ny-Ålesund (N-1)	Norway	Ny-Ålesund	Below Zeppelinfjellet	34	2006	AKB, HK	78.9130	11.9235	12.35 %
Ny-Ålesund (N-2)	Norway	Ny-Ålesund	Brøggerhalvøya	33	2006	AKB, HK	78.9340	11.8353	11.11 %
Ny-Ålesund (N-3)	Norway	Ny-Ålesund	Brøggerhalvøya	40	2006	AKB, HK	78.9394	11.8020	14.81 %
Greenland (AK-1071)	Greenland	Mestervig	Mestervig	10	2003	OG	72.2425	-23.8975	-
Greenland (AK-112)	Greenland	Dronning Margrethe II Land	Ardencaple Fjord	10	2003	MKH	75.3000	-20.8500	-
Greenland (AK-359)	Greenland	Jameson Land	Constable Point	10	2002	IS, LL	70.7456	-22.6898	-
Russia (AK-144)	Russia	Yamalo-Nenetskiy AO	South Yamal	10	2002	MK	68.2000	68.9000	-
Russia (AK-3713)	Russia	Nenetskiy AO	Nenetskaya Gryada	11	2004	DE	68.3360	53.3000	-
Russia (AK-3508)	Russia	Taymyrskiy AO	Ary-Mas nature reserve	11	2004	AT, PS	72.4644	101.8636	-
Russia (AK-4436)	Russia	Komi Republic	Balbanyu	11	2004	AT, ES, IGA	65.3410	60.7120	-
Russia (AK-4484)	Russia	Yamalo-Nenetskiy AO	Chernaya mountain	11	2004	MK	66.8410	65.5000	-

*AKB: A.K. Brysting, AT: A. Tribsch, DE: D. Ehrich, ES: E. Shobnitsina, HK: H. Kauserund, IGA: I.G. Alsos, IN: I. Nordal, IS: I. Skrede, LL: L. Lund, MFMB: M.F.M. Bjorbækmo, MK: M. Kapralov, MKH: M.K. Holte, OG: O. Gilg, PS: P. Schönswetter, UV: U. Vik.

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Table 2. Characterization of the ten *Dryas octopetala* microsatellite markers developed for this study. F gives the forward 5´-3 primer and R the reverse 5´-3´ primer. Repeat types were observed in sequenced clones. T_A gives annealing temperatures. Number of alleles is based on the 471 individuals in this study. Mix A and B represent the markers which were co-loaded on the ABI-sequencer. Scoring error was calculated from two independent scorings of the dataset, and the allelic dropout was calculated from replicate runs. Marker D20 was left out of the analysis, and number of alleles, scoring error and allelic dropout thus not calculated.

Locus	Primer sequences 5´- 3´	Repeat types	M13 lable	T_A	Size range (bp)	MIX	No. of alleles	Scoring error	Allelic dropout
D3	F: TTTGCAAAACAACAAACAGTTG R: GTGTGGCAAGACTCGAGAGC	$(GTAT)_9$	PET	60	144-198	A	22	0.37%	2.17%
D5	F: AGCGTGCCTTAATTGCATTC R: TCTGTGGTTCCACAAAGTGC	$(GATA)_6$	NED	56	194-213	A	8	0.18%	0.86%
D8	F: CTTGCTGCGACCAGATTTTC R: TAGGGCGCTCTAAGAACCAC	$(TC)_{28}$	NED	56	166-241	B	39	1.84%	4.16%
D10	F: AAGGCTACGGAAAAGCTTGC R: CACAGCAATGATATATGTTGAGAGG	$(CT)_7\,GTCA\,(CT)_5\,(CA)_{12}$	FAM	56	222-314	A	43	1.48%	2.05%
D11	F: GCGATTCCGAATTTTACAGG R: TCCCGAAGAACTCTTCCTAGC	$(CT)_{20}$	PET	56	169-209	B	19	0.59%	7.80%
D13	F: ACCTGAATGGTCTTCCCAAG R: ATGCCGTTTTATGCTTCGTG	$(GTAT)_7$	FAM	56	90-192	B	39	0.39%	1.95%
D14	F: GTTAGGCATCACCACAATGC R: CAAACTGTTGTTGCAAAGATGG	$(CT)_{21}$	FAM	56	172-230	B	10	0.78%	4.23%
D17	F: CAGCAGCATGAGCTGAAAAG R: AAGCACTTAAAAGCATGTGTGC	$(AT)_4(GT)_{13}$	VIC	56	144-174	B	11	0.00%	0.00%
D20	F: TTTTTGGTTACTGTTATCTGCATC R: AATCCCGTACCAGGAAAACC	$(AT)_5\,(GTAT)_9\,(GT)_{11}$	FAM	56	144-168	A	-	-	-
D21	F: CCGGAAAGCACCATTAGTTG R: TCATGTGGGACTAAGGGATTC	$(TACA)_6$	VIC	50	155-246	A	23	0.20%	0.00%

Statistical analysis

The microsatellite markers were scored using the program GENEMAPPER version 3.7 (Applied Biosystems). One marker (D20) was left out from the following analyses because it was too difficult to score. Individuals where more than two peaks were detected for a single locus and more than three of the nine microsatellite loci failed to amplify were removed from the dataset. To calculate a scoring error, the entire dataset was scored twice and the ratio between observed number of allelic differences and total number of allelic comparisons obtained. The last scored dataset was used further in all analyses. Calculation of the ratio between observed number of allelic differences and the total number of allelic comparisons was also performed on replicates and on negative controls to estimate the error between and within runs and to detect null alleles (allelic dropout).

NTSYS$_{PC}$ version 2.1 (Rohlf, 2000) was used to visualize genetic structure in the data by running a principal coordinates analysis (PCO) on an allelic presence/absence dataset with simple matching similarity.

The dataset was clone-corrected by removing all individuals from the same sub-localities with identical multi-locus genotypes, and converted into ARLEQUIN

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(Excoffier et al., 2006) and STRUCTURE (Pritchard et al., 2000) input format using the program CONVERT (Glaubitz, 2004).

STRUCTURE version 2.2 is a model-based clustering method for multi-locus genotype data for inferring population structure and assigning individuals to populations, developed by Pritchard et al. (2000). A number of potential clusters (K) between one and eighteen were tested, with simulations that were run for 10^6 iterations following a burn-in length of 10^5 iterations using the freely available Bioportal computer service (University of Oslo, http://www.bioportal.uio.no). For each K, ten rounds were run. STRUCTURE was set up to use an admixture ancestry model and run twice on the data, the first time using a correlated allele frequency model (Falush et al., 2003), where the frequencies in the different populations are likely to be similar, and the second time using an independent allele frequency model, where the model assumes that the allele frequencies in each populations are independent. A hierarchical structure analysis was run on all groups detected after the first run to identify further sub-structure within these groups, using the same parameters as for the first runs, but restricting K to nine and only using a correlated allele frequency model. The statistical package R version 2.8.0 (http://cran.r-project.org/) was used to summarize outputs of STRUCTURE using a collection of R functions, STRUCTURE-SUM (Ehrich, 2006).

ARELQUIN version 3.1 was used to infer various population genetic statistics. A standard analysis of molecular variance (AMOVA) was used to analyze at which level the genetic variation was distributed, i.e., within populations, between populations, or between regions. The significance of covariance components associated with the different possible levels of genetic structure was tested using non-parametric permutation procedures (Excoffier et al., 1992). To compute the dissimilarities between the populations, pairwise F_{ST} values were calculated for all pairs of populations. Expected heterozygosity and the number of alleles were computed as standard diversity indices for each locus, as well as a mean for the populations. To test the hypothesis that the observed diploid genotypes are product of a random union of gametes, observed and expected heterozygosity with associated P-vaules were calculated for each marker in each sub-population from Troms, Finse, Langesund, Longyearbyen and Ny-Ålesund, where the populations were sampled in a standardized fashion, using a clone-corrected dataset. The calculations were performed by an exact locus by locus test of Hardy-Weinberg equilibrium using 10^5

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steps in the Markov chain and 10^3 dememorization steps. A likelihood ratio test of linkage disequilibrium was performed for the sub-populations using 10^4 permutations and ten initial conditions (Excoffier and Slatkin, 1998).

To address the taxonomical issue of *Dryas* on Svalbard, i.e. whether the three morphotypes of *Dryas* (*octopetala*, *punctata* and x *vagans*) can be assigned to three taxa, a PCO analysis was conducted on a clone-corrected dataset consisting of the 178 Svalbard individuals in NTSYS_{PC} version 2.1 with simple matching similarity. An AMOVA was performed using ARELQUIN version 3.1 to investigate whether the genetic variation was distributed among or within the three morphotypes.

Results

A total of 471 individual plants were successfully scored for six or more of the nine microsatellite markers, and 214 different alleles were detected. Among the 471 plants, the scoring error varied from 0.0 % (D17) to 1.8 % (D8), and the allelic dropout varied from 0.0 % (D17, D21) to 7.8 % (D11; Table 2). Ninety plants shared the same multi-locus genotype with another plant and were removed in the clone-corrected dataset. Noteworthy, 95.2 % of the plants that shared multi-locus genotype came from the same sub-locality. Six plants from different sub-localities but within the same main locality (Troms) shared multi-locus genotypes, while no plants from different main localities had a similar multi-locus genotype.

Phylogeography

The PCO analysis of the total dataset structured the plants largely according to their geographic origin (Fig. 2). Plants from Svalbard and mainland Norway appeared at the extremes of component axis one (explaining 12.0 % of the variation) with plants from Russia and Greenland grouping in between. The
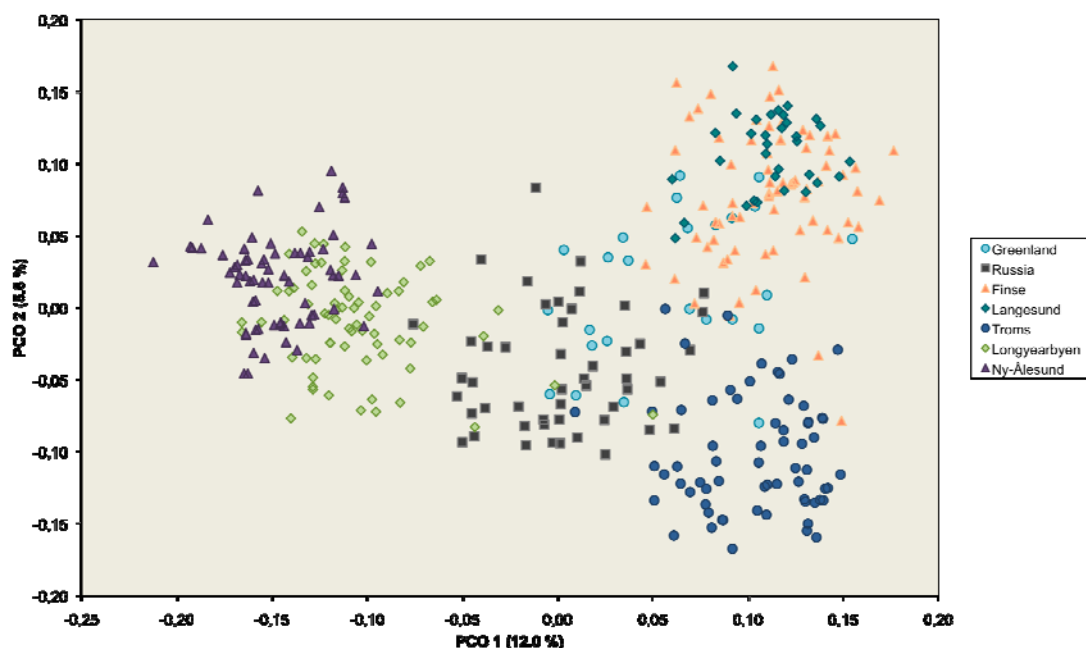

Figure 2. Principal coordinate analysis of 471 *Dryas octopetala* individuals based on the allelic presence/absence dataset of the nine microsatellites and using simple matching similarity.

second axis, explaining 5.6 % of the variation, separated plants from mainland Norway into two more or less distinct groups, one including plants from

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Langesund and Finse (both South Norway) and the other including plants from the Troms population (North Norway).

The STRUCTURE analysis, run on the clone-corrected dataset (391 plants), grouped the analysed material into three clusters, both when analyzed with independent and correlated allele frequency models (Fig. 3a-b, Fig. 4a-b). One cluster included plants from the Greenland, Troms and Russian populations, a second cluster included plants from Langesund and Finse (southern Norway cluster), and a third cluster included the plants from Longyearbyen and Ny-Ålesund (Svalbard cluster). Some individuals in each cluster were not allocated unambiguously. This was especially prominent in the analysis using the independent allele frequency model (Fig. 3b, Fig. 4b), where some individuals from the Greenland, Troms and Russian populations were more closely related to individuals from the southern Norwegian cluster.
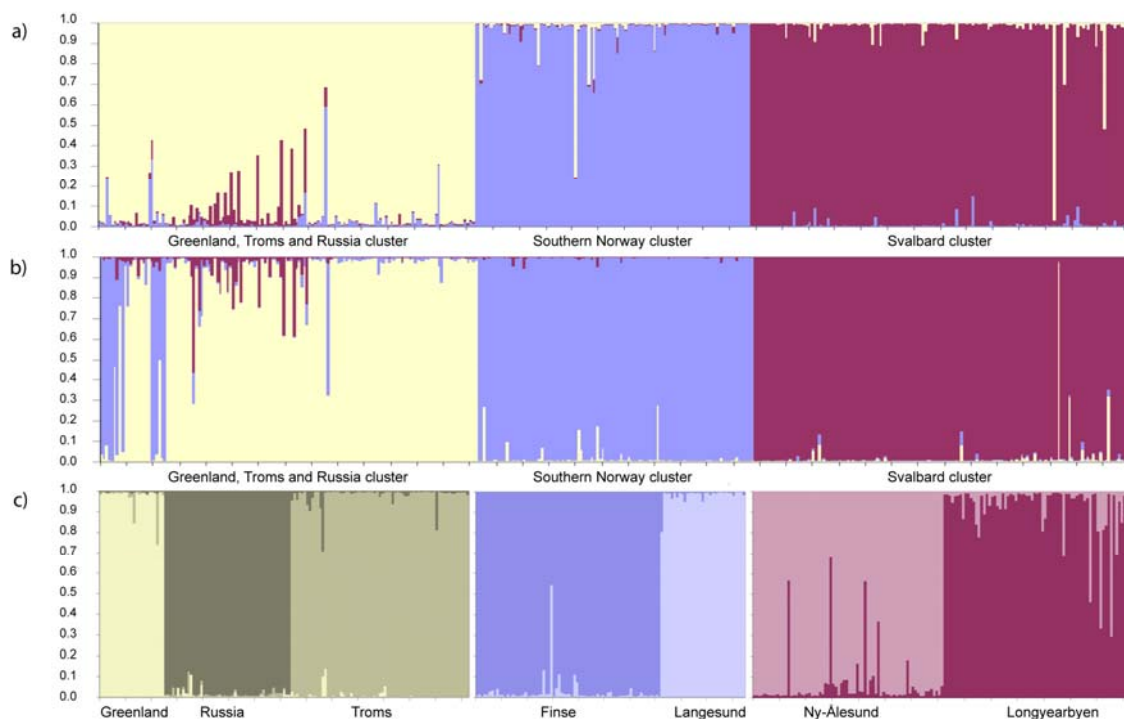
Figure 3. Summary plot of cluster estimates identified from the STRUCTURE analysis based on the clone-corrected dataset consisting of 391 individuals of *Dryas octopetala*. Each individual is represented by a single vertical line broken into K colored segments, with lengths proportional to each of the K inferred clusters. Structure clusters are based on a) correlated allele frequency model, b) independent allele frequency model and c) from hierarchical structure with correlated allele frequency model.

The hierarchical structure analyses further divided the identified clusters into smaller sub-clusters, where plants from each main locality constituted separate sub-clusters (Fig. 3c, Fig. 4 c-e). Noteworthy, the Longyearbyen and Ny-Ålesund

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sub-clusters (both Svalbard) had higher proportions of plants allocating to each other compared to any of the other sub-clusters (Fig. 3c).
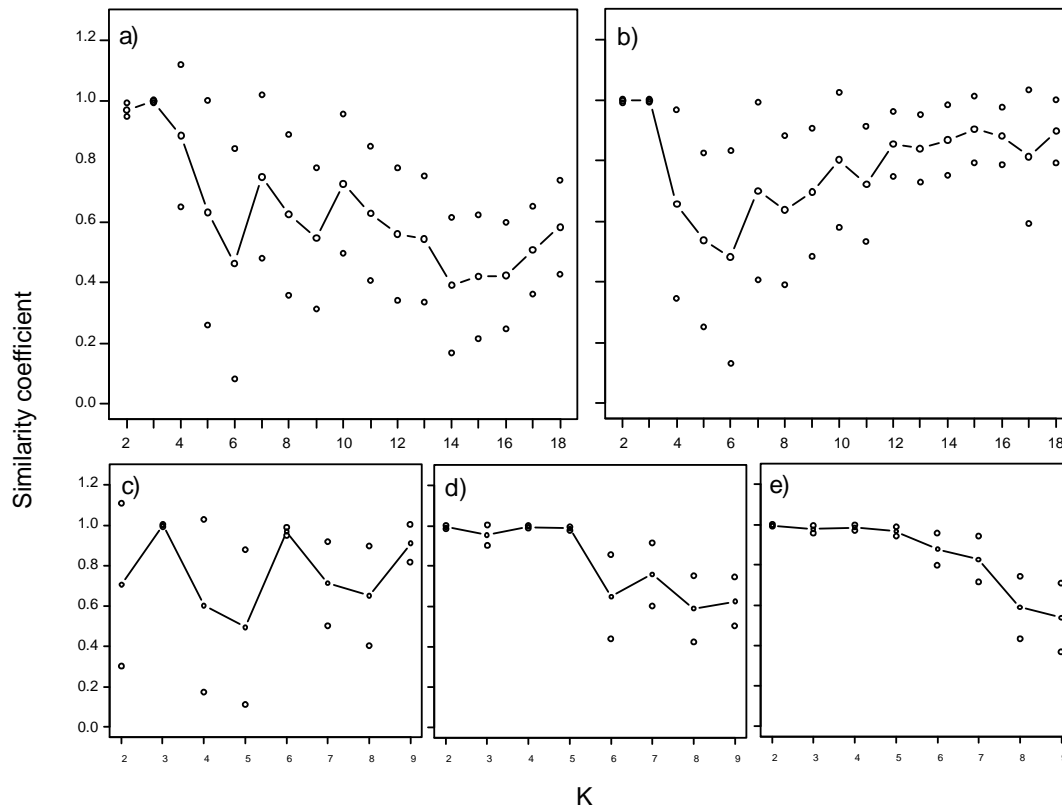

Figure 4. Plot of estimated similarity coefficients for the STRUCTURE clusters of 391 individuals of *Dryas octopetala*. Both the correlated run (a) and the independent run (b) identified three clusters, while the hierarchical structure run on individuals from Greenland, Troms and Russia identified three sub-clusters (c), the hierarchical structure run on individuals from Finse and Langesund identified two sub-clusters (d), and the hierarchical structure run on individuals from Svalbard identified two sub-clusters (e).

The AMOVA of the three main groups identified in the STRUCTURE analysis using correlated allele frequencies resulted in 12.89 % variation among groups, 11.37 % among populations within groups and 75.74 % within populations (Table 3). When no grouping of populations was inferred, 22.10 % of the variation was among populations, and 77.90 % within populations. Separate AMOVAs of the different groups were also conducted. In the group including Greenland, Troms and Russia, 15.13 % of the variation was among populations and 84.81 % within populations. The southern Norway group had 15.19 % variation among populations and 84.81 % within populations, and for the Svalbard group, the AMOVA resulted in 7.77 % variation among populations and 92.23 % within populations.

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Table 3. Analysis of molecular variance (AMOVA) of *Dryas octopetala* based on nine microsatellite markers for the whole dataset (a), the clusters detected in STRUCTURE (b-e) and the three morphotypes (i.e., *octopetala*, *punctata* and x *vagans*) on Svalbard (f).

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
a) All populations	Among populations	6	537.481	0.79166	22.10
	Within populations	775	2162.530	2.79036	77.90
b) Three clusters	Among groups	2	354.337	0.47486	12.89
	Among populations within groups	4	183.144	0.41886	11.37
	Within populations	775	2162.530	2.79036	75.74
c) Greenland, Troms and Russia cluster	Among populations	2	99.181	0.52669	15.13
	Within populations	283	836.193	2.95474	84.87
d) Southern Norway cluster	Among populations	1	51.809	0.53912	15.19
	Within populations	208	626.215	3.01065	84.81
e) Svalbard cluster	Among populations	1	32.154	0.20762	7.77
	Within populations	284	700.122	2.46522	92.23
f) Morphotypes on Svalbard	Among taxa	2	13.669	0.08280	3.09
	Within taxa	303	787.252	2.59819	96.91

Pairwise F_{ST} values were calculated among all populations and ranged from 0.078 between Longyearbyen and Ny-Ålesund, to 0.379 between Ny-Ålesund and Langesund (Table 4), indicating that Ny-Ålesund and Longyearbyen are the least genetic separated populations, whilst Ny-Ålesund and Langesund are the most genetically differentiated populations.

Out of the standard diversity indices calculated for the different populations (Table 5), Greenland (the three Greenland populations treated as one population) had the highest expected heterozygosity with 0.7740, whilst the lowest expected heterozygosity was found for Ny-Ålesund and for Langesund (0.5425 and 0.5482, respectively). Out of the populations sampled in the same standardized way using 6 x 6 m grids (i.e. Langesund, Finse, Troms, Longyearbyen and Ny-Ålesund), Finse had the highest heterozygosity (0.7401), whilst Langesund and Ny-Ålesund had the least. The highest mean number of alleles was found for the Russian population (the five Russian populations treated as one population) with 13.2 alleles and the lowest for Langesund with 4.78 alleles.

Table 4. Pairwise F_{ST} values calculated for the *Dryas octopetala* populations. All populations were significantly differentiated from each other (p<0.05).

	Greenland	Russia	Troms	Finse	Langesund	Ny-Ålesund
Russia	0.136	0.000				
Troms	0.179	0.145	0.000			
Finse	0.116	0.158	0.149	0.000		
Langesund	0.246	0.256	0.279	0.152	0.000	
Ny-Ålesund	0.265	0.215	0.331	0.268	0.379	0.000
Longyearbyen	0.217	0.165	0.281	0.231	0.339	0.078

Table 5. Standard diversity indicies: Expected heterozygosity (H_E) and mean number of alleles for the *Dryas octopetala* populations.

Population	H_E mean + s.d.	# of alleles + s.d
Greenland	0.7740 ± 0.1081	6.889 ± 2.892
Russia	0.7322 ± 0.2052	13.222 ± 8.182
Finse	0.7401 ± 0.1051	10.667 ± 4.472
Langesund	0.5482 ± 0.2276	4.778 ± 2.279
Ny-Ålesund	0.5425 ± 0.2693	8.000 ± 5.292
Longyearbyen	0.5770 ± 0.3001	8.444 ± 6.803
Troms	0.6010 ± 0.2556	9.444 ± 6.064

Population structure

In the analyses of plant population structure, only plants from the southern Norwegian, Troms and Svalbard populations, where plants had been sampled in a grid fashion, were included. The 13 different sub-populations were analysed separately. Plants within sub-populations with identical multi-locus genotypes (presumably representing the same genet) were mapped onto the grid in order to visualize the distribution of genets (Fig. 5). In general, plants sharing the same multi-locus genotype occurred adjacent in the grid. As can be observed in Fig. 5, the proportion of ramets in each grid varied among the sub-populations but there was apparently no systematic trend across populations.

Table 6. Results from tests of deviations from Hardy-Weinberg expectations (P-values) for the 13 sub-populations. Numbers in bold indicate significant deviations from the expected genotype frequencies. Heterozygote excesses are indicated with plus, and deficits with a minus sign. Loci that were monomorphic in a population are indicated with "-".

| Locus	F-1	F-2	F-3	L-1	T-1	T-2	T-3	S-1	S-2	S-3	N-1	N-2	N-3
D3	+ 0.235	- 0.088	+ 0.618	+ 0.129	+ 0.807	- 0.162	+ 0.623	- 0.552	+ 0.957	- 0.336	**- 0.002**	+ 0.155	+ 0.652
D5	+ 0.663	+ 0.970	- 0.587	+ 1.000	- 0.528	+ 1.000	- 1.000	+ 1.000	+ 1.000	+ 0.128	+ 1.000	+ 0.671	+ 1.000
D8	+ 0.584	+ 0.966	+ 0.739	+ 0.853	- 0.051	+ 0.412	+ 0.168	- 0.177	- 0.496	**- 0.029**	+ 0.504	+ 0.802	- 0.090
D10	- 0.390	- 0.106	- 0.346	- 0.190	**- 0.004**	**- 0.000**	+ 0.642	- 0.122	**- 0.000**	**- 0.001**	+ 0.248	**- 0.000**	+ 0.326
D11	**- 0.000**	- 0.209	**- 0.026**	**- 0.002**	**- 0.044**	+ 0.368	+ 0.488	**- 0.002**	**- 0.001**	+ 0.261	**- 0.001**	- 0.134	+ 0.245
D13	- 0.457	- 0.283	**+ 0.027**	**- 0.003**	+ 0.353	- 0.385	**- 0.032**	- 0.343	+ 0.480	- 0.053	+ 0.631	+ 0.132	- 0.210
D14	+ 0.495	+ 0.761	- 0.195	+ 1.000	**- 0.018**	**- 0.001**	+ 0.736	-	-	1.000	+ 1.000	+ 1.000	+ 1.000
D17	- 0.612	+ 0.364	+ 0.591	- 1.000	-	-	-	+ 1.000	- 0.540	- 0.099	- 0.205	+ 1.000	-
D21	+ 0.640	- 0.090	+ 0.111	- 0.510	- 0.067	- 0.483	-	- 0.693	+ 0.051	+ 0.454	+ 0.764	+ 0.185	**+ 0.004**

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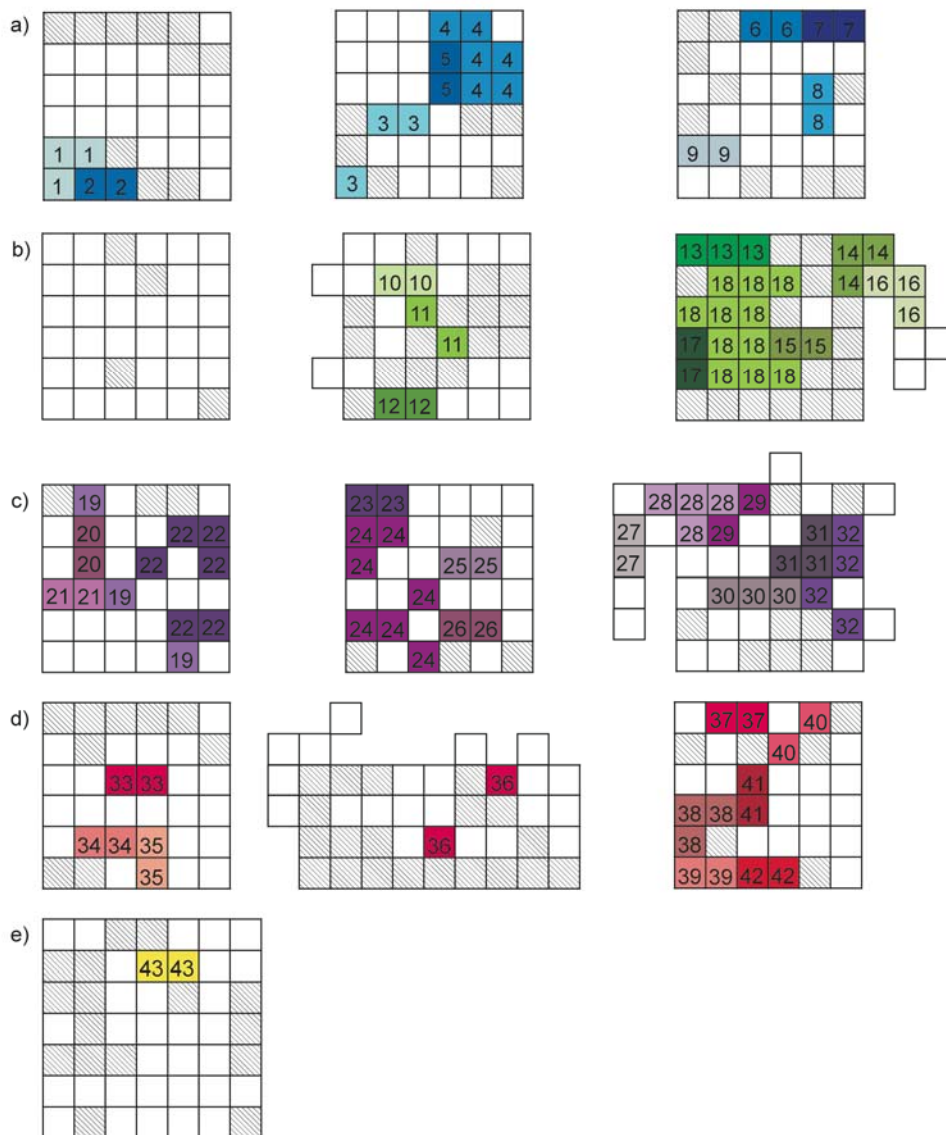
Figure 5. Distribution of 124 *Dryas octopetala* genets mapped onto the grid of each sub-population. Uncoloured squares represent samples with unique multi-locus genotype. Coloured and numbered squares represent samples with identical multi-locus genotype. Different shades of blue represent the distribution of clones in the Finse population (a), green in the Troms population (b), purple in the Ny-Ålesund population (c), red in the Longyearbyen population (d) and yellow in the Langesund population (e). Hatched squares are those where *D. octopetala* was not growing and thus not sampled, or represent samples that were not included in the analyses.

In general, the allele frequencies at the nine loci were in accordance with Hardy-Weinberg expectations across all sub-populations with a few deviations (Table 6). Both significant heterozygote excesses (1.7 % of the loci across all populations) and deficits (14.5 % of the loci across all populations) occurred.

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The likelihood ratio test of linkage disequilibrium between all combinations of loci within sub-populations, revealed that in average 10.54 % of the combinations were in linkage disequilibrium, ranging from 3.70 % in the S-1 (Longyearbyen) sub-population to 14.81 % in the L-1 (Langesund) and N-3 (Ny-Ålesund) sub-populations (Table 1).

Taxonomy

In a PCO analysis of the three morphotypes found on Svalbard (i.e., *octopetala*, *punctata* and x *vagans*), six individuals were removed as they had missing data for the D11 marker, and consequently grouped as outliers. One of the removed individuals was of *punctata* type, whilst the others were of *octopetala* type. The two Svalbard populations (Longyearbyen and Ny-Ålesund) were separated along component axis one, which explained 10.27 % of the variation (Fig. 6a). Component axis two explained 7.99 % of the variation. Individuals of *punctata* and x *vagans* type mainly occurred within the Longyearbyen group, but were here intermingled with individuals of *octopetala* type, and no structuring relating to morphotypes was observed (Fig. 6b). The AMOVA of the three morphotypes resulted in 3.09 % variation among the morphotypes, and 96.91 % variation within the morphotypes (Table 3f).
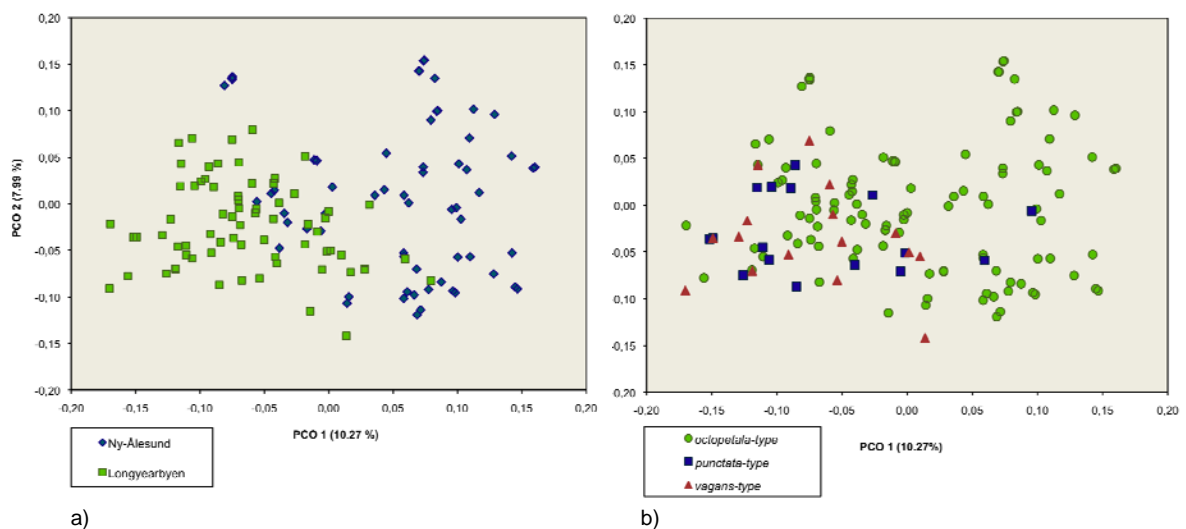

Figure 6. Principal coordinate analyses of the 178 *Dryas octopetala* individuals sampled from Svalbard using an allelic presence/absence dataset of the nine microsatellites and simple matching similarity coefficient. Information on a) geographic origin of the samples (i.e., Longyearbyen and Ny-Ålesund) and b) the three morphotypes (i.e., *octopetala*, *punctata* and x *vagans*) is mapped on to the PCO plot.

Discussion

New microsatellite markers for *Dryas octopetala*

In this study ten newly developed microsatellite markers for *D. octopetala* have been tested and evaluated. Of the ten markers developed, nine were used in the analyses and found to be polymorphic, including from eight to 43 alleles per loci analysed. In six of the nine Troms and Svalbard sub-populations, one or two markers were fixated and thus did not contribute with any genetic information. Overall, the microsatellites seemingly provided a resolution that was high enough to distinguish between individuals (see below), as well as providing a genetic pattern at a larger geographic scale. Whereas phylogeographic studies of animals often are based on microsatellites (e.g., Adams et al., 2006; Goropashnaya et al., 2007; Nittinger et al., 2007; Rossiter et al., 2007), plant studies are often based on AFLPs, except for studies of plants closely related to the genome sequenced *Arabidopsis thaliana* (L.) Heynh (e.g., Carlsen et al. 2007; Jørgensen et al., 2008). Few microsatellite makers have been developed for plants in general, and especially for arctic-alpine plants. The polyploidy of many arctic-alpine plants causes most microsatellite loci to be multi-allelic, making it difficult to analyse the information generated with existing software. However, microsatellites have been developed for some diploid arctic-alpine plants (e.g., Mariette et al., 2001; Skrede et al., In press). The microsatellites developed for this study provide a useful tool for further studies of *D. octopetala,* and perhaps close relatives*,* both at a larger, phylogeograpic scale, and at a smaller population scale where standard population genetics can be applied.

Phylogeography in North Europe as revealed by microsatellite markers

The STRUCTURE analyses group the *D. octopetala* material into three main groups related to latitude; one southern group, one intermediate group and one northernmost group. At a smaller scale, the hierarchical STRUCTURE analyses showed that all the sampled regions (i.e., Greenland and Russia) and populations were genetically differentiated. Only a very small proportion of the individuals allocated to other clusters than the cluster containing the population they were sampled from. However, in spite of this differentiation, most of the genetic

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variation occurred within populations/regions. As judged from the microsatellite markers, Greenland is the genetically most variable group, followed by the Finse population and Russia. However, even though they were treated as one group in several of the analyses, individuals from Greenland and Russia were sampled from much broader geographic regions and in another manner than the other populations, making direct comparisons of genetic diversity difficult.

At the last glacial maximum, the European ice sheet extended south to 52° N and permafrost south to 47° N. As the ice melted, species from the tundra, cool temperate and warm temperate habitats migrated northwards. Most temperate species in North Europe had their last glacial maximum refugia in the south of Europe, and the colonization northwards was rapid (Hewitt, 1999). In addition, an eastern refugium has been suggested for several organisms (e.g., Taberlet et al., 1998; Skrede et al., 2006). As noted by Taberlet et al. (1998), the combination of intraspecific polymorphism and fossil data makes it possible to identify postglacial immigration routes. The past distribution of *Dryas* in Europe has long been particularly well known because of its abundant occurrence as a macrofossil in Quaternary deposits in northern and central continental European lowlands (Tralau, 1963). This past distribution in the lowlands contrasts strongly with its modern distribution in Europe, where it is restricted to mountain areas (Birks, 2008). When *Dryas* populations were isolated in refugia during glacial periods probably only minor differentiation took place due to the long generation time and thereby slow evolution, and when the distribution area expanded during warmer periods, plants from different refugia met and could still freely exchange genes. Low level of interpopulational isozyme variation (Max et al., 1999; Philipp and Siegismund, 2003) and the high level of shared microsatellite variation across populations observed in this study support this scenario.

The restricted sampling of this study with regard to number of populations, especially when it comes to potential refugium areas in central, southern and eastern Europe (Skrede et al., 2006), limits the inference of possible immigration routes of *D. octopetala* to the North Atlantic area. Nevertheless, two main scenarios may be suggested from the observed microsatellite patterns. The high level of shared microsatellite variation between populations, the existence of three main groups related to latitude and generally lower diversity in the north (i.e., on Svalbard) than further south could support a scenario of a stepwise immigration on

a broad front from the south. However, the results could also point towards two or possibly three main immigration routes following the glacial retreat. Major expansions of *D. octopetala* from the south as well as from the east are likely immigration scenarios for the southern and the intermediate group, respectively.

It seems likely that the two southern Norwegain populations, Finse and Langesund, share an immigration route from the south closely following the ice-edge as it withdrew. The Langesund population occurs today in a small isolated coastal area within the potential forest zone as a relict population from when the ice retreated (Dyring, 1911; Lid, 1958), which is reflected in the low genetic diversity found in this population.

The close link between individuals from Greenland, Troms and Russia supports a shared immigration route, probably from a refugium in the east. However, in the STRUCTURE analysis using an independent allele frequency model, some individuals from Greenland allocated to the southern Norwegian cluster, suggesting that there might have been several dispersal events to Greenland, both from the east and from the south. A contact zone between immigrants from east and south could be part of the explanation why high genetic diversity is found in Greenland. However, opposed to the clustering from the STRUCTURE analysis, the PCO analysis groups the three mainland Norway populations together, suggesting a close genetic relationship and a possible shared immigration route from the south.

Possible *in-situ* survival during the last glacial maximum cannot be ruled-out for the northernmost group (Svalbard), based on generally high pairwise F_{ST} values as well as the distinct grouping in the STRUCTURE analyses. However, a possible colonization route from the east seems more likely, based on the fact that the Svalbard individuals group close to Russia in the PCO, that the pairwise F_{ST} values are lower between Longyearbyen and Russia than between any other region/population (except Ny-Ålesund), and that a small proportion of Svalbard individuals allocates to the intermediate group (Greenland, Troms and Russia) in the STRUCTURE analyses.

Several other studies have investigated the phylogeography and immigration routes of *D. octopetala* in the North Atlantic area. Based on leaf morphology, Elkington (1965) proposed that *D. octopetala* had immigrated to East Greenland from Svalbard. This was supported by Philipp and Siegismund (2003),

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who used a combination of morphological and genetic characters for their interpretation, and noted that the high genetic diversity in East Greenland could be the result of immigration from Svalbard combined with possible hybridization with the North American *D. integrifolia* M.Vahl. Chlebicki et al. (2005) used observations of microfungi growing on *D. integrifolia* and *D. octopetala* as a method to explain the immigration routes of the two species to Greenland. They suggested that the similarity of the Greenland and the Ural Mountains mycobiota could indicate a migrational model of colonization of *D. octopetala* to Northeast Greenland from Ural by wind, driftwood, and migratory birds.

Skrede et al. (2006) studied the phylogeography of *D. octopetala* in Eurasia using AFLPs. Their results indicated two main immigration routes for the North Atlantic plants. One group, referred to as the European group, probably survived in a major southern refugium between the Scandinavian Ice Sheet and the Alps during the last glaciation, and expanded northwards as the ice retreated and colonized northwestern Europe, Iceland and East Greenland. A second group, referred to as the Eastern group, probably survived the last glaciation in a major refugium in northern Russia/eastern Europe. Their data provided strong evidence of repeated colonization of Svalbard by this eastern linage. The predominant source area was also found to be northwestern Russia for several other plant species occurring on Svalbard, whereas colonization from Scandinavia to Svalbard seemed to be rare (Alsos et al., 2007). Although *in-situ* glacial survival of *D. octopetala* on Svalbard could not be excluded, Skrede et al. (2006) concluded that it did not provide a reasonable explanation for the high diversity they observed for this area. Tremblay and Schoen (1999) investigated the phylogeography of the closely related *D. integrifolia* in North America, and suggested that this species survived in glacial refugia south of the American ice sheets as well as in the Canadian Arctic.

The results from this study are congruent with the hypothesis of several immigration routes of *D. octopetala* into the North Atlantic region, as suggested by Skrede et al. (2006). However, the main patterns revealed by microsatellites and AFLPs differed in several aspects, first of all with regard to populations from Svalbard and Greenland. The microsatellite patterns did not show the same strong support for an eastern immigration route to Svalbard or for a mainly southern immigration route to Greenland, as was suggested by Skrede et al. (2006). The

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close association between Greenland and especially Russia, which was indicated by microsatellite patterns, was contradicted by AFPLs, which grouped Greenland together with North American populations. However, it cannot be ruled out that the Greenland populations would have clustered differently if North American samples had been included in the present study.

The fact that the phylogeographic structure obtained in this study differs somewhat from that observed by Skrede et al. (2006) might of course be related to the different sampling of the two studies, but it might also be due to the different markers used. As Gaudeul et al. (2004) noted, two extreme strategies can be adopted when choosing a molecular technique for genetic diversity assessment; sampling numerous poorly informative markers (AFLPs) or sampling few highly informative markers (microsatellites). Several studies have compared AFLPs and microsatellites at smaller, regional scales (Mariette et al., 2001; Gaudeul et al., 2004; Woodhead et al., 2005; Sønstebø et al., 2007). However, at larger geographic scales, fewer studies have inferred a direct comparison of the two marker systems. Skrede et al. (2008) compared genetic structuring and diversity in three circumpolar species of *Draba* L. using 160 AFLP markers and 10 microsatellite loci. They found more distinct genetic clustering in the three *Draba* species using AFLP markers than microsatellite loci, and suggested that AFLPs are better suited for large-scale phylogeographic studies than microsatellites, as microsatellites may evolve too fast and homoplasy may blur the main structure. They also found that intrapopulation genetic diversity measures based on AFLPs and microsatellites were not correlated. Quite different rarity measures were also obtained for the two types of markers. Owing to the intrinsic differences in mutation rates between the two marker systems, within population diversity estimated with microsatellites should according to Gaudeul et al. (2004) be higher than with AFLPs. In Skrede et al. (2006), where AFLPs were used to infer the phylogeography of *D. octopetala*, AMOVA generally revealed a higher percentage variation among populations compared to the present study based on microsatellite variation. However, this may also be related to the fact that more geographically distinct populations from North America and Central Asia were included in Skrede et al. (2006).

Population structure of *Dryas octopetala* populations

The results show that the nine microsatellite markers to a large extent are able to separate between different genets of *D. octopetala*; 95.2 % of the plants with similar multi-locus genotypes belonged to the same sub-localities and these plants were in most cases sampled adjacent in the grid system. No plants from different main localities shared similar multi-locus genotype. However, between two sub-localities in Troms, T-1 and T-2, six individuals shared the same multi-locus genotype, indicating that the markers did not give a high enough resolution to distinguish between individuals in all cases. One of the markers was fixed in the Troms population, making it more likely that different genets obtain similar multi-locus genotypes in this population.

In general, the results indicate that on a very local scale (i.e. within the sampled 6 x 6 m grids), most plants represent different genets. However, *D. octopetala* individuals can locally also reach a larger spatial distribution by vegetative growth and spread by lateral ramets. For example, one individual in one of the Troms sub-localities covered at least 11 m^2 (T-3). Wookey et al. (1995) suggested that clonal growth of *D. octopetala* enables individuals to persist and grow in an extreme environment where sexual proliferation often is unsuccessful, but no systematic latitudinal trend was found in this study concerning the proportion of genets and ramets in the grids.

The population genetic analyses revealed that genotype frequencies of the local *D. octopetala* populations were mainly in Hardy-Weinberg equilibrium, indicating that the Hardy-Weinberg expectations in general were met. However, most of the loci that deviated significantly from the expected genotype frequency distributions, showed a deficiency of heterozygotes, indicating a small extent of inbreeding or genetic drift. There was no latitudinal trend concerning the heterozygote deficiency; the northernmost populations from Longyearbyen and Ny-Ålesund were, as the other populations, mainly in accordance with Hardy-Weinberg expectations. Furthermore, most of the genetic variation detected in this study occurred within populations, rather than between populations. The obtained results correspond with the fact that *D. octopetala* is a sexual reproducing, predominantly outcrossing, wind-dispersed, long-lived species, which occurs in a late successional stage and consists of large and stable populations (Elkington, 1971; McGraw, 1987; Molau, 1993; Wookey et al., 1995); all factors that

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promotes a high genetic variation within groups and corresponding low genetic divergence between populations. Small amounts of gene-flow as the result of long-distance migration by seeds or even by pollen can prevent divergence between populations and thus reduce the genetic structure among populations. The long-lived habit of *Dryas* may also reduce effects of drift and increase chances of migration and thus also hinder divergence among populations (Loveless and Hamrick, 1984).

Only one *Dryas* species occur on Svalbard

A higher proportion of genetic variation was found between the two sampled Svalbard populations (i.e., Longyearbyen and Ny-Ålesund) than between the three morphotypes based on glands on the leaves. Thus, no support is found for the existence of three *Dryas* taxa on Svalbard as proposed by Yurtsev (1997). This is in accordance with a study by Siegismund and Philipp (1999), where morphological and isozyme characters were investigated for the morphotypes *octopetala* and *punctata* in four populations on Svalbard. As in the present study, they found a larger genetic divergence between geographic populations than between the two groups recorded as *octopetala* and *punctata*, and concluded that there is only one species on Svalbard, i.e., *D. octopetala*. Also the analyses by Skrede (2004) indicate a strictly geographic structuring of the genetic variation in *D. octopetala* on Svalbard. The recognition of only one *Dryas* species on Svalbard is in agreement with the latest version of the Panarctic Flora Checklist (Elven et al., 2008). Here, *D. punctata* is recognised as a species in a more restricted sense than done by Yurtsev (1997), with its distribution limited to North Siberia.

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