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ORIGINAL ARTICLE

Genetic differentiation and postglacial migration of the *Dactylorhiza majalis* ssp. *traunsteineri/lapponica* complex into Fennoscandia

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Abstract Eight variable regions (microsatellites, insertion/deletion and duplication regions) from the plastid DNA genome were analyzed for 91 populations belonging to *Dactylorhiza majalis* ssp. *traunsteineri* and closely related taxa. A total of 36 composite plastid haplotypes were found. The two dominating haplotypes had a clear geographic distribution suggesting at least two separate immigration routes into Scandinavia after the last glaciation: one southwestern route and one or two southeastern routes. *D. majalis* ssp. *traunsteineri* could not be clearly separated from any of the other taxa included in the study except for *D. majalis* ssp. *sphagnicola*. The morphologically similar taxa *D. majalis* ssp. *traunsteineri*, *D. majalis* ssp. *lapponica* and *D. majalis* ssp. *russowii* showed no genetic differentiation, and therefore we suggest an amalgamation of the three taxa into one broadly circumscribed subspecies; *D. majalis* ssp. *lapponica*. The plastid data also revealed incidents of hybridization and possible introgression between *D. majalis* ssp. *lapponica* and other members of the genus, e.g., *D. incarnata*.

Keywords Phylogeography · Plastid DNA · Hybridization · Narrow-leaved marsh-orchid · Lapland marsh-orchid · *Dactylorhiza traunsteineri* · *Dactylorhiza russowii* · *Dactylorhiza lapponica*

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Introduction

Fennoscandia was covered with ice during the last glaciation (c. 22,000 to 17,000 cal yrs BP) and was not completely ice-free until approximately 8,000 years ago. Gradually, plants hibernating in refugial areas outside the ice sheet recolonized the open habitats left by the ice. Immigration routes and histories of Fennoscandian populations have been described for various species (e.g., *Picea abies* in Lagercrantz and Ryman 1990; *Picea abies* in Kullman 1996; *Silene dioica* in Malm and Prentice 2002; *Calluna vulgaris* in Rendell and Ennos 2002; *Carex digitata* and *Melica nutans* in Tyler et al. 2002; *Betula pendula* in Palmé et al. 2003; *Dactylorhiza maculata* s.l. in Ståhlberg 2007). Populations in previously glaciated areas may be genetically depleted as a consequence of repeated bottlenecks during stepwise migration, a pattern that has been described for several species of deciduous forest trees (e.g., Ferris et al. 1998; King and Ferris 1998). Such species seem to have been located in distant southern refugia during the last ice age. Plants with a more temperate distribution have, however, been shown to be equally genetically variable in Fennoscandia as populations of the same taxon in other parts of Europe (Tyler et al. 2002; Borgen and Hultgård 2003; Palmé et al. 2003; Skrede et al. 2006; Ståhlberg 2007). These temperate species may have had refugia close to the ice sheet (Stewart and Lister 2001) and seem to have kept most of their genetic variation during the migration process. Additionally, many plant species have colonized Fennoscandia through more than one immigration route (Hultén 1950; King and Ferris 1998; Nordal and Jonsell 1998; Malm and Prentice 2005; Ståhlberg 2007), which may have further contributed to comparatively high levels of genetic diversity.

Another factor affecting the genetic diversity of immigrated temperate species is the occurrence of polyploidy. Polyploid plant species are thought to increase in number with latitude in the Northern Hemisphere (Löve and Löve 1974; Grant 1981; Otto and Whitton 2000; Brochmann et al. 2004) and, for example, 44% of the plant taxa in boreal areas of the Arctic are polyploids compared to 82% in the polar desert further north (Brochmann et al. 2004). Several studies have shown that polyploids have higher levels of genetic diversity than their related diploids (e.g., Soltis and Rieseberg 1986; Lumaret and Barrientos 1990; Luttikhuizen et al. 2007) and due to their multiple chromosome complements they might have a higher potential for storing their genetic diversity during repeated bottleneck episodes associated with recolonization of previously glaciated areas.

Dactylorhiza Necker ex Nevski (Orchidaceae) is an example of a temperate plant genus with members of different ploidy levels. It is dominated by a polyploid complex consisting of diploid and tetraploid taxa. The complex must have originated well before the Weichselian glaciation (Hedrén et al. 2007) and is now found in large parts of Europe and Asia Minor (Pridgeon et al. 2001; Delforge 1995). During the ice ages, the Balkans (Hedrén et al. 2007), central Europe, and parts of central Russia (Ståhlberg 2007) acted as the most important refugia for the complex. Subsequent migration to formerly glaciated areas probably happened rapidly due to efficient seed dispersal by small seeds (Dressler 1993) and availability of suitable habitats (Adams 1997; Adams and Faure 1997).

D. maculata (L.) Soó s.l. and *D. incarnata* (L.) Soó s.l. are the present-day representatives of the parental lineages that built up the polyploid complex of *Dactylorhiza* in Europe (Hedrén 1996). Repeated hybridizations between the parental lineages have given rise to several allotetraploid derivatives (Hedrén 2003). This reticulate evolution has yielded a large amount of morphological variation in the complex which makes species delimitation difficult. Hybridization between present day representatives of the complex has often been considered as an additional factor contributing to further taxonomic confusion (Mossberg and Nilsson 1987; Baumann and Künkele 1988; Delforge 2001). Moreover, it could be discussed whether morphologically distinct populations represent independent evolutionary lineages arising from separate polyploidization events, or they have differentiated from widespread taxa as a consequence of genetic drift or selection. In the former case it may be motivated to recognize deviating forms as species, whereas in the latter case they may merely be seen as slightly deviant local forms without taxonomic value. Depending on underlying principles of species delimitation, different authors have recognized between one (Pedersen et al. 2003) and 23 (Delforge 1995) tetraploid species. Here we mainly follow the taxonomic delimitation of taxa as in Delforge (2001) but treat all allotetraploid taxa as subspecies of *D. majalis* (Rchb.) P. F. Hunt & Summerh. as suggested by Pedersen et al. (2003). One of the most common and variable allotetraploid is *D. majalis* (Rchb.) P. F. Hunt & Summerh. ssp. *traunsteineri* (Saut.) H. Sund., which was originally described on basis of material collected at Schwarzsee, Kitzbühel, Austria. Populations referable to this taxon have subsequently been reported from a wide area of northern Europe, including the Fennoscandian-Baltic region and the British Isles, although the taxonomic status of material from these latter regions has often been discussed and there is no consensus among present authors. It is characterized by having bluish-green leaves that are usually spotted on the upper surface, acute leaf apex, and a few-flowered, lax inflorescence with fairly large purple flowers provided with a prominent median lip lobe (Delforge 2001; Mossberg and Stenberg 2003). The value of leaf spotting as key character has however been questioned as it often varies within and between populations (Hylander 1966). *D. traunsteineri* has repeatedly been divided into subspecies and varieties (Hylander 1966; de Soó 1980) and morphologically distinct forms from certain areas have often been recognized (Hylander 1966; Hansson 1994; Andersson 1995). In the Fennoscandian-Baltic area two further taxa have been recognized that are clearly linked to *D. majalis* ssp. *traunsteineri*; *D. majalis* (Rchb.) P. F. Hunt & Summerh. ssp. *lapponica* (Laest.) H. Sundermann (Hylander 1966; Senghas 1968; de Soó 1980; Andersson 1996) and *D. majalis* (Rchb.) P. F. Hunt & Summerh. ssp. *russowii* (Klinge) H. Sund. (Senghas 1968; Mossberg and Nilsson 1987; Andersson 1994). *D. majalis* ssp. *lapponica* has a northern and alpine distribution whereas *D. majalis* ssp. *russowii* can be found in the Baltic countries and in Russia. The three taxa are not only morphologically similar but also grow in similar habitats. For instance, both *D. majalis* ssp. *traunsteineri* from southern Sweden and ssp. *russowii* from the East of the Baltic sea are found in calcareous fens growing in association with, e.g., *Carex lepidocarpa* Tausch ssp. *lepidocarpa*, *Schoenus ferrugineus* L., *Primula farinosa* L. and *Epipactis palustris* Crantz. The above-mentioned members of *Dactylorhiza* are the central focus for this study and subsequently denoted "the core complex". The core complex may sometimes be hard to delimit from other allotetraploid taxa in the Fennoscandian-Baltic area. For example, *D. majalis* (Rchb.) P. F. Hunt & Summerh. ssp. *sphagnicola* (Höppner) H. A. Pedersen & Hedrén (Hylander 1966; Ekman 1985; Bjurulf 2005) has some flower characters in common with ssp. *traunsteineri* but is normally found in poor fens and is not associated with indicator species of calcareous fens (Mossberg and Nilsson 1987; Baumann et al. 2006).

As mentioned above several morphological studies have been made to clarify the relationships between the different taxa in the core complex but no fine scale genetic analyses have been performed up to now. To be able to elucidate variation patterns and taxonomic limits within this group of closely related taxa we used fast-evolving plastid markers of microsatellite type for this study. The plastid genome is uniparentally inherited (presumably maternally in orchids, Corriveau and Coleman 1988; Cafasso et al. 2005) and markers thereof are commonly used in phylogeographic and phylogenetic studies (summarized in Lowe et al. 2004). Another important aspect of plastid markers, especially when studying allopolyploids, are the infrequence of recombination (Wolfe and Randle 2004), a problem associated with data sets from the nuclear genome.

The specific aims of this study were (1) to describe the genetic differentiation within the core complex in Fennoscandia and the Baltic area, (2) to describe limits between the core complex and other allotetraploid members of *Dactylorhiza* occurring in the area, and (3) to describe geographic variation patterns and relate these patterns to potential recolonization routes into Scandinavia after the last ice age.

Materials and methods

Plant material

Six hundred and fifty individuals from 91 populations were included in this study (Appendix). Two or more floral buds or apparently un-pollinated flowers with bracts were collected from each specimen and immediately dried in silica gel (Chase and Hills 1991). A majority of the populations (79) belonged to the core complex (*D. majalis* ssp. *traunsteineri*, ssp. *russowii* and ssp. *lapponica*) and the sampling covered most of its distribution in Fennoscandia (mainly Sweden, Norway and Finland) and Estonia. Additionally, five populations of *D. majalis* ssp. *traunsteineri* were sampled in Russia, Lithuania and Austria. One of the Austrian populations was from the type locality of ssp. *traunsteineri* near Kitzbühel. We did not separate out the subordinate taxon sometimes associated with *D. majalis* ssp. *traunsteineri*, ssp. *curvifolia*, but plants in agreement with this form were included from populations in both Finland and Sweden.

Most of the material in the core complex was easy to identify whereas some populations were more or less intermediate between the three subspecies. Accordingly, there are both typical *D. majalis* ssp. *traunsteineri* populations and ambiguous populations within the core complex included in this study. Moreover, 11 populations from Norway and middle Sweden were classified as *D. majalis* ssp. *traunsteineri* in local floras or by field botanists, but approached ssp. *sphagnicola* in our opinion. Morphologically, the two subspecies can be separated by differences in the spur: ssp. *sphagnicola* is characterized by a narrow, cylindrical and decumbent spur, whereas ssp. *traunsteineri* is characterized by a slightly wider, conical and straight spur (Delforge 2001; Bjurulf 2005; Baumann et al. 2006). The ambiguity of the populations motivated the inclusion of some populations of *D. majalis* ssp. *sphagnicola* as reference material. As further reference material, populations of all the remaining allotetraploid species of Fennoscandia were included: *D. majalis* (Rchb.) P. F. Hunt & Summerh. sspp. *majalis*, *praetermissa* (Druce) D. M. Moore & Soó, *purpurella* (T. Stephenson & T. A. Stephenson) D. M. Moore & Soó and *baltica* (Klinge) H. Sund. Earlier studies (Devos et al. 2003; Hedrén 2003; Shipunov et al. 2005; Pillon et al. 2007) of plastid DNA variation in *Dactylorhiza* have shown that allotetraploid *Dactylorhiza* have inherited their plastid genomes from *D. maculata* s.l, which means that *D. maculata* s.l and not *D. incarnata* s.l. is the seed parent of the allotetraploids. If more recent hybridization between an allotetraploid and *D. incarnata* has occurred, and *D. incarnata* has served as the seed parent, the *D. incarnata* plastid haplotype can be seen in the allotetraploid, but probably only in a fraction of plants within populations. In order to discover such secondary hybridization we also included one population of *D. incarnata* as a reference. The taxonomic classifications of the plant material in this study were based on scientific floras (Hylander 1966; de Soó 1980), field floras (Krok and Almquist 1994; Mossberg and Stenberg 2003) and local expertise. Voucher material of all populations (dried flowers) has been deposited at the Lund botanical museum (LD).

Molecular methods

Total DNA was extracted by the CTAB (cetyltrimethyl ammonium bromide) method (Doyle and Doyle 1990). Eight polymorphic plastid loci were analyzed for each sample. Five of the loci were mononucleotide repeats, two were duplicated regions and one was an insertion/deletion region. All loci were selected because of their high polymorphism for the genus, (previously tested by Hedrén et al. unpublished). All the alleles were defined according to size and combined into haplotypes. Detailed information of the loci and primers is given by Table 1. PCR conditions for all fragments were as follows: 5.6 ng DNA was amplified using 0.26 μM Cy5 labeled primer, 0.26 μM unlabeled primer, 1.6 mM $MgCl_2$, 210 μM dNTP's, 1× PCR buffer (Applied Biosystems) and 0.12 U Amplitaq Gold polymerase (Applied Biosystems), in a total volume of 5 μL. The PCR profile was: 94°C 1 min, T_a (Table 1) 1 min,

Table 1 Detailed information on the loci and primers used in this study. T_a = annealing temperature

Locus	Type	Approximate fragment size	Location	Primers	Primer sequence (5′-3′)	T_a (°C)
1	polyA[a]	183–186	*trn*T–*trn*L intergenic spacer	Cy5trnL5[e]	CGAAATCGGTAGACGCTACGC	57
				trnLR5	CGTTAGAACAGCTTCCATTG	
6	dupl region[b]	177–367	*psb*C–*trn*S pseudospacer	Cy5trnS2	AGAGTTTCAGGTCCTACCTA	54
				psbC2	GTGTTCCTAACTGCCCACTT	
6B	dupl region[b]	460–610	*psb*C–*trn*S pseudospacer	Cy5trnS1[f]	GGTTCGAATCCCTCTCTCTC	54
				trnS2f	TAGGTAGGACCTGAAACTCT	
8	polyT[c]	73–76	*rps*19–*psb*A intergenic spacer	Cy5HK7F	CACCTAGACACTTATCATTC	54
				HK8R	CCGATTTCTCCAAATTTTCG	
9	indel region[c]	171–202	*rps*19–*psb*A intergenic spacer	Cy5HK9R	CTAGCTTCTGTGGAAGTTCC	54
				HK8F	CGAAAATTTGGAGAAATCGG	
10B	polyA-AT-T[c]	138–163	*psb*A–*trn*K exon 1 intergenic spacer	Cy5trnK1A[g]	CCGACTAGTTCCGGGTTCGA	56
				HK10F	GAAAGGCTTGTTATTTCACAG	
11B	polyA[c]	82–87	*rpl*16 intron	Cy5F71[h]	GCTATGCTTAGTGTGTGACTCGTTG	53
				F71R2	AGTTTATAGTGGGGTCAGCC	
19	polyT[d]	137–149	*trn*S–*trn*G intergenic spacer	Cy5trnSGf3	GAGTAATAGTGTTCTAATAAGAG	58
				trnSGr3	CAGACGCAGTCAAGATAGCA	

[a] Soliva and Widmer (1999)

[b] Hedrén (2003)

[c] Hedrén et al. (unpublished)

[d] Pillon et al. (2007)

[e] Hedrén et al. (unpublished); Taberlet et al. (1991)

[f] *trn*S, Demesure et al. (1995)

[g] *trn*K, Demesure et al. (1995)

[h] F71, Jordan et al. (1996)

72°C 1 min 30 s for 40 cycles. PCR fragments were labeled and separated on an ALF Express II automated sequencer (Amersham Biosciences). Size determination was performed using ALFwin Fragment Analyser 1.03.01 software.

Data analysis

Population differentiation patterns were summarized in two multidimensional scaling diagrams (MDS; Kruskal 1964a, b) using the computer program NT-SYSpc 2.2 (Rohlf 2005). In both calculations Nei's average number of differences between populations (Nei and Li 1979) were calculated between all pairs of populations in the computer program Arlequin 3.01 (Excoffier et al. 2005) and used as input matrix for the multidimensional scaling analysis.

Relationships between all haplotypes were illustrated in a Median-joining network assisted by the program NETWORK 4.5 (Bandelt et al. 1999) with all options set to default.

To test whether genetic distances were correlated to geographic distances between populations a Mantel test (Mantel 1967) was performed using NT-SYSpc 2.2 (Rohlf 2005). A geographic distance matrix based on Euclidian distances was compared with a genetic distance matrix based on Nei's average number of differences between populations (Nei and Li 1979). The geographically distant Russian and Austrian populations were excluded from the test, as we were primarily interested in the correlation between genetic and geographic distances in the Scandinavian/Baltic area.

Results

Characterization of haplotypes

Using eight variable loci, 36 haplotypes were found among the 650 analyzed individuals (annotated H1–H36; Table 2). Two of the haplotypes (H1 and H4) counted for more than 60% of the individuals and 13 of the haplotypes (H1, H2, H4–H8, H10–H12, H17, H18, and H21) counted for more than 91% of the individuals. The relationships between haplotypes and their relative frequencies can be seen in the haplotype network (Fig. 1). Three distinct haplotype groups appeared in the network. The first group, denoted

Table 2 Combined haplotypes detected in the present study by means of markers described in Table 1. *N* is numbers of individuals found with a particular haplotype

No.	Locus (bp)								*N*
	1	6	6B	8	9	10B	11B	19	
1	185	222	470	75	196	145	84	149	246
2	185	222	470	75	196	146	84	149	10
3	185	367	610	73	171	138	86	137	1
4	185	222	470	76	189	148	84	148	150
5	185	222	470	75	196	143	84	149	17
6	185	281	560	73	177	146	85	149	13
7	186	281	560	73	177	146	85	149	6
8	185	281	560	73	177	147	85	149	31
9	185	281	560	73	177	148	85	149	5
10	185	222	470	73	177	150	85	149	23
11	184	367	610	73	171	138	85	137	21
12	184	367	610	73	171	138	86	137	13
13	185	191	560	76	189	140	84	149	5
14	184	367	610	73	171	138	84	137	1
15	186	222	470	75	196	145	84	149	1
16	185	222	470	73	196	138	84	137	1
17	185	222	470	75	196	145	84	148	32
18	185	222	470	75	196	146	84	148	11
19	185	222	470	75	196	148	84	149	1
20	185	222	470	76	196	148	84	148	1
21	185	222	470	76	202	140	84	149	25
22	185	367	610	75	196	145	84	149	4
23	185	222	470	75	185	145	84	149	3
24	183	367	610	73	171	143	86	137	2
25	185	222	470	75	189	143	85	148	7
26	185	281	560	75	177	148	85	149	1
27	185	222	610	73	171	138	86	137	1
28	185	281	560	74	177	147	85	149	4
29	184	367	610	73	171	141	86	137	2
30	184	367	610	73	171	140	85	137	1
31	185	222	470	76	189	148	84	149	3
32	184	367	610	73	171	142	86	137	2
33	186	222	470	76	189	148	84	148	1
34	184	367	610	73	171	140	86	137	2
35	185	222	470	74	196	146	84	149	1
36	185	281	560	74	177	148	85	149	2

group I, included most of the haplotypes and was divided into two subgroups, dominated by haplotypes 1 and 4, respectively. The second group, to the left in Fig. 1, consisted mainly of haplotype 6 to haplotype 10 and was denoted group II. In the right end of the network several rare haplotypes, e.g., H11 and H12, clustered into the third group, denoted *incarnata* group. Haplotype 1 was the most common haplotype and a lot of rare haplotypes were connected to it, e.g., H15, H22 and H23. In contrast, haplotype 4, which was the second most common haplotype, only connected to a few rare haplotypes, e.g., H20. Haplotype 21 had a somewhat distant position in relation to the other haplotypes of the second group with only haplotype 13 connected to it.

Population differentiation

The MDS diagram based on average pairwise differences between haplotypes and including all populations is given as Fig. 2. Three main clusters appeared in the MDS. The

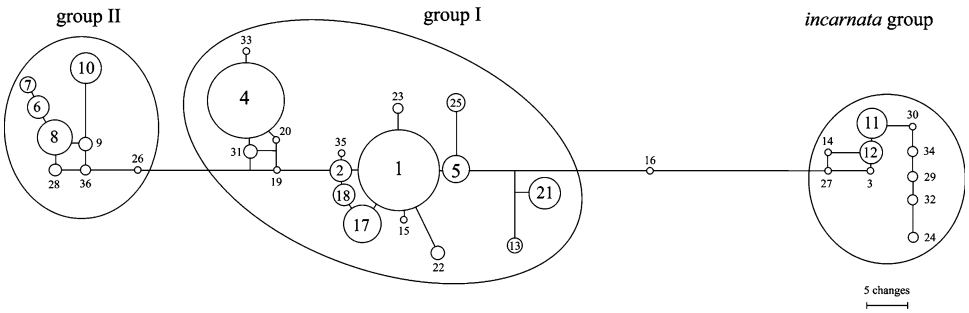

Fig. 1 Median-joining network of all haplotypes included in this study. Distances correspond approximately to the number of changes between haplotypes. All the haplotypes are numbered and the three haplotype groups are indicated

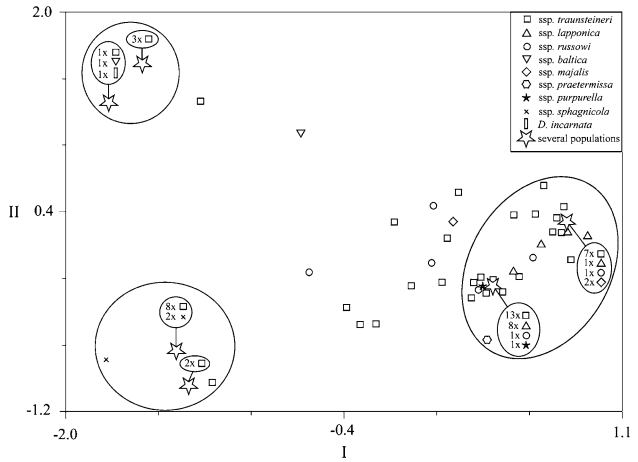

Fig. 2 Multidimensional scaling analysis of all populations included in this study. The three clusters mentioned in the text are indicated. Stress = 0.17

cluster to the upper left included the reference population of *D. incarnata* and five allotetraploid populations fixed for *incarnata* haplotypes (H11 and H12). Less than four samples were examined from any of these allotetraploid populations. The cluster to the lower left contained the three reference populations of *D. majalis* ssp. *sphagnicola* and eight other allotetraploid populations dominated by group II haplotypes. The larger cluster to the lower right contained more than 60% of the examined populations. Populations observed in between the three main clusters were either constituted by two or more different haplotypes from the three clusters or carried some of the odd haplotypes described below. Two dense subclusters that appeared in the large cluster to the lower right were included in a separate MDS given as Fig. 3. Haplotype 1 was prevalent in the populations in the left half of the diagram in Fig. 3, whereas haplotype 4 dominated to the right.

Taxonomic differentiation in haplotypes

The MDS diagram of all populations (Fig. 2) revealed no separation of populations labeled as *D. majalis* ssp. *traunsteineri* from other allotetraploid populations. The *D. majalis* ssp. *traunsteineri* populations were evenly distributed along the axes and were interspersed by other taxa. Other members of the core complex were also not distinct. The populations of *D. majalis* ssp. *lapponica* did not form a separate group in any of the MDS ordinations (Figs. 2, 3), although all populations were included in the right cluster of Fig. 2. The same result appeared for the ssp. *russowii* populations (Figs. 2, 3). Similarly, the three *D. majalis* ssp. *majalis* populations as well as the two populations of ssp. *purpurella* were located among ssp. *traunsteineri* populations (Figs. 2). However, the reference population of *D. majalis* ssp. *praetermissa* was located below the right cluster and the populations of ssp. *baltica* were placed within the upper left cluster or half-way between this cluster and the right cluster (Fig. 2). All the populations of *D. majalis* ssp. *sphagnicola* were situated in the lower left cluster in Fig. 2. This cluster also contained the 11 ambiguous populations with uncertain affinity to *D. majalis* ssp. *traunsteineri* from Norway and middle Sweden (populations 24, 26, 28, 29–32, 42, 49–51), which all carried some of the typical ssp. *sphagnicola* haplotypes from haplotype group II. The three populations of *D. majalis* ssp. *majalis* had different haplotypes including H4, H21 and H25. Haplotype 21 was also present in four populations of *D. majalis* ssp. *traunsteineri* and two of ssp. *lapponica*, all from the mixed region in Northern Lapland. Haplotype 25 is a rare haplotype unique to one of the *D. majalis* ssp. *majalis* populations and has not been found elsewhere. The single population of *D. majalis* ssp. *praetermissa* from Denmark contained the unique haplotype H13.

Geographic variation within the core complex

The MDS ordination given as Fig. 3 was based on 52 populations of the core complex. (All the populations in the two groups of the lower right cluster in Fig. 2 plus the 21 adjacent populations, populations of *D. majalis* ssp. *praetermissa* and ssp. *majalis* were excluded). Groups separated along the horizontal axis had different geographic origins. Notably, the left side contained all populations from the southern Swedish mainland whereas all populations from Gotland but one were located on the

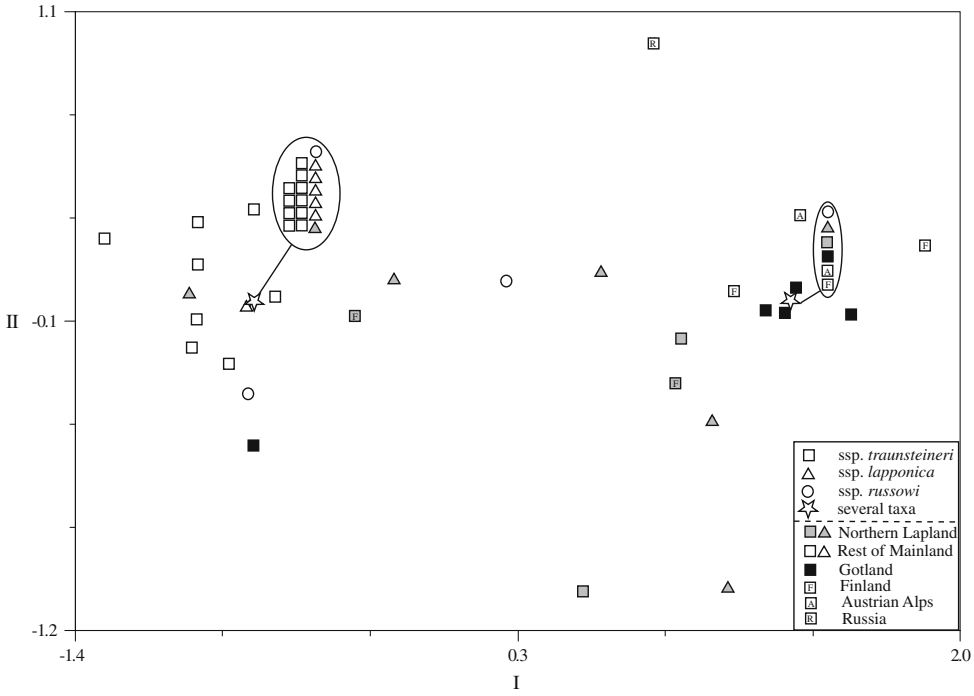

Fig. 3 Multidimensional scaling analysis of populations of the right cluster of Fig. 2. Only populations of the core complex are included. Stress = 0.10

right side. The right group was more geographically widespread than the left and contained populations from Russia, Austrian Alps and all but one of the Finnish populations.

Some populations were located between the main clusters, including populations from Finland, Sweden and Estonia. The Swedish populations were all from north of the southern border of Lapland (64°15′N) and the Finnish populations were north of the Gulf of Bothnia.

The geographic pattern seen in Fig. 3 was also evident in Fig. 4a, where core complex populations containing haplotypes 1 and 4 were marked:

Haplotype 1 was totally dominating on the Swedish mainland apart from northern Scandinavia. It was not found in central Finland and only rarely encountered in Norway. On Gotland we only found two individuals containing haplotype 1 in one single population (out of seven populations examined). This haplotype was also present in Estonia and Lithuania. Haplotype 4 had a more eastern distribution and was most common in Finland and on Gotland. Additionally, it was found in Estonia and Austria. There were also populations from northern Sweden and northern Norway containing haplotype 4 and the haplotype could be found as far south as southern Lapland. This distribution of haplotypes created a mixed zone in northern Fennoscandia where both haplotypes 1 and 4 were present and sometimes observed in the same populations. There were other meeting zones of the two haplotypes in Estonia (Hiiumaa and Saaremaa) and on Gotland (Sweden). Only one population (*D. majalis* ssp. *traunsteineri*) fixed for haplotype 1 was found on Gotland while two such populations were encountered in Estonia (ssp. *russowii*). Moreover, there was a population of *D. majalis* ssp. *russowii* on Saaremaa carrying this haplotype together with haplotype 4.

The map given as Fig. 4b shows the distribution of a less common haplotype, H10, which was found in *D. majalis* ssp. *traunsteineri* populations on Gotland and in ssp. *russowii* populations on Saaremaa and Hiiumaa. Haplotype 10 was strongly divergent from the other haplotypes encountered in the core complex and most closely related to the *D. majalis* ssp. *sphagnicola* haplotypes (Fig. 1). Except for Gotland and Estonia it was also found in Austria.

Almost all populations initially labeled as *D. majalis* ssp. *traunsteineri* from Norway and four populations from central Sweden were fixed for typical *D. majalis* ssp. *sphagnicola* haplotypes (Fig. 4c).

A weak but highly significant positive correlation, 0.18975, was observed between geographic and genetic distances among populations from the Fennoscandic-Baltic area (Mantel test; $p < 0.001$).

Discussion

Haplotype evaluation

Plastid markers are commonly used in phylogeographic studies due to their relatively slow mutation rate and

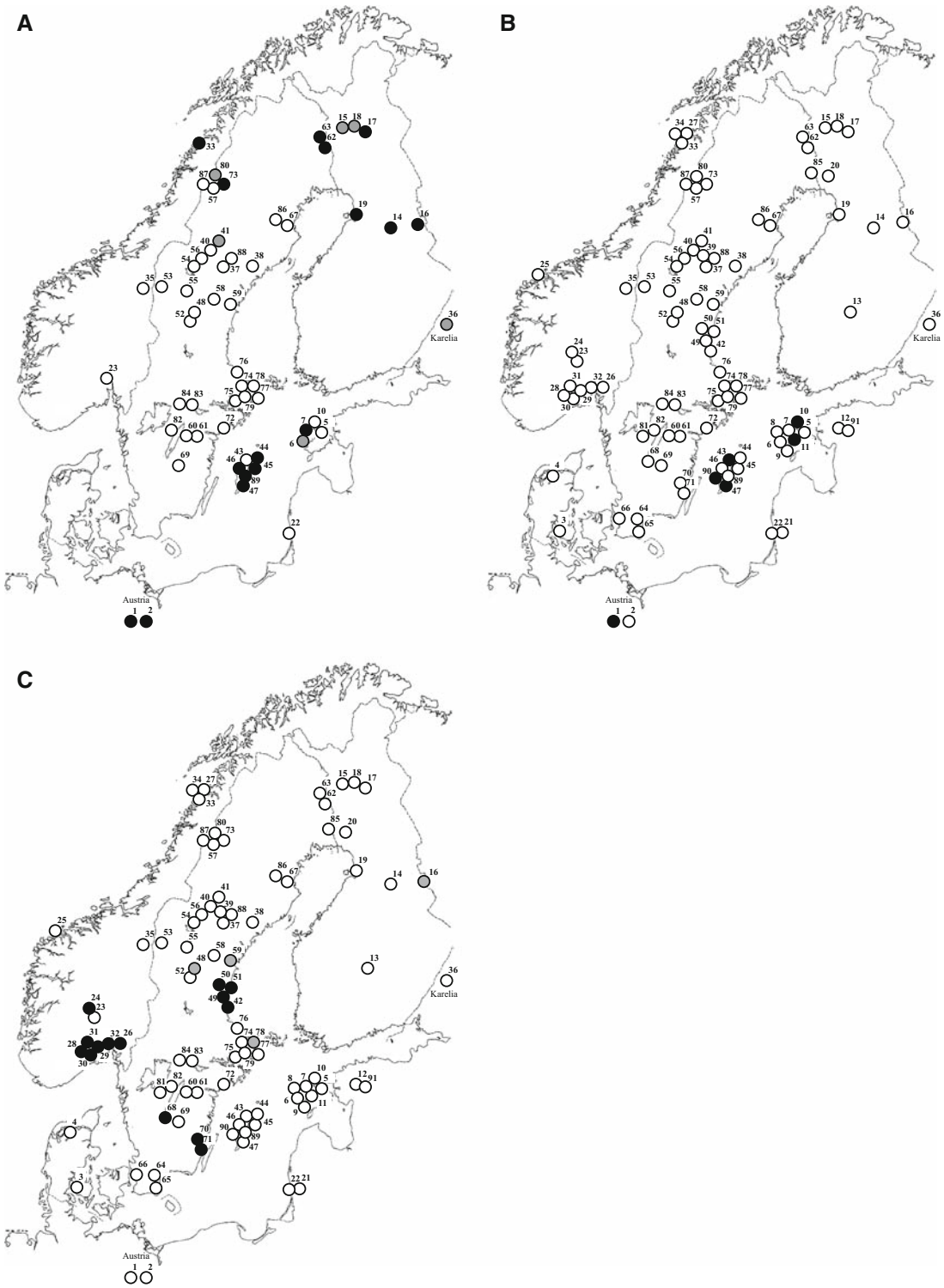

Fig. 4 Geographic distribution of haplotypes. **a** Core complex populations containing haplotype 1 and/or 4. *Black symbols* populations containing haplotype 4. *White symbols* populations containing haplotype 1. *Grey symbols* populations with individuals carrying haplotype 1 as well as individuals carrying haplotype 4. **b** Populations containing haplotype 10. *Black symbols* populations carrying haplotype 10. **c** Populations containing group II haplotypes. *Black symbols* populations totally dominated by individuals with group II haplotypes (100% carry the haplotypes). *Grey symbols* populations with a lesser content of individuals carrying group II haplotypes (less than 50% carry the haplotypes). *White symbols* populations with no individuals of group II haplotypes. Populations 68, 70 and 71 are allopatric *D. majalis* ssp. *sphagnicola* populations, whereas the other populations with black symbols are identified as *D. majalis* ssp. *traunsteineri* populations

uniparental origin. However, for the same reasons, plastid markers may not be suitable for distinguishing between closely related taxa. Introgression of plastid DNA as a result of interspecific hybridization may further complicate phylogeographic studies based on plastid data. However, the mutation rate may differ substantially between plastid microsatellite loci and when combining several loci with different mutation rates into combined haplotypes a more structured pattern may appear. Furthermore, the fact that orchids have very small seeds and thus have the capacity to spread over long distances suggests that the gene flow by seeds may be more important than pollen flow (discussed in Cozzolino et al. 2003). In this study, the eight chloroplast markers proved to be very informative when answering our questions on both taxonomy and phylogeography.

Thirty-six haplotypes were found in the distribution area out of which two (H1 and H4) were present in more than 60% of the samples. Despite the high frequencies of these two haplotypes, we found variation within populations and often variation within a small defined area.

Gene flow by hybridization

Most allotetraploid populations in this study had plastid haplotypes previously characterized as typical *D. maculata* s.l. haplotypes. This finding confirms the common notion that *D. maculata* s.l. has acted as donor of the plastid genome at the polyploidization event (Devos et al. 2003; Hedrén 2003; Shipunov et al. 2005; Pillon et al. 2007). Further, the finding that two clearly defined and large subgroups of haplotypes dominate in the core complex (characterized by H1 and H4, respectively) supports earlier results suggesting multiple origins of allotetraploids in *Dactylorhiza* (Hedrén 1996, 2003; Devos et al. 2006). However, no present day representatives of *D. maculata* s.l. carrying haplotype 4 have been found in spite of a large sample surveyed from the major part of the European distribution area (Ståhlberg 2007), and a *D. maculata* s.l. ancestor carrying this haplotype should have been found if it still existed. The high number of allotetraploid populations carrying haplotype 4 and the wide distribution of this haplotype in the allotetraploids suggest that haplotype 4 might be an old haplotype originating well before the last glaciations and that different allotetraploid taxa carrying this haplotype share a common history. The alternative explanation to the observed pattern is that haplotype 4 has differentiated from another allotetraploid haplotype, for example haplotype 1 which is also widespread in present day *D. maculata* s.l. However, 4 to 12 mutations at four loci out of eight studied is required for that conversion and we consider this a less likely scenario.

Some of the allotetraploid populations had haplotypes typical of *D. incarnata* (Fig. 2). It is hypothetically possible that these populations have inherited the *incarnata* plastids at some polyploidization events. However, since the number of individuals carrying the *incarnata* haplotypes is low, only eight individuals in altogether five populations, the presence of *incarnata* haplotypes rather indicate backcrossing between the allotetraploids and *D. incarnata* at a later stage. Introgression with *D. maculata* s.l. may also take place, but is more difficult to detect since the allotetraploids normally inherit the plastids from this lineage. Nevertheless, the *traunsteineri* population 78 in southern Sweden may show such a history. It contains two haplotypes, the common *traunsteineri* haplotype 1 and one individual with H7, a group II haplotype. In addition, individuals of *D. maculata* s.str. that grow at the same locality are dominated by haplotype 7 (Hedrén et al. unpublished). It is however difficult to separate between hybridization with *D. maculata* s.str. and hybridization with *D. majalis* ssp. *sphagnicola* since they both carry group II haplotypes. The same conclusions about introgression could be drawn for the two *traunsteineri* populations 48 and 59 which carry a few individuals with typical *sphagnicola* haplotypes. Previous studies on *Dactylorhiza* have also reported introgression of allotetraploids by their parental lineages (Hedrén 2003; Aagaard et al. 2005; Shipunov et al. 2005) as well as hybridization between allotetraploids (Heslop-Harrison 1954; Hedrén 2003).

Taxonomy

We conclude that the pattern of plastid haplotype composition found in this study does not support the subdivision of the core complex into three taxa. This conclusion is strongly supported by previous (Andersson 1996) and unpublished (Pedersen unpublished) findings on variation in morphology of the core complex as well as earlier genetic studies of the taxa using allozymes, AFLPs and chloroplast data (Hedrén 1996; Hedrén et al. 2001; Hedrén 2003; Pillon et al. 2007). Therefore we suggest an amalgamation of the three taxa into one with the oldest name applied: *D. majalis* ssp. *lapponica*.

Core complex individuals with the two most common haplotypes (H1 and H4) sometimes grew at adjacent sites or even in mixed populations. When examining such populations in the field we found no obvious differences in morphology between the two haplotypes. However, our genetic data is in congruence with previous findings on differentiation in morphology (Andersson 1994; Hansson 1994) and allozymes (Hedrén 1996) between populations of *D. majalis* ssp. *traunsteineri* on Gotland and mainland Sweden. Our results are also congruent with the finding

that *D. majalis* ssp. *traunsteineri* on Gotland and ssp. *russowii* in Estonia are closely similar in morphology (Andersson 1994). All individuals of the taxonomically ambiguous populations (belonging to either *D. majalis* ssp. *traunsteineri* or ssp. *sphagnicola*) from the area around Oslo (Norway) and Gästrikland and Hälsingland (Sweden), showed typical *sphagnicola* haplotypes. Therefore we suggest that they should be treated as this taxon. In general, all populations of *D. majalis* ssp. *sphagnicola* in this study are well separated from the rest of the allotetraploids (Fig. 2) which is in agreement with earlier studies (Hedrén 2003; Devos et al. 2006) and further strengthens the position of ssp. *sphagnicola* as a separate subspecies. In contrast, our data indicates no clear differentiation between *D. majalis* ssp. *traunsteineri* on the one hand and ssp. *majalis*, ssp. *purpurella* or ssp. *praetermissa* on the other. To confirm this finding additional analysis including more material of all the different tetraploid taxa is needed.

As the interpretation of plastid markers could be hampered by chloroplast capture and that the results only show genetic variation from one of the parents, nuclear markers would better describe the reticulate evolution of polyploid complexes. Consequently, newly developed nuclear microsatellites (Nordström and Hedrén 2007) might be useful for future studies of the problematic species delimitations in *Dactylorhiza*.

Geographic pattern

The distribution of the common haplotypes 1 and 4 in this study shows a clear geographic pattern (Fig. 4a). The most common haplotype (H1) is dominating the Swedish mainland, particularly the southern parts, while haplotype 4 is prevalent in Finland, on Gotland (Sweden) and in northern Scandinavia. The high frequencies of these two haplotypes may be explained by rapid expansion after the last glaciations of populations carrying these haplotypes (cf. Hewitt 1996). On the basis of the present distribution of the two haplotypes the immigration history for the Scandinavian populations can be described. Two immigration routes are seen, carrying haplotype 1 and 4, respectively: One southwestern route of haplotype 1 originating in continental Europe and entering the Swedish and Norwegian mainland probably via Denmark, and one path of haplotype 4 with a more eastern direction through Estonia and Finland to northern Sweden and Norway. This immigration scenario is similar to results from earlier studies of phylogeography in Scandinavia on both plants (Nordal and Jonsell 1998; Berglund and Westerbergh 2001; Malm and Prentice 2005) and animals (Jaarola and Tegelström 1995; Taberlet et al. 1995).

Interestingly, the distribution of haplotype 4 also reveals a connection between the Estonian islands Saaremaa and Hiiumaa, and Gotland (Sweden) (Fig. 4a). The same connection is revealed by the distribution of the rare haplotype 10 (Fig. 4b). We interpret this pattern such that Gotland, as well as Saaremaa and Hiiumaa are all colonized by the same refugial population located somewhere to the southeast of the Baltic Sea. The dispersal to Gotland over the Baltic Sea seems surprising since Gotland is located more than 100 km from the eastern shore. However, the small air-filled seeds of orchids may travel thousands of kilometers by wind (Jersáková and Malinová 2007) and supposedly also by epizoochory. Haplotype 4 is also found in *D. majalis* ssp. *majalis* from southernmost Sweden and it may be hypothesized that the haplotype has spread to the core complex on Gotland from these populations. However, the flowering times of *D. majalis* ssp. *majalis* and ssp. *traunsteineri* are different and together with the fact that no other population of ssp. *traunsteineri* on the Swedish mainland carries the haplotype, such a spreading route does not seem likely.

Recent studies on immigration history in Nordic plants have revealed eastern refugia during the last glaciations (e.g., Lagercrantz and Ryman 1990; Skrede et al. 2006; Ståhlberg 2007). Haplotype 4 may have such an origin, or alternatively it may have had refugia further south since it is present in Austrian populations. Haplotype 1 is ubiquitous in Europe including Russia (Ståhlberg 2007). A study at a larger geographical scale may have the potential to identify the refugial areas of the haplotypes found in this study.

Haplotypes 1 and 4 meet in northern Fennoscandia. Similar contact zones between colonizing genotypes are seen for other species with southeastern and northwestern immigration routes into Scandinavia (Berglund and Westerbergh 2001; van Rossum and Prentice 2004; Ståhlberg 2007) and could be explained by glacial history. The ice cover in northern Scandinavia melted slowly and was completely gone much later than in the adjacent areas (Berglund 2004). This led to an accumulation of immigrating species on both sides of the ice sheet and as the last remains of the ice melted populations of different origin came into contact in approximately the same area. Another meeting zone of haplotypes 1 and 4 is in Estonia, where both haplotypes occur in close vicinity together with several others. Perhaps the genetic variation in Estonia could be explained by its geographic location close to refugia.

To conclude, we did not find any support in plastid haplotypes for separating *D. majalis* ssp. *traunsteineri*, ssp. *lapponica* and ssp. *russowii* into three taxa. Considering also the high degree of morphological similarity and results

from previous studies (Andersson 1994; Hedrén 1996; Hedrén et al. 2001; Hedrén 2003; Pillon et al. 2007) we suggest an amalgamation of the three taxa into *D. majalis* ssp. *lapponica*. Furthermore, this broadly defined subspecies was not separated from other allotetraploids in the study area why additional studies should be made on a larger scale and with additional taxa. Newly developed nuclear microsatellites (Nordström and Hedrén 2007) may be used to bring clarity to the relationships of the genus. The genetic variation in the core complex had a clear geographic component and we have identified a minimum of two immigration routes into Scandinavia after the last glaciation.

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Appendix

Table 3

Table 3 Identification and geographic origin of populations of *Dactylorhiza* Neck. ex Nevski used in this study. Latitude and longitude references are approximate

No.	Taxon	Country	Locality	Latitude	Longitude	Number of individuals
1	*D. majalis* (Rchb.) P.F.Hunt & Summerh. ssp. *traunsteineri* (Saut.) H. Sund.	Austria	Tirol, Kitzbühel	47°27	12°23	10
2	*D. majalis* ssp. *traunsteineri*	Austria	Steiermark, Gusswerk	47°45	15°20	10
3	*D. majalis* ssp. *purpurella* (T. & T.A. Stephenson) D.M. Moore & Soó	Denmark	ØJy, Assens	55°16	09°54	6
4	*D. majalis* ssp. *praetermissa* (Druce) D.M Moore & Soó	Denmark	VJy, Sjørups sø	56°52	09°24	8
5	*D. majalis* ssp. *russowii* (Klinge) H. Sund.	Estonia	Hiiumaa, Järvamaa	58°48	22°50	2
6	*D. majalis* ssp. *russowii*	Estonia	Saaremaa, Kuusnömme	58°19	21°59	2
7	*D. majalis* ssp. *russowii*	Estonia	Saaremaa, Ninase	58°32	22°14	2
8	*D. majalis* ssp. *baltica* (Klinge) H. Sund.	Estonia	Saaremaa, Vesiku	58°20	21°58	7
9	*D.* majalis ssp. *baltica*	Estonia	Saaremaa, Karala	58°16	21°55	3
10	*D. majalis* ssp. *russowii*	Estonia	Hiiumaa, Heistesoo	58°56	22°17	12
11	*D. majalis* ssp. *russowii*	Estonia	Saaremaa, Viidume	58°17	22°08	14
12	*D. majalis* ssp. *russowii*	Estonia	Jalase, Pedasmaa	58°28	22°07	1
13	*D. incarnata* (L.) Soó	Finland	Jyväskylä	62°14	25°44	12
14	*D. majalis* ssp. *traunsteineri*	Finland	Kn, Paltamo	64°19	28°03	10
15	*D. majalis* ssp. *traunsteineri*	Finland	KiL, Kaukonen	67°32	24°51	11
16	*D. majalis* ssp. *traunsteineri*	Finland	Kn, Korpijärvi	64°45	29°57	10
17	*D. majalis* ssp. *traunsteineri*	Finland	SoL, Moskuvaara	67°36	26°53	10
18	*D. majalis* ssp. *traunsteineri*	Finland	KiL, Mustavaara	67°37	25°23	10
19	*D. majalis* ssp. *traunsteineri*	Finland	OP, Haukipudas	65°13	25°25	5
20	*D. majalis* ssp. *traunsteineri*	Finland	PeP, Tervola	66°05	25°02	11
21	*D. majalis* ssp. *traunsteineri*	Lithuania	Kretinga, Kartena	55°55	21°28	1
22	*D. majalis* ssp. *traunsteineri*	Lithuania	Palanga, Nemirseta	55°53	21°04	1
23	*D. majalis* ssp. *traunsteineri*	Norway	Bu, Gjellebekk	59°49	10°18	10
24	*D. majalis* ssp. *traunsteineri*	Norway	Bu, Grimsrudsbrenna	59°43	10°06	2
25	*D. majalis* ssp. *purpurella*	Norway	MR, Giske, Molnes	62°34	6°05	5
26	*D. majalis* ssp. *traunsteineri*	Norway	Ak, Marimyr	59°46	10°51	2
27	*D. majalis* ssp. *traunsteineri*	Norway	No, Bodø	67°20	14°29	1

Table 3 continued

No.	Taxon	Country	Locality	Latitude	Longitude	Number of individuals
28	*D. majalis* ssp. *traunsteineri*	Norway	Te, Skien, Limitjern	59°16	09°30	6
29	*D. majalis* ssp. *traunsteineri*	Norway	Te, Siljan	59°18	09°49	6
30	*D. majalis* ssp. *traunsteineri*	Norway	Te, Skien	59°16	09°30	1
31	*D. majalis* ssp. *traunsteineri*	Norway	Vf, Djupedalsmyr	59°30	10°10	6
32	*D. majalis* ssp. *traunsteineri*	Norway	Vf, Holmestrand	59°30	10°10	1
33	*D. majalis* ssp. *traunsteineri*	Norway	No, Inndyr, Holtan	67°02	14°04	1
34	*D. majalis* ssp. *traunsteineri*	Norway	No, Inndyr, Øya	67°02	14°01	1
35	*D. majalis* ssp. *lapponica* (Laest.) H. Sundermann	Norway	ST, Røros, Sølendet	62°34	11°23	3
36	*D. majalis* ssp. *traunsteineri*	Russia	Karelia, Petrozavodsk	61°47	33°48	9
37	*D. majalis* ssp. *traunsteineri*	Sweden	Ång, Edsele	63°16	16°36	14
38	*D. majalis* ssp. *traunsteineri*	Sweden	Ång, Överå	63°35	17°11	10
39	*D. majalis* ssp. *traunsteineri*	Sweden	Ång, Ramsele	63°31	16°25	2
40	*D. majalis* ssp. *traunsteineri*	Sweden	Ång, Svedbergsviken	63°37	15°58	10
41	*D. majalis* ssp. *lapponica*	Sweden	Jmt, Kalvberget	64°44	15°21	10
42	*D. majalis* ssp. *traunsteineri*	Sweden	Gstr, Axmarbruk	61°02	17°08	2
43	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Agmyr	57°49	18°39	4
44	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Harudden	57°55	18°42	15
45	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Hoburgsmyr	57°49	18°50	4
46	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Kauparve	57°49	18°53	14
47	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Lojsthajd	57°20	18°19	15
48	*D. majalis* ssp. *traunsteineri*	Sweden	Hls, Los	61°37	14°54	13
49	*D. majalis* ssp. *traunsteineri*	Sweden	Hls, Marmaverken	61°16	16°52	2
50	*D. majalis* ssp. *traunsteineri*	Sweden	Hls, Nybo-stomyran	61°32	16°44	4
51	*D. majalis* ssp. *traunsteineri*	Sweden	Hls, Tröne, Berge	61°28	16°50	2
52	*D. majalis* ssp. *lapponica*	Sweden	Hrj, Hamra	62°34	12°15	6
53	*D. majalis* ssp. *lapponica*	Sweden	Hrj, Klinken	62°43	12°18	5
54	*D. majalis* ssp. *traunsteineri*	Sweden	Jmt, Handog	63°19	15°01	1
55	*D. majalis* ssp. *lapponica*	Sweden	Jmt, Skalberget	62°34	14°27	15
56	*D. majalis* ssp. *lapponica*	Sweden	Jmt, Vackermyran	63°28	15°20	10
57	*D. majalis* ssp. *lapponica*	Sweden	LyL, Blomstervallen	65°41	15°10	10
58	*D. majalis* ssp. *traunsteineri*	Sweden	Mpd, Granboda	62°34	15°42	7
59	*D. majalis* ssp. *traunsteineri*	Sweden	Mpd, Måckelmyran	62°15	17°27	18
60	*D. majalis* ssp. *traunsteineri*	Sweden	Ög, Hagebyhöga	58°27	14°56	9
61	*D. majalis* ssp. *traunsteineri*	Sweden	Ög, Kärna mosse	58°25	15°32	11
62	*D. majalis* ssp. *traunsteineri*	Sweden	Nb, Jupukka	67°17	23°14	9
63	*D. majalis* ssp. *lapponica*	Sweden	Nb, Suksijoki	67°20	23°13	4
64	*D. majalis*. ssp. *majalis*	Sweden	Sk, Eskeröd	55°46	14°07	11
65	*D. majalis* ssp. *majalis*	Sweden	Sk, Örup	55°30	13°55	10
66	*D. majalis* ssp. *majalis*	Sweden	Sk, Saxtorp	55°49	12°56	10
67	*D. majalis* ssp. *traunsteineri*	Sweden	Vb, Slätmyran	64°56	20°27	7
68	*D. majalis* ssp. *sphagnicola* (Höppner) H. A. Pedersen & Hedrén	Sweden	Sm, Dumme mosse	57°45	14°00	5
69	*D. majalis* ssp. *traunsteineri*	Sweden	Sm, Eksjö	57°38	14°46	11
70	*D. majalis* ssp. *sphagnicola*	Sweden	Sm, Jonsbo fly	57°03	15°50	2
71	*D. majalis* ssp. *sphagnicola*	Sweden	Sm, Sjömaden	56°53	15°52	3
72	*D. majalis* ssp. *traunsteineri*	Sweden	Srm, Sjösa	58°45	17°07	12

Table 3 continued

No.	Taxon	Country	Locality	Latitude	Longitude	Number of individuals
73	*D. majalis* ssp. *lapponica*	Sweden	LyL, N. Fjällnäs	65°45	15°30	10
74	*D. majalis* ssp. *traunsteineri*	Sweden	Upl, Bladåker	59°59	18°18	2
75	*D. majalis* ssp. *traunsteineri*	Sweden	Upl, Ed	60°17	18°19	2
76	*D. majalis* ssp. *traunsteineri*	Sweden	Upl, Gårdskär	60°37	17°37	7
77	*D. majalis* ssp. *traunsteineri*	Sweden	Upl, Gunnarsmaren	59°42	19°02	8
78	*D. majalis* ssp. *traunsteineri*	Sweden	Upl, Norrmarjum	59°53	18°40	9
79	*D. majalis* ssp. *traunsteineri*	Sweden	Upl, Rimbo	59°44	18°19	9
80	*D. majalis* ssp. *lapponica*	Sweden	LyL, Rödingsträsket	65°42	15°07	3
81	*D. majalis* ssp. *traunsteineri*	Sweden	Vg, Jättened	58°19	14°41	17
82	*D. majalis* ssp. *traunsteineri*	Sweden	Vg, Sjogerstad	58°19	13°46	3
83	*D. majalis* ssp. *traunsteineri*	Sweden	Vsm, Myggkärret	59°24	14°46	6
84	*D. majalis* ssp. *traunsteineri*	Sweden	Vsm, Röjängen	59°26	14°47	8
85	*D. majalis* ssp. *lapponica*	Sweden	Nb, Armasjärvi	66°19	23°30	10
86	*D. majalis* ssp. *lapponica*	Sweden	Vb, Bjurträsk	64°59	19°34	10
87	*D. majalis* ssp. *lapponica*	Sweden	LyL, Joeström	65°45	14°57	11
88	*D. majalis* ssp. *traunsteineri*	Sweden	Ång, Junsele	63°35	17°11	10
89	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Kallgatburg	57°41	18°40	10
90	*D. majalis* ssp. *traunsteineri*	Sweden	Gtl, Klinte	57°35	18°21	10
91	*D. majalis* ssp. *russowii*	Estonia	Jalase, Parka	59°00	24°33	

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