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Plastid DNA variation in the *Dactylorhiza incarnata/maculata* polyploid complex and the origin of allotetraploid *D. sphagnicola* (Orchidaceae)

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Abstract

To obtain further information on the polyploid dynamics of the the *Dactylorhiza incarnata/maculata* polyploid complex and the origin of the allotetraploid *D. sphagnicola* (Orchidaceae), plastid DNA variation was studied in 400 plants from from Sweden and elsewhere in Europe and Asia Minor by means of polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLPs) and sequencing. Allotetraploid taxa in Europe are known have evolved by multiple independent polyploidization events following hybridization between the same set of two distinct ancestral lineages. Most allotetraploids have inherited the plastid genome from parents similar to *D. maculata sensu lato*, which includes, e.g. the diploid *D. fuchsii* and the autotetraploid *D. maculata sensu stricto*. *D. sphagnicola* carries a separate plastid haplotype different from the one found in other allotetraploid taxa, which is in agreement with an independent origin from the parental lineages. Some of the remaining allotetraploids have local distributions and appear to be of postglacial origin, whereas still other allotetraploids may be of higher age, carrying plastid haplotypes that have not been encountered in present day representatives of the parental lineages. Introgression and hybridization between diploids and allotetraploids, and between different independently derived allotetraploids may further have contributed to genetic diversity at the tetraploid level. Overall, the *Dactylorhiza* polyploid complex illustrates how taxon diversity and genetic diversity may be replenished rapidly in a recently glaciated area.

Keywords: *Dactylorhiza sphagnicola*, genetic diversity, hybridization, PCR-RFLP, plastid DNA, polyploid evolution

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Introduction

The evolutionary dynamics of a polyploid complex, including the rate of hybridization and polyploidization, provide essential background data for understanding genetic variation patterns in polyploid plants, niche width, niche separation and competition potentials when diploid parental and polyploid derivative taxa are compared with each other (Thompson & Lumaret 1992; Soltis & Soltis 1993). Data accumulated during the last two decades have shown that polyploid complexes may be quite dynamic systems, and that polyploid taxa often contain astonishingly high

levels of genetic diversity (Soltis & Soltis 1993, 1999). These levels of genetic diversity indicate that polyploids are comparable to diploids in their ability to respond and adapt to changing conditions in the environment. Also, several studies have described considerable reorganization of the polyploid genome (Song *et al.* 1995; Leitch & Bennett 1997; Soltis & Soltis 1999), which thus provides an arena for combining traits that would be incompatible at the diploid level. Accordingly, polyploidy in itself may confer evolution of unique traits of high adaptive importance.

Polyploidy is a widespread phenomenon in higher plants. Based on chromosome number variation within genera, Stebbins (1971) estimated that about a third of the angiosperms are polyploids. It is also well known that the frequency of polyploids in the flora increases with higher latitude and higher altitude (Löve & Löve 1974). The areas

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with the highest numbers of polyploids are also those areas that were most seriously affected by climate changes during Pleistocene ice ages, in particular those covered with ice sheets during glacial maxima. The great majority of plants in these areas must have reimmigrated after the retreat of the ice approximately 15–10 000 years ago. Formation of polyploids may be a rapid process, and of the relatively few endemic taxa in such areas a high proportion is usually polyploids. This pattern indicates that the formation of polyploids may be seen as an important evolutionary process conferring adaptative novelty.

A high number of polyploid taxa, mainly tetraploids, have been described within the *Dactylorhiza incarnata/maculata* complex (de Soó 1980; Averyanov 1990; Buttlar 1991; Delforge 2001), which constitutes the major part of the Eurasian orchid genus *Dactylorhiza*. Several of these tetraploids have their entire, or their main, distribution in areas that were ice-covered, encouraging the hypothesis that they may have evolved after the last ice age. The main structure of the complex has been described by means of chromosome numbers, genome analysis (Heslop-Harrison 1953), codominant allozyme markers (Hedrén 1996a,b,c, 2001a) and amplified fragment length polymorphisms (AFLPs; Hedrén *et al.* 2001). Most of the tetraploid taxa occurring in Europe are allotetraploids that have been formed by hybridization between two principal ancestral lineages, the *D. incarnata* s.l. and the *D. maculata* s.l. lineages. The first of these lineages, the diploid marsh orchids, contains a range of morphologically differentiated taxa such as *D. ochroleuca* (Boll) J. Holub and *D. cruenta* (O. F. Müller) Soó with scattered distribution in Europe, in addition to the widespread *D. incarnata* (L.) Soó s.s. However, this lineage contains surprisingly low levels of genetic variation in Europe, a finding revealed by both allozymes (Hedrén 1996a, 2001b; Pedersen 1998) and AFLPs (Hedrén *et al.* 2001). The second lineage, the spotted orchids, is more variable in these markers and also in ploidy level (e.g. Hagerup 1938; Gathoye & Tyteca 1989). Whereas the widespread *D. fuchsii* (Druce) Soó and the southeastern European *D. saccifera* (Brongn.) Soó are both diploids, *D. maculata* (L.) Soó s.s. is considered as an autotetraploid derived from a diploid member of this lineage (Hedrén 1996a). This interpretation is supported by (i) a high degree of morphological similarity between *D. maculata* s.s. and diploid members of the lineage, (ii) a close correspondence in allozyme marker distribution and frequencies between *D. maculata* s.s. and *D. fuchsii* and (iii) genotype distribution patterns in populations of *D. maculata* s.s. that do not deviate from those expected by tetrasomal segregation (Hedrén 1996a). However, AFLP fingerprints indicate that *D. maculata* is somewhat differentiated from *D. fuchsii*, or at least the *D. fuchsii* that is present in Sweden today (Hedrén *et al.* 2001).

A third diploid lineage, *D. euxina* (Nevski) S.K. Cherepanov, occurs in Asia Minor and has given rise to additional

allotetraploids restricted to this area. Thus, *D. urvilleana* (Steud.) H. Baumann & Künkele has been formed by hybridization between members of the *D. euxina* and *D. maculata* s.l. lineages, whereas *D. armeniaca* Hedrén has been formed by hybridization between the *D. euxina* and *D. incarnata* s.l. lineages (Hedrén 2001a).

Allozyme studies have shown that the whole group of European allotetraploids, and also many of the separate allotetraploid taxa, must have multiple origins from their ancestral lineages, because they share more than two alleles with the *D. maculata* s.l. lineage at several investigated loci. However, due to restricted numbers of alleles identified at each studied locus, and a high degree of polymorphism within populations, allozymes have been of limited use in describing the exact origins of most of the allotetraploids.

In the present study, I used markers from the plastid genome in an attempt to obtain more detailed information on origins and variability of tetraploid taxa in the *D. incarnata/maculata* polyploid complex. Plastid DNA has been studied in several other polyploid complexes to provide such information (Soltis & Soltis 1989; Soltis *et al.* 1989; Wolf *et al.* 1990; Brochmann *et al.* 1996; Segraves *et al.* 1999; Renno *et al.* 2001). Multiple origins of polyploids would be indicated if (i) different allotetraploid taxa were characterized by different cpDNA haplotypes and if (ii) these haplotypes were also found in the parental lineages. Such hypotheses would be strengthened if some haplotypes had small distributions, but were found in both a parental lineage and an allotetraploid derivative occurring in the same area, or even in the same habitat (Segraves *et al.* 1999). However, several processes may act to conceal such patterns, e.g. the evolution of new haplotypes, and hybridization and introgression between allotetraploids and the parental species or between different allotetraploids.

The study is focused particularly on one of the allotetraploids with a relatively wide distribution in Northern Europe, *D. sphagnicola* (Höppner) Soó. Whereas most allotetraploids grow in wetlands with high pH, *D. sphagnicola* is confined to poor fens, often within bog complexes. The species has sometimes been associated with *D. incarnata* due to its many-flowered inflorescences and narrow unspotted leaves (Sundermann 1975, 1980), but allozyme markers show that it is clearly of allotetraploid origin (Hedrén 1996a). However, in contrast to other allotetraploids which exhibit fixed heterozygosity at all examined allozyme loci, it is characterized by free recombination at the cytosolic glucose-6-phosphate isomerase locus [GPI (= PGI), E.C. 5.3.1.9] and populations of *D. sphagnicola* often depart from other allotetraploids in allele frequencies (Hedrén 1996a). A study based on AFLP fingerprints also confirmed the somewhat deviating position of *D. sphagnicola* (Hedrén *et al.* 2001) being slightly more similar to *D. incarnata* than the other examined allotetraploids.

Table 1 Summary of haplotype data given for each genome group, taxon and country. For haplotype definitions, see Fig. 2

Taxon/site	Genome	Country	<i>n</i> loc	<i>n</i> ind	Haplotypes												
					A	B	C	D	E	F	G	H	I	J	K	L	M
<i>fuchsii</i>	FF	Sweden	10	48			38		8	2							
<i>fuchsii</i>	FF	France	2	5			5										
<i>saccifera</i>	FF	Greece	1	10	6	4											
<i>maculata</i> s.s.	FFFF	Sweden	17	64			9		19	7	11	1	7	9		1	
<i>maculata</i> s.s.	FFFF	Norway	1	1			1										
<i>maculata</i> s.s.	FFFF	France	1	1					1								
<i>maculata</i> s.s.	FFFF	UK	2	2					2								
<i>incarnata</i> s.s.	II	Sweden	11	24												24	
<i>cruenta</i>	II	Sweden	9	15												15	
<i>ochroleuca</i>	II	Sweden	3	3												3	
<i>incarnata</i> s.l.	II	Turkey	6	6												6	
<i>incarnata</i> s.l.	II	UK	3	4												4	
<i>majalis</i>	FFII	Sweden	2	11			11										
<i>traunsteineri</i>	FFII	Sweden	11	82			77	1								4	
<i>lapponica</i>	FFII	Sweden	5	17			17										
<i>sphagnicola</i>	FFII	Sweden	9	37					37								
Björnekulla	FFII	Sweden	1	10			2		8								
Dimbo	FFII	Sweden	1	6					4		1		1				
Djurgårdsängen	FFII	Sweden	1	1			1										
Viklau	FFII	Sweden	1	2			2										
Gylvik	FFII	Sweden	1	11			9									2	
Hide	FFII	Sweden	1	8			7									1	
Kneknäset	FFII	Sweden	1	4			4										
Långbromossen	FFII	Sweden	1	5			5										
Knupbodarna	FFII	Sweden	1	11					11								
<i>baltica</i>	FFII	Estonia	1	1			1										
<i>russowii</i>	FFII	Estonia	2	2			2										
<i>alpestris</i>	FFII	France	4	9			6	3									
<i>elata</i> s.s.	FFII	France	2	4											4		
<i>majalis</i>	FFII	France	1	1				1									
<i>traunsteineri</i>	FFII	UK	2	2												2	
<i>purpurella</i>	FFII	UK	2	2			1									1	
<i>nieschalkiorum</i>	FFII	Turkey	1	1			1										
<i>euxina</i>	EE	Turkey	3	4												4	
<i>urvilleana</i>	EEFF	Turkey	6	7			7									1	
<i>armeniaca</i>	EEII	Turkey	1	2												2	

Materials and methods

Plant material

The *D. incarnata/maculata* polyploid complex forms a variable group of orchids with notoriously ill-defined species limits (e.g. Delforge 2001). Although many taxa, particularly the allotetraploids, often approach each other in external morphology, they are usually treated as separate species (Buttler 1991; Delforge 2001); this practice is followed here.

A total 423 samples was examined in the study, 359 from Sweden and 64 from more southern parts of the distribution range (Table 1). The material was determined according to standard floras covering these areas, notably Hylander (1966), de Soó (1980) and Renz & Taubenheim

(1984). All major taxa present in Sweden were sampled, i.e. *D. fuchsii* and *D. maculata* s.s. representing the *D. maculata* s.l. parental lineage, *D. incarnata* s.s., *D. ochroleuca* and *D. cruenta* representing the *D. incarnata* s.l. parental lineage and *D. sphagnicola*, *D. majalis* (Rchb.) P.F. Hunt & Summerhayes, *D. traunsteineri* (Rchb.) Soó and *D. lapponica* (Hartm.) Soó, which are all allotetraploids. In addition, a number of local populations that could not be matched with any of the described allotetraploids were included and treated by the name of their localities. Two of these populations, Kneknäset and Långbromossen, are somewhat similar to *D. sphagnicola* in external morphology, i.e. narrow, unspotted leaves and many-flowered inflorescences, but do not exhibit the allozyme characters of *D. sphagnicola* (Hedrén 1996a). Also the Björnekulla population,

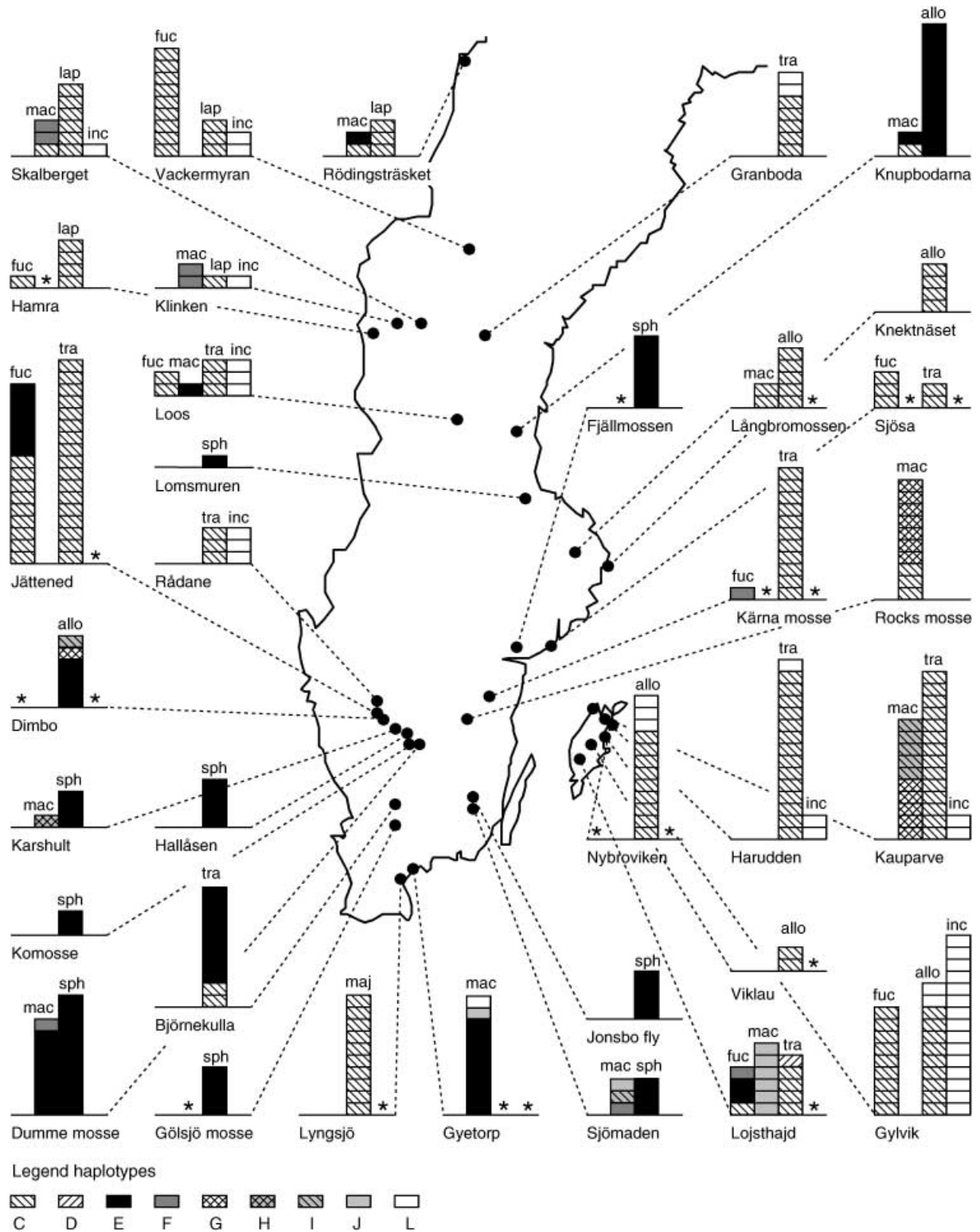


Fig. 1 Haplotype variation at Swedish *Dactylorhiza* localities with more than four studied individuals, or else with particularly interesting material. Sampled material of *D. fuchsii* (2 ×, genome composition FF), *D. maculata* s.s. (4 ×, genome composition FFFF), allotetraploids (4 ×, genome composition FFII) and *D. incarnata* s.l. (2 ×, genome composition II) are reported as separate bars from the left to the right for each locality. If these taxa were present at the localities, but not collected for haplotype analysis, their presence have been indicated by a star. Species abbreviations: ‘fuc’ *D. fuchsii*, ‘mac’ *D. maculata* s.s., ‘lap’ *D. lapponica*, ‘maj’ *D. majalis*, ‘sph’ *D. sphagnicola*, ‘tra’ *D. traunsteineri*, ‘inc’ *D. incarnata* s.l.; ‘allo’ denote local allotetraploid populations of unclear affinities.

which is usually regarded as *D. traunsteineri*, contained some plants similar to *D. sphagnicola*.

Most taxa are confined in calcareous habitats except for, i.e. *D. maculata* s.s. and *D. sphagnicola*. *D. maculata* s.s. grows at drier sites than *D. sphagnicola*, but is often found at margins or at elevated tussocks in the poor fen–bog complexes which are the typical habitat for *D. sphagnicola*. In this study, both taxa were often sampled from the same localities.

NonSwedish material was examined to obtain a background to the haplotype variation observed in Sweden. This material comprised a variety of taxa from mainly more southern parts of the distribution range. The location of the Swedish populations with more than four examined plants, or else with particularly interesting material, are shown in Fig. 1. The haplotype variation in the entire material is summarized in Table 1, in which the material has been subdivided by taxon and area of origin. A more detailed list in which each population is given as a separate entry may be obtained from the author upon request.

All taxa and most populations included in the present study have been characterized previously for allozyme variation (Hedrén 1996a,b,c, 2001a; unpubl. data). Because *D. incarnata* s.l. and *D. maculata* s.l. have completely different alleles at most of the variable allozyme loci, allozymes have been used to define a haploid genome I derived from the former and a genome F derived from the latter (Hedrén 1996a). Thus members of the *D. incarnata* lineage could be characterized by genome composition II, and members of the *D. maculata* s.l. lineage by genome composition FF or FFFF, depending on ploidy level. The European allotetraploids typically contain two I alleles and two F alleles at each locus, and are denoted accordingly by genome composition FFII. Similarly, for material included from Asia Minor, the diploid *D. euxina* may be denoted by genome composition EE, and the two allotetraploids derived from this lineage by EEFF (*D. urvilleana*) or EEII (*D. armeniaca*; Hedrén 2001a). The genome composition of all studied taxa is given in Table 1.

The ploidy levels previously reported for *Dactylorhiza* have been based on chromosome counts, and allozyme variation patterns have also been used to verify the ploidy levels of the studied populations and taxa. Diploids may not express more than two alleles at any locus as they are composed of two chromosome sets, whereas tetraploids may express up to four different alleles. Because allozyme expression is proportional to allele copy number at some of the examined loci, tetraploids may alternatively be identified by having unbalanced expression, corresponding to 3:1 or 2:1:1 allelic ratios. Allotetraploids normally express two alleles from each parental lineage at each locus.

Methods

Total-DNA was extracted by the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1990) from

either deep-frozen leaf samples kept in a -80°C freezer, or from dry flowers preserved by the silica gel method (Chase & Hills 1991). The sampling should have small effects on long-term survival of the sampled plants, as 2–10 flowers or part of a leaf were collected for analysis.

Initially, a subsample of 10 plants, representing the full diversity of the *D. incarnata/maculata* polyploid complex, was amplified for 12 different plastid DNA regions using the three primer pairs reported by Taberlet *et al.* (1991) and nine of those reported by Demesure *et al.* (1995). Regions that were amplified successfully from most of the samples were examined subsequently for size variation and variation in fragment patterns when cut by a set of 11 different restriction endonucleases, including *AluI*, *CfoI*, *EcoRI*, *HaeIII*, *HindIII*, *HinfI*, *MspI*, *NdeII*, *RsaI*, *TaqI* and *Tru9I*. Four variable primer pair/endonuclease combinations were then selected to screen the entire material. The *trnL-trnF* intergenic spacer (Taberlet *et al.* 1991) was amplified by an initial round of denaturing at 95°C for 4 min, followed by 41 cycles of denaturing at 92°C for 45 s, annealing at 63°C for 45 s and extension at 72°C for 3 min, ended by a final extension at 72°C for 10 min. The *trnC-trnD* intergenic spacer (Demesure *et al.* 1995) was amplified by an initial round of denaturing at 95°C for 4 min, followed by 41 cycles of denaturing at 92°C for 45 s, annealing at 57.5°C for 45 s and extension at 72°C for 4 min, ending with a final extension at 72°C for 10 min. The *psbC-trnS* and the *trnS-trnfM* intergenic spacers (Demesure *et al.* 1995) were amplified by an initial round of denaturing at 95°C for 4 min, followed by 41 cycles of denaturing at 92°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 2 min, ending with a final extension at 72°C for 10 min. The *trnL-trnF* and *trnC-trnD* amplification products were cut with *RsaI* to reveal variable fragments, whereas the *trnS-trnfM* amplification product was cut with *HinfI*. The *psbC-trnS* amplification resulted in two fragments, the shorter of which was examined for size variation. For each region, 10 μL of the amplification product was cut with 0.7 units of restriction enzyme. Fragments were separated in 1.5% agarose gels at 5 V/cm and visualized by ethidium bromide staining.

Plastid DNA sequencing

Because questions on homology arose when summarizing the variation found at different regions of the *Dactylorhiza* plastid DNA, it was also found necessary to characterize two of these regions for sequence variation using a subset of the material. The *trnL-trnF* spacer was amplified by means of universal primers e and f reported by Taberlet *et al.* (1991). The PCR product was purified using Qiagen PCR purification columns. The dideoxy cycle sequencing reactions included Cy5 dye-labelled primers using the Thermo sequenase primer cycle sequencing kit (Amersham

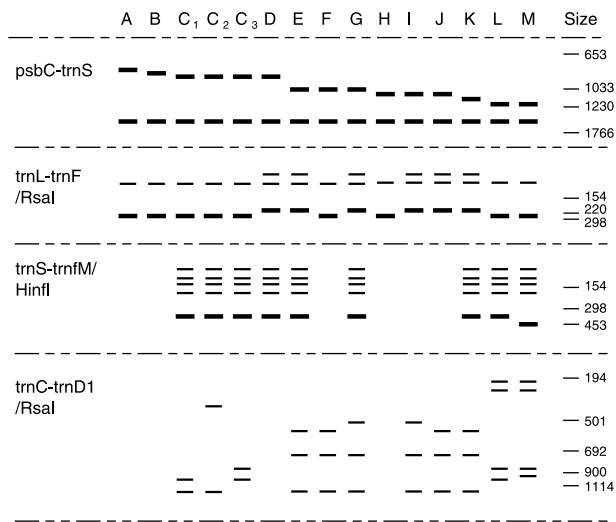


Fig. 2 Phenotypes found at the four studied plastid DNA regions. Haplotypes were defined by the combined pattern observed for these regions. Three different variants of haplotype C were observed in the *trnC-trnD1* region, but as most samples sorted as C did not yield any amplification product for the region, this variation was not possible to use to further subdivide the C haplotype.

Biosciences). The *psbC-trnS* gene and pseudogene were amplified by the primers published by Demesure *et al.* (1995), and separated by size on 1.5% agarose gels. Excised gel slices were purified by means of Qiagen gel extraction kit. Dye-labelled dideoxy nucleotides were incorporated during cycle sequencing using the Thermo sequenase Cy5 dye terminator cycle sequencing kit (Amersham Biosciences). Two internal primers were designed specifically for cycle sequencing of the mid-portion of the pseudogene and were used in addition to the PCR primers. All cycle sequencing reactions were purified by means of ethanol precipitation. Dye-labelled fragments were separated on an ALF Express II automated sequencer (Amersham Biosciences). Alignments were performed by eye.

Results

Characterization of plastid DNA haplotypes

Thirteen different haplotypes were recognized on the basis of the combined patterns seen at the four plastid DNA regions that were examined for restriction site and fragment length variation (Fig. 2).

The *psbC-trnS* amplification product consisted of two fragments, one large fragment of about 1500 base pairs (bp) and one smaller fragment that was variable in length. The larger fragment (not shown) demonstrated a close sequence similarity with *psbC-trnS* region reported for maize (GenBank Accession no. X86563), and consisted of the major portion of the *psbC* gene, a small spacer and a 5' portion of the *trnS* gene. The smaller fragment was inter-

preted as a pseudogene. It showed some resemblance to the *psbC* gene close to the *psbC* primer site, but the remaining portion (Table 2) contained considerable size variation due to deletions and insertions, some of which were duplications of other parts of the same fragment. Seven different size variants were identified by separation on agarose gels. Sequencing of a restricted number of samples showed that fragments of the same size class are generally homologous, as were those that were found in different taxa. The exceptions were the L and M haplotypes, which were interpreted as the same size variant when separated on agarose gels, but which in fact differed by 8 bp and are characterized by different indels (Table 2).

The *trnL-trnF* amplified region was cut into two or three fragments when cut with *RsaI* (Fig. 2). Sequences from this region are compared with some other species of *Dactylorhiza* and one of the sequences reported for *Gymnadenia conopsea* by Soliva & Widmer (1999) in Table 3. The *Dactylorhiza* haplotypes D, E, I, J and K differed from haplotypes A, B, C, F and H by a single point mutation at position 193, rendering the former with an additional restriction site for *RsaI*. The latter had the same base (C) as haplotype L and M at this position, but differed by two point mutations at positions 108 and 174 and a 4-bp deletion at positions 207–210. None of the variations seen at the two sequenced regions was due to microsatellite variation.

The *trnS-trnM* amplified region gave rise to two fragments when cut with *HinfI* (Fig. 2), the larger of which was examined for size variation. All taxa were identical, except for *D. euxina* which had a larger fragment; thus, it was not regarded as necessary to characterize all non-*euxina* samples for this region.

The *trnC-trnD* amplified region gave rise to complex fragment patterns when cut with *RsaI* (Fig. 2). Six different patterns were recognized. Most samples sorted as haplotype C did not yield any amplification product for this region, and although three different patterns were observed in those samples that did amplify, this information could not be used to subdivide the material further.

Haplotype variation in species and populations

Among the diploids, a single haplotype (L) was found in *D. incarnata* s.l. (2 ×, genotype II) and a single haplotype (M) in *D. euxina* (2 ×, genotype EE; Table 1). *Dactylorhiza fuchsii* (2 ×, FF) contained haplotypes C, E and F, whereas a single population of the related *D. saccifera* (2 ×, FF) from Greece contained the unique haplotypes A and B. The autotetraploid *D. maculata* s.s. (4 ×, FFFF) shared haplotypes C, E and F with *D. fuchsii*, but it also contained haplotypes G, H, I and J, which were not found in any of the investigated diploids. Furthermore, the frequency of haplotype C was lower, but the frequency of haplotype E higher, than in *D. fuchsii*. Several local populations of *D. maculata* s.s.

Table 2 Summary of sequence alignments of the *psbC-trnS* pseudogene in *Dactylorhiza*. Each symbol denote a gene fragment of unique sequence. Accession numbers for each extract are given within parentheses

Taxon and sample #	Origin	Haplotype	Length of fragment	Total length	GenBank accession number
			3 2 1 1 111 111 1 1 1 1 1 1 1 1 0 4 25 20 7 2 2 7 578178617815081517873368733687389443366367242374377431		
<i>D. saccifera</i> (202)	Pavliani, Greece	A	ab-----cdidef-----opqr---tv--yz#fz----\$	873	AY283524
<i>D. saccifera</i> (198)	Pavliani, Greece	B	ab-----cdi---kl-----mfopqr---tv--yz#fz----\$	888	AY283525
<i>D. fuchsii</i> (416)	Lojsthajd, Sweden	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	AY283526
<i>D. maculata</i> (210)	Rocks mosse, Sweden	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	
<i>D. maculata</i> (387)	Ed, Sweden	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	
<i>D. purpurella</i> (508)	Anglesey, UK	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	AY283527
<i>D. alpestris</i> (381)	Eygas, France	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	
<i>D. baltica</i> (285)	Pirita, Estonia	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	
<i>D. traunsteineri</i> (125)	Harudden, Sweden	C	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	
<i>D. majalis</i> (65)	La Batie, France	D	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	AY283528
<i>D. alpestris</i> (383)	La Batie, France	D	ab-----cdidefkl-----mfopqr---tv--yz#fz----\$	914	
<i>D. maculata</i> (389)	Knupbodarna, Sweden	E	abcdefghb---cdidefkl-----mfopq-stustv--yz#fz----\$	1049	
<i>D. maculata</i> (398)	Rödingsträsket, Sweden	E	abcdefghb---cdidefkl-----mfopq-stustv--yz#fz----\$	1049	
<i>D. maculata</i> (502)	Anglesey, UK	E	abcdefghb---cdidefkl-----mfopq-stustv--yz#fz----\$	1049	AY283529
<i>D. sphagnicola</i> (296)	Jonsbo fly, Sweden	E	abcdefghb---cdidefkl-----mfopq-stustv--yz#fz----\$	1049	
<i>D. maculata</i> (396)	Klinken, Sweden	F	abcdefghb---cdidefkl-----mfopq-stustv--yz#fz----\$	1049	AY283530
<i>D. maculata</i> (371)	Karshult, Sweden	H	abcdef-hb---cdidefkl-----mfopq-stustv--yz#fz##fz\$	1084	AY283531
<i>D. elata</i> (385)	Val de Trebans, France	K	abcdef-hb---cdidefklm-----fkmfopq-stustv--yz#fz##fz\$	1128	AY283532
<i>D. incarnata</i> (447)	Gylvik, Sweden	L	abcdefghbcdijcdidefklmn-----fkmfo-q-stustv-xyz#fz##fz\$	1172	AY283533
<i>D. euxina</i> (3)	Sekersu, Turkey	M	abcdefghbcdijcdidefklmnfklmnfkmfo-q-stustv--yz#fz----\$	1180	AY283534
<i>D. viridis</i> (497)	Skalberget, Sweden		abcdef-hb---cdidefkl-----mfopq-stustvwxvxyz#fz----\$	1049	AY283535
<i>D. iberica</i> (496)	Exarhos, Greece		?bcdefghbcdijcdidefklmn-----fkmfo???????????????????		AY283536

Table 3 Summary of variable positions at the *trnL-trnF* region in *Dactylorhiza*. The *Dactylorhiza* sequences are compared to the corresponding sequence reported for *Gymnadenia conopsea* by Soliva & Widmer (1999; GenBank Accession no. AF105325). Recognition sequences for *RsaI* are underlined. Accession numbers for each extract are given within parentheses

Taxon and sample no.	Origin	Haplotype	position after primer <i>trnL</i> 011111111122222 900478999900013 289546123478907 +	GenBank Accession Nr.
<i>Gymnadenia conopsea</i>	St Moritz, Switzerland		GTACGGGTCCCTGCC	AF105325
<i>D. saccifera</i> (202)	Pavliani, Greece	A	TTGCG-GTCC----T	AY284952
<i>D. saccifera</i> (198)	Pavliani, Greece	B	TTGCG-GTCC----T	
<i>D. fuchsii</i> (416)	Lojstahjd, Sweden	C	TTGCG-GTCC----T	AY284953
<i>D. maculata</i> (210)	Rocks mosse, Sweden	C	TTGCG-GTCC----T	AY284954
<i>D. alpestris</i> (381)	Eygas, France	C	TTGCG-GTCC----T	
<i>D. alpestris</i> (383)	La Batie, France	D	TTGCG- <u>GTAC</u> ----T	
<i>D. traunsteineri</i> (413)	Lojstahjd, Sweden	D	TTGCG- <u>GTAC</u> ----T	AY284955
<i>D. maculata</i> (389)	Knupbodarna, Sweden	E	TTGCG- <u>GTAC</u> ----T	
<i>D. fuchsii</i> (418)	Lojstahjd, Sweden	F	TTGCG-GTCC----T	
<i>D. maculata</i> (245)	Dumme mosse, Sweden	F	TTGCG-GTCC----T	AY284956
<i>D. maculata</i> (190)	Kauparve, Sweden	G	TTGCG- <u>GTAC</u> ----T	
<i>D. maculata</i> (371)	Karshult, Sweden	H	TTGCG-GTCC----T	
<i>D. maculata</i> (364)	Sjömaden, Sweden	I	TTGCG- <u>GTAC</u> ----T	AY284957
<i>D. maculata</i> (422)	Lojstahjd, Sweden	J	TTGCG- <u>GTAC</u> ----T	
<i>D. elata</i> (385)	Val de Trébans, France	K	TTGCG- <u>GTAC</u> ----T	
<i>D. incarnata</i> (4)	Erzurum, Turkey	L	TCGCT-GTCCCTTTT	
<i>D. incarnata</i> (447)	Gylvik, Sweden	L	TCGCT-GTCCCTTTT	AY284958
<i>D. incarnata</i> (504)	Anglesey, UK	L	TCGCT-GTCCCTTTT	
<i>D. armeniaca</i> (39)	Savsat, Turkey	L	TCGCT-GTCCCTTTT	
<i>D. euxina</i> (3)	Sekersu, Turkey	M	TCGCGGGTCCCTTTT	AY284959
<i>D. iberica</i> (496)	Exarhos, Greece		TCGCG-GTCCCTTTT	AY284960
<i>D. viridis</i> (497)	Skalberget, Sweden		TCGAG-GTCCCTTTT	AY284961

and *D. fuchsii* were polymorphic and contained different haplotypes (Fig. 1).

Among the allotetraploids, *D. sphagnicola* (4 ×, FFII) was fixed for haplotype E (Table 1). Haplotype C dominated in the remaining allotetraploids, except for the two local, unnamed populations from Dimbo and Knupbodarna (Fig. 1) and *D. traunsteineri* (4 ×, FFII) from Björnekulla. Two haplotypes were confined to the allotetraploids. Haplotype K was unique to *D. elata* (Poir.) Soó (4 ×, FFII) from France, whereas haplotype D was found in *D. alpestris* (Pugsley) Aver. (4 ×, FFII) and *D. majalis* (4 ×, FFII) from France as well as in a single specimen of *D. traunsteineri* from Lojstahjd on the island of Gotland. Populations of *D. traunsteineri* from the United Kingdom contained haplotype L. *Dactylorhiza urvilleana* (4 ×, EEFF) from Turkey contained haplotypes C and M and *D. armeniaca* (4 ×, EEII) contained haplotype L. Some populations of allotetraploids were polymorphic. The Björnekulla population contained haplotypes C and E, whereas populations from Gylvik, Nybroviken, Harudden and Granboda contained low frequencies of haplotype L in addition to C. The *D.*

traunsteineri population at Lojstahjd contained one sample with haplotype D in addition to samples with haplotype C.

Discussion

Haplotype variability and differentiation of the parental lineages

The diploid *D. fuchsii* (FF) and the tetraploid *D. maculata* s.s. (FFFF) shared several of the recognized haplotypes. It is possible that this pattern may be a result of recurrent polyploidization and continuous gene flow from the diploid to the tetraploid level such as described for, e.g. the grass *Dactylis glomerata* (Lumaret & Barrientos 1990). However, it is also possible that both taxa have retained much of the haplotype polymorphism that may have been present in a common ancestor.

The single haplotype variant found in *D. incarnata* (2 ×, II), L, differed by several indels and point mutations from the *D. maculata* s.l. haplotypes (Tables 2 and 3). The low degree of haplotype variability in *D. incarnata* is in agreement

with previous findings from allozyme studies (Hedré 1996a) and AFLP fingerprints (Hedré *et al.* 2001) that genetic variability in European *D. incarnata* is restricted.

Haplotype L was similar to the haplotype found in *D. euxina* (2 ×, EE), denoted as M. A relatively close position of these species was also suggested by allozyme patterns (Hedré 2001a). Judging from the fact that the 4 bp indel in the *trnL-trnF* spacer at positions 191–194 was present in these two species and in *Gymnadenia* (Table 3), but absent from *D. maculata* s.l. and *D. elata* (4 ×, FFII), the haplotypes found in the latter two species (A–K) should be more closely related to each other than to that of *D. incarnata*.

Gene flow between the *D. incarnata* s.l. and *D. maculata* s.l. parental lineages appears to be very rare. The majority of examined *D. maculata* s.s. from the Gyetorp locality had haplotype E, but a single specimen was encountered with the typical *D. incarnata* s.l. haplotype, L (Fig. 1). However, *D. incarnata* was also very common at the locality, and it seems likely that the L haplotype has been introduced into *D. maculata* s.s. by local hybridization and introgression (in spite of the fact that no such hybrids were observed when the material was collected). A clear separation of *D. incarnata* s.l. and *D. maculata* s.l. was also observed in a study based on allozyme loci (Hedré 1996a).

Patterns of origin of allotetraploid *Dactylorhiza*

All members of the Orchidaceae that have been examined inherit their plastid genome from the maternal parent (Harris & Ingram 1991) and it is assumed that this is also true for *Dactylorhiza*. If so, the frequent occurrence of *D. maculata* s.l. haplotypes in the investigated allotetraploids suggests that this lineage served most frequently as the maternal parent in the hybridization events that preceded allopolyploid formation in *Dactylorhiza*. A possible exception is *D. traunsteineri* from the United Kingdom, which contained the *D. incarnata* haplotype L, but only two specimens were examined from this area (Table 1).

The polyploidization process has not been studied experimentally in *Dactylorhiza*. However, it is the most common pattern in other polyploid complexes that new polyploids arise by stepwise increase in chromosome number by means of unreduced gametes (deWet 1980; Ramsey & Schemske 1998). According to this scenario, already established polyploids may backcross with related diploids and contribute genetic material to hybrids at intermediate ploidy levels from which new polyploids may arise.

Some lines of reasoning suggest that the maternal parent to *D. sphagnicola* (4 ×, FFII) may have been related to the autotetraploid *D. maculata* s.s. (4 ×, FFFF). First, the E haplotype that characterizes *D. sphagnicola* is more frequent in *D. maculata* s.s. than in *D. fuchsii* (2 ×, FF), which predominantly contain haplotype C. Second, these are the only members of *Dactylorhiza* in Sweden that are found in acidic habitats,

and *D. sphagnicola* may have inherited the ability to grow and compete in such habitats from *D. maculata* s.s. However, it should be kept in mind that haplotype E is also found in high frequencies in some populations of *D. fuchsii*, e.g. at Jättened. It should also be observed that primary hybrids between *D. incarnata* and *D. maculata* s.s. are uncommon, but may be found at sites where acidic habitats are mixed with calcareous fens, e.g. at Kauparve (Fig. 1).

The remaining allotetraploids generally carry the same haplotype that is most frequent in *D. fuchsii*, and they may have arisen by hybridization between diploid *D. fuchsii* and *D. incarnata*. These two diploids are often found in close vicinity of each other in calcareous areas, and diploid hybrids between them are common.

Multiple origins

D. sphagnicola (4 ×, FFII) shares at least four different alleles with *D. maculata* s.l. (4 ×, FFFF) at the allozyme locus phosphogluconate dehydrogenase, PGD (E.C. 1.1.1.44; Hedré 1996a), which reveals that *D. sphagnicola* must have had multiple origins, either by multiple polyploidization events or by hybridization and backcrossing between the original allotetraploid and the *D. maculata* s.l. parent. However, *D. sphagnicola* is fixed for a single plastid DNA haplotype, which means that either (i) the same haplotype was transferred to the allotetraploid on several occasions, or (ii) that the additional alleles were introduced by pollen, or (iii) that the species was originally polymorphic, but has subsequently lost the other plastid DNA variants. The effective population size of the plastid genome is smaller than that of the nuclear genome, and accordingly the plastid genome is more likely to become fixed during population bottlenecks (cf. Moritz 1996).

Several of the remaining allotetraploid taxa also have more than two *D. maculata* s.l. alleles at one or several loci, and may also have evolved by multiple polyploidization events or backcrossing with the *D. maculata* s.l. parental lineage. However, most of these taxa are characterized by haplotype C, and it cannot be excluded that these allozyme alleles have been spread among the allotetraploids by means of hybridization and introgression at the tetraploid level (see below).

The local allotetraploid at Dimbo contained both the common *D. maculata* s.s. haplotype E and the rare G and I haplotypes. This pattern may indicate that the population itself has evolved by at least three independent polyploidization events, but it is also possible that some of the haplotypes was introduced by subsequent introgression from *D. maculata* s.s. after the allotetraploid was formed.

Backcrossing with the parental lineages

Several of the localities sampled for *D. sphagnicola* in this study were also sampled for *D. maculata* s.s. Primary

hybrids between these taxa ($4 \times$, FFFI) were often encountered and their identity has been verified by allozyme analysis (Hedrén 1996a). However, in spite of the fact that *D. maculata* s.s. contained a variety of haplotypes at these sites, only haplotype E was present in the *D. sphagnicola* samples. Accordingly, it is possible that F_n hybrid formation and backcrossing may be relatively uncommon between these taxa. However, as the E haplotype is the most common one in *D. maculata*, lack of other haplotypes in *D. sphagnicola* does not provide evidence that introgression has not occurred.

Moreover, the haplotype data indicate that some populations of the allotetraploid *D. traunsteineri* and two of the local allotetraploids on Gotland may be affected by introgression from the diploid *D. incarnata*. Although most plants in these populations contained haplotype C, which predominates in *D. fuchsii*, a few individuals contained haplotype L which they shared with *D. incarnata*. *Dactylorhiza incarnata* also appeared in large numbers at several of these sites, and it seems likely that the *incarnata* haplotype has been transferred to the allotetraploids by local hybridization and introgression.

Hybridization between allotetraploids

Roberts (1966) argued that because apparent hybrids were not encountered in some mixed populations of *D. purpurella* (T. & T.A. Stephenson) Soó and *D. traunsteineri*, and *D. purpurella* and *D. majalis* (= *D. occidentalis* (Pugsley) P. Delforge) in Britain, hybridization probably rarely takes place between allotetraploids. However, Bateman & Denholm (1983) pointed out that many allotetraploid taxa contain such large intrapopulation variability that their morphologies overlap considerably and, accordingly, any hybridization and introgression may remain undetected. Hybridization between allotetraploids is suspected at the Björnekulla locality. This material contained both the C and the E haplotypes (Tables 1 and 3). These plants exhibited varying degrees of intermediacy between *D. traunsteineri* and *D. sphagnicola* in morphology, and the observed pattern of plastid DNA haplotype variation may be taken as evidence for local hybridization and introgression at the tetraploid level between these two taxa.

Because the widespread allotetraploids (except for *D. sphagnicola*) contained mainly haplotype C, it is not possible to deduce from haplotype data whether they have separate origins from each other or whether some of them have themselves multiple origins. Because of this pattern it is also possible that gene flow between them, or introgression from the diploid *D. fuchsii* (Lord & Richards 1977), would remain undetected if it occurs. It would be valuable to develop a more detailed data set, either by sequencing or investigating other cpDNA regions for restriction site variation in these allotetraploids to find data that would give increased knowledge about any introgression.

Haplotype distribution and age of allotetraploid taxa

Some of the haplotypes found in the allotetraploids, i.e. haplotypes D and K, were not encountered in any of the samples representing their parental lineages. It is possible that the parents carrying these haplotypes may have become extinct, or may be present in other areas than those sampled here, but it is also possible that these haplotypes have arisen by mutation at the tetraploid level. Extended sampling over a wider part of the distribution area of *Dactylorhiza* may help to solve this question.

Haplotype D, as well as haplotypes C and E, were found to be widespread in the distribution area of the genus *Dactylorhiza* in Europe and they were found in several recognized taxa that have smaller distribution areas. Thus, these haplotypes may be older than many of the taxa in which they are harboured today. The fact that some of the haplotypes are shared between the parental lineages and local allotetraploid taxa distributed in the same geographical areas is consistent with the idea that the latter may be of local origin, but because these haplotypes are widespread, it is also possible that the local allotetraploids have originated somewhere else and migrated to their present localities afterwards.

Conclusions and evolutionary implications

The distribution of plastid DNA haplotypes in *Dactylorhiza* combined with previous knowledge on allozyme variation patterns indicate that allotetraploids have evolved by hybridization between the parental *D. maculata* s.l. and *D. incarnata* s.l. lineages on several separate occasions. Furthermore, some populations characterized by haplotype polymorphisms may have evolved by repeated local polyploidization. In most of the studied allotetraploids, the *D. maculata* s.l. lineage contributed the plastid genome. However, *D. incarnata* s.l. haplotypes also appear to have entered populations of *D. traunsteineri* locally by hybridization and introgression, and at least in one case hybridization at the tetraploid level appear to have taken place between the latter and *D. sphagnicola*. The Swedish allotetraploids do not contain any unique haplotypes. They may have evolved recently from parental lineages in Scandinavia, or may be derived from allotetraploid taxa outside the area. Some of the allotetraploids from other parts of Europe may be of greater age.

Considering the relative importance of these processes, it may be hypothesized that the *D. incarnata/maculata* polyploid complex constitutes an unusually dynamic system of polyploid speciation and extinction in which polyploids evolve continuously from the same set of broadly defined parental lineages. Some polyploids may inherit by chance portions of the parental genomes that in combination have a high adaptive value in a particular habitat. Given enough

time and availability of this habitat, they may disperse over large areas, and perhaps they did so after the Weichselian when previously glaciated areas became available for colonization and refugial populations of their parental taxa encountered each other and hybridized. However, the eventual fate of the many local polyploids is unknown. Most of them are likely to go extinct, a few may establish themselves at new localities, and yet others may be swamped into the gene pool of already well-established species, thereby widening the genetic basis for further adaptation and evolutionary change at the polyploid level. Genetic variation could also be added by introgression from the parental lineages or by restructuring of the polyploid genome, but these processes may be of minor importance, given the high incidence of polyploidization in *Dactylorhiza*.

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The author is interested in fine-scale systematics, colonization and conservation of Scandinavian plant groups with complex variation patterns. This particular study was carried out as a part of a major project aiming at detailed understanding of patterns and evolutionary consequences of polyploid evolution in the orchid genus *Dactylorhiza*.
