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# Systematics, phylogeography and polyploid evolution in the *Dactylorhiza maculata* complex (Orchidaceae)

**David Ståhlberg**

AKADEMISK AVHANDLING
som för avläggande av filosofie doktorsexamen vid naturvetenskapliga fakulteten, Lunds universitet, kommer att offentligen försvaras vid Ekologiska institutionen, Avdelningen för växtekologi och systematik, fredagen den 27 april 2007 kl 10.00.
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Fakultetsopponent: Professor Hilde Nybom, Sveriges lantbruksuniversitet, Balsgård.

Lund 2007

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in ms).

Cover: Satyrium Basilicum alterum (vel femina)
[*Dactylorhiza maculata* ssp. *maculata*]
Picture from Rembert Dodoens (1583): Stirpium historiae pemptades sex. Antuerpiae.

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This thesis is based on the following papers, which are referred to by their Roman numerals:

I Hedrén M., Nordström S., Ståhlberg D. Plastid DNA variation and the origin of allopolyploid *Dactylorhiza* (Orchidaceae) in Scandinavia. Submitted.

II Ståhlberg D., Hedrén M. Systematics and phylogeography of *Dactylorhiza maculata* s.l. (Orchidaceae) in Scandinavia: insights from cytological, morphological and molecular data. Manuscript.

III Ståhlberg D. Habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. (Orchidaceae) in Scandinavia. Manuscript.

IV Ståhlberg D., Hedrén M. Phylogeography of the *Dactylorhiza maculata* polyploid complex (Orchidaceae): high genetic diversity in the north and indications of plastid DNA recombination. Manuscript.

# Systematics, phylogeography and polyploid evolution in the *Dactylorhiza maculata* complex (Orchidaceae)

## Introduction and background

### *Influence of Quaternary climatic changes on speciation*

Quaternary climatic changes have had a profound impact on speciation, structuring of genetic diversity and the shaping of the present-day distributions of plant and animal taxa (Vuilleumier, 1971; Hewitt, 1996, 2000, 2004; Avise, 2000). In Europe, the repeated cycles of glacials and interglacials during the Pleistocene (c. 2 Ma until 10 000 BP) have caused massive fluctuations in the distributions of taxa. Fragmentation and isolation of populations during the long-lasting glacials and expansion during the short interglacials have resulted in marked differences among regions in intraspecific diversity. Oscillations of population sizes, bottle necks, founder events and other population historical events associated with climatic shifts have further contributed to differentiation among regional population groups. As a combined effect of range shifts and population differentiation, divergent lineages have occasionally formed contact zones, leading to reticulate speciation by means of hybridization and polyploidization (Grant, 1981; Stebbins, 1984; Hewitt, 1988, 2001). A high frequency of polyploid taxa may be a particularly important feature of regions harbouring young floras and faunas, including Fennoscandia and adjacent areas of northern Europe that were covered by thick ice sheets during the maximum of the last (Weichselian) glaciation (LGM; c. 22 000 to 18 000 BP), as compared to more climatologically stable regions (Löve and Löve 1974; Stebbins, 1971; Hewitt, 1988; Soltis et al., 2003).

Analysis of macrofossil and pollen data together with organellar markers have shown that populations of many temperate species in the European flora and fauna survived the LGM in various southern refugia in the Mediterranean region (the Iberian, Apennine and Balkan peninsulas), and in the Caspian/Caucasian region (Huntley and Birks, 1983; Petit et al., 1993; Demesure et al., 1996; Hewitt, 2004). Similarly, patterns of postglacial migration have been reconstructed for many temperate species, and a general picture of high intraspecific genetic diversity in refugial areas in the south and low diversity in previously glaciated areas in the north has been established (Ferris et al., 1999). However, this picture may be too simple (cf. Widmer and Lexer, 2001). Increasing evidence suggests that the southern refugia for temperate species were supplemented by more northern refugia during the LGM (reviewed by Stewart and Lister, 2001), which clearly would have resulted in more complex patterns of the distribution of intraspecific genetic diversity. Areas of sheltered topography in mountainous parts of Central Europe may have provided suitable stable microclimates for thermophilous plant and animal species (Litynska-Zajac, 1995; Willis and van Andel, 2004; Magri et al., 2006; Ursenbacher et al., 2006; Sommer and Nadachowski, 2006). Populations of more cold tolerant species may as well have survived close to the southern and eastern edges of the Fennoscandian ice sheet together with arctic-alpine species (Rendell and Ennos, 2002; Palmé et al., 2003; Alsos et al., 2005; Skrede et al., 2006).

Increased intraspecific genetic diversity also occurs in contact zones where divergent populations from separate refugia meet (Petit et al., 2003). Such zones of secondary contact have been demonstrated for both plants and animals at intermediate latitudes in Central Europe (Petit et al. 2003). Polyploidization appears to be common at these latitudes (Stebbins, 1984). Several studies have indicated that central-northern Scandinavia may be another area of secondary contact between divergent populations immigrating from the northeast and the south (Jaarola and Tegelström, 1995; Fredga, 1996; Nyberg-Berglund and Westerbergh, 2001).

## *Polyploid evolution*

Polyploid speciation has long been recognized as an important process in plant evolution (Müntzing, 1936; Stebbins 1950; Grant, 1981). Recent genomic studies have made it clear that angiosperms possess genomes with considerable gene redundancy, indicating that "most (if not all) plants have undergone one or more episodes of polyploidization" (Soltis et al., 2003). Many taxa are ancient polyploids that have become secondarily diploidized due to gene silencing and other genomic processes (e.g. Lynch and Conery, 2000). Functional polyploids in the traditional sense refer to polyploids that are integral parts of polyploid complexes consisting of closely related taxa at various ploidy levels (cf. Grant, 1981; Soltis et al., 2003). Polyploidization is probably the most common mechanism of sympatric speciation (Otto and Whitton, 2000), and it is widely accepted that a single taxon may arise several times by independent polyploidization events, via both allopolyploidization and autopolyploidization (Soltis and Soltis, 1993, 1999; Soltis et al., 2003).

Chromosome doubling is an instantaneous mode of speciation that results in effective postzygotic barriers between new polyploids and their diploid progenitors (Ramsey and Schemske, 1998). On the other hand, new polyploid plants are subjected to competitive constraints because they are also exposed to pollen from diploid plants, which results in triploid offspring that have low fitness (minority cytotype disadvantage; Levin, 1975). Adaptive strategies are needed for establishment and persistence of new polyploids. Such strategies include apomixis, self-pollination, pollinator shift and habitat differentiation (Soltis et al., 2003). However, even though the literature on polyploidy is comprehensive there are relatively few studies that focus on the ecological aspects of polyploidization.

## *Variation in* Dactylorhiza

*Dactylorhiza* Necker ex Nevski is one of the most taxonomically investigated genera in the orchid family (e.g. Klinge, 1898; Vermeulen, 1947; Soó, 1960; Senghas, 1968; Nelson, 1976; Averyanov, 1990; Pedersen, 1998). The genus is widespread (Eurasia, Northern Africa, Alaska), and consists of a confusing variety of forms that are difficult to sort into discrete taxa. Consequently, the number of species varies strongly among authors, ranging from 12 (Klinge, 1898) to 75 (Averyanov, 1990). Part of the taxonomic complexity could be explained by the frequent interpretation of aberrant populations and specimens as separate taxa (Bateman and Denholm, 2003), but more important explanations may be innate factors such as phenotypic plasticity, or that many taxa are young and have not yet acquired good separating characters. Since long, hybridization and polyploidization have been recognized as critical factors for the understanding of the diversification in *Dactylorhiza* (Hagerup, 1938; Heslop-Harrison, 1957). During the last decade, molecular tools have provided deeper insights into the evolutionary history of *Dactylorhiza* and the intricate patterns of speciation that characterize the genus (Hedrén, 1996, 2001, 2003; Hedrén et al., 2001, 2007; Pridgeon et al., 1997; Bateman et al., 1997; Pedersen, 1998, 2004, 2006; Bullini et al., 2001; Devos et al, 2003, 2005, 2006a, b; Shipunov et al., 2004, 2005; Pillon et al., 2006, 2007).

Most species of *Dactylorhiza* form a polyploid complex that has undergone extensive reticulate evolution (summarized by Hedrén, 2002). In Europe, a large number of allotetraploid taxa (i.e. taxa belonging to the *D. majalis* [Rchb.] P.F. Hunt & Summerh. group) have evolved repeatedly by hybridization between two broadly defined parental lineages: the *D. incarnata* (L.) Soó s.l. lineage and the *D. maculata* (L.) Soó s.l. lineage. Extensive studies of these two parental lineages are necessary to achieve a detailed comprehension of polyploid evolution in *Dactylorhiza*. A better understanding of the variation patterns in *D. incarnata* s.l. and *D. maculata* s.l. is urgent also for proper decisions about conservation priorities, since many allotetraploid taxa in Europe are threatened by habitat loss (e.g. Janečková et al., 2006; Pillon et al., 2006).

It appears that more information will be gained by studying the *D. maculata* s.l. parental lineage, rather than the *D. incarnata* s.l. parental lineage. Nearly all investigated allotetraploids with *D. incarnata* s.l. x *D. maculata* s.l. origin have inherited their plastid genomes from the *D. maculata* s.l. parent (Hedrén, 2003; Hedrén et al., 2007; Devos et al, 2003, 2006a; Shipunov et al., 2004, 2005; Pillon et al., 2007). It is therefore of particular interest to investigate *D. maculata* s.l. for plastid DNA variation. Also at nuclear marker loci it appears more profitable to analyze *D. maculata* s.l. rather than *D. incarnata* s.l. *Dactylorhiza maculata* s.l. is considerably more variable than *D. incarnata* s.l. at all nuclear marker loci investigated so far: allozymes (Hedrén, 1996, 2001; Pedersen, 1998, 2004, 2006), amplified fragment length polymorphisms (AFLPs; Hedrén et al. 2001, 2007) and internal transcribed spacers of nuclear ribosomal DNA (ITS nrDNA; Shipunov et al., 2004, 2005; Devos et al, 2005, 2006a; Pillon et al., 2007). Since *D. maculata* s.l. is morphologically very variable and occupies a wide range of habitats, molecular genetic studies should ideally be combined with morphometric and ecological studies.

## The *Dactylorhiza maculata* complex

*Dactylorhiza maculata* s.l. is a morphologically and genetically variable polyploid complex consisting of diploid (2n = 40) and tetraploid (2n = 80) cytotypes (Averyanov, 1990; Hedrén, 1996; Hedrén et al., 2001; Tyteca, 2001; Bateman and Denholm, 2003; Shipunov et al., 2004; Devos et al., 2005). At least 30 taxa at various taxonomic levels have been described (Soó, 1960; Delforge, 1995), but most contemporary authors distinguish between three or four morphologically and largely cytologically defined taxa: (1) *D. maculata* ssp. *fuchsii* (Druce) Hyl., a predominantly diploid taxon that typically grows in semi-open woodlands on fertile soils throughout most of northwestern Eurasia (absent or rare in southern and southeastern Europe); (2) *D. maculata* ssp. *saccifera* (Brongn.) Diklic, a diploid taxon that gradually replaces ssp. *fuchsii* on the Apennine peninsula and in southeastern Europe; (3) *D. maculata* ssp. *maculata*, a tetraploid taxon that characteristically is found in more open habitats such as grasslands, coastal moorlands and boreal-subarctic peatlands in western and northern Eurasia (absent in southeastern Europe); (4) *D. foliosa* (Sol. ex Lowe) Soó, a geographically isolated Madeiran diploid. It should be observed that tetraploid populations of *D. maculata* ssp. *fuchsii* are common in the mountain areas of Central Europe (e.g. Groll, 1965; Vöth and Greilhuber, 1980), and that ssp. *maculata* is rare or absent in the same region (e.g. Klein and Kerschbaumsteiner, 1996).

Chromosome counts and ploidy level determinations of almost 400 populations of *D. maculata* s.l. from throughout all of the distribution range have been reported in the literature (Table 1). The distributions of diploid and tetraploid populations with respect to geography and taxonomy are shown in Figure 1. This compilation shows that there is a clear correlation between ploidy level and taxonomy, except for populations of ssp. *fuchsii*. Of more than 150 cytologically investigated populations of ssp. *fuchsii* from the Alps, northern Apennines, western Carpathians and adjacent mountain areas in eastern Germany and the Czech Republic, 80 % have turned out to consist of tetraploid plants; in the Alps the proportion is even higher. Outside Central Europe, populations of ssp. *fuchsii* consist of diploid plants.

Morphometric studies have shown that ssp. *fuchsii* and ssp. *maculata* are relatively distinct in southern and western Europe (Heslop-Harrison, 1951; Bateman and Denholm, 1989, Dufrêne et al., 1991; Tyteca and Gathoye, 2004). In contrast, other studies have indicated that the morphological differentiation decreases towards the north (Averyanov, 1990; Shipunov et al., 2004, 2005). In general, hybridization between ssp. *fuchsii* and ssp. *maculata* is considered to be common (e.g. Delforge, 1995), and triploid hybrids are occasionally reported (Table 1). So far, no taxonomic studies of the *D. maculata* complex have incorporated detailed ecological data.

Using allozyme markers it has been shown that tetraploid populations of *D. maculata* s.l. have originated by autopolyploidization (Hedrén, 1996). Regionally focused studies based on plastid DNA and/or nrDNA markers (e.g. Devos et al, 2003, 2005, 2006a; Shipunov et al., 2004; Hedrén, 2003; Pillon et al., 2007), as well as on morphometry and/or cytometry (e.g. Heslop-Harrison, 1951; Vöth, 1978; Reinhard, 1985; Jagiełło, 1986-1987; Bateman and Denholm, 1989; Dufrêne et al., 1991; Tyteca and Gathoye, 2004) suggest, when considered together, that tetraploid populations of *D. maculata* s.l. may include at least three separate autotetraploid lineages: (i) *D. maculata* ssp. *maculata* from southern and western Europe, (ii) *D. maculata* ssp. *maculata* from northern and eastern Europe and (iii) *D. maculata* ssp. *fuchsii* from the mountain areas of Central Europe. According to more or less obvious morphological differences between ssp. *maculata* and present-day diploids, the first two lineages may be relatively ancient. Moreover, there are differences in chromosome size (Jagiełło and Lankosz-Mróz, 1986-1987) and in AFLP banding patterns (Hedrén et al., 2001) between ssp. *maculata* and present-day diploids. In contrast, the third lineage may be relatively young since diploid and tetraploid populations of ssp. *fuchsii* are morphologically indistinguishable (Groll, 1965; Vaucher, 1966; Scharfenberg, 1977; Vöth, 1978; Vöth and Greilhuber, 1980; Jagiełło, 1986-1987; Jagiełło and Lankosz-Mróz, 1986-1987; Reinhardt, 1988; Gölz and Reinhard, 1997; Bertolini et al., 2000).

## Aims of this thesis

The association between taxonomic complexity and universal biological issues such as Quaternary migration history and polyploid evolution makes the foundation for this thesis. The objective was to investigate and describe different aspects of variation in the *Dactylorhiza maculata* complex and relate the variation patterns to underlying biological processes. Four separate studies are included in the thesis. In the first study (**Paper I**) focus was on the origin of allopolyploid taxa in Scandinavia. This study was also a methodological study in which plastid DNA markers with appropriate variation were developed and examined. The next study (**Paper II**) had a more taxonomic approach. Molecular markers from both the plastid and nuclear genomes were combined with cytological and morphological data in order to unravel patterns of differentiation in the *D. maculata* complex in Scandinavia. The third study (**Paper III**) was a detailed investigation of habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid ssp. *fuchsii* and tetraploid ssp. *maculata*. Plastid and nuclear DNA markers, as well as cytological, morphological and ecological data were used for this investigation. The last study (**Paper IV**) was a large-scale overview of population genetic structure, postglacial migration and polyploidization in the *D. maculata* complex. This study was based on both plastid and nuclear DNA markers.

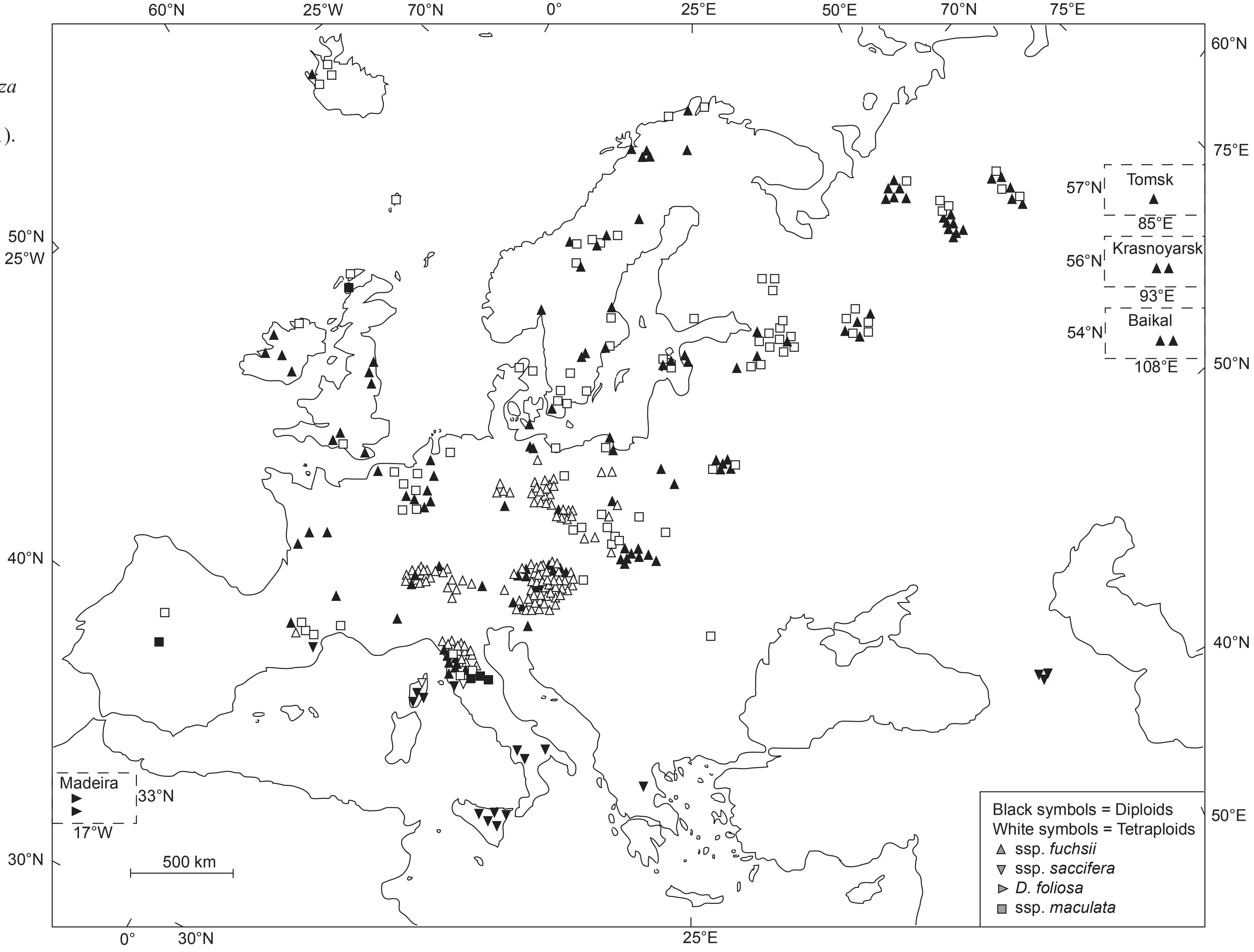


**Figure 1.** Distribution of diploid and tetraploid populations of *Dactylorhiza maculata* s.l. based on literature data (see Table 1).

## Material and methods

### *Sampling*

The variation patterns in the *Dactylorhiza maculata* complex were investigated at various geographical levels.

For the study of allotetraploid speciation (Paper I) plant material was mainly collected from northern Europe, but some reference material from other parts of Europe was included as well. More than 1 000 individuals from c. 150 populations of *D. majalis* s.l. (allotetraploids) and *D. maculata* s.l. (the maternal lineage) were sampled for this study. In the taxonomically focused study of the *D. maculata* complex in Scandinavia (Paper II) we investigated about 30 pure populations of either diploid ssp. *fuchsii* or tetraploid ssp. *maculata*. The populations were chosen to cover as much as possible of the biogeographic variation in Scandinavia. One population from the adjacent Kola Peninsula was included to improve the representation of morphologically controversial northern populations. Between 5 and 20 individuals from each population were analysed. In the third study (Paper III), which had an ecological perspective, I investigated two mixed populations of diploid ssp. *fuchsii* and tetraploid ssp. *maculata*. The populations were located in the coastal lowland of central Sweden (Sjösa nature reserve; 58°45′N, 17°07′E) and the central part of the Scandinavian mountain ridge (Hamra nature reserve; 62°34′N, 12°15′E). I chose these two sites since they represent contrasting biogeographic regions with different environmental conditions. About 50 samples/sample plots at each site were analysed. The fourth study (Paper IV) had a broad geographic perspective. Almost 2 000 samples of *D. maculata* s.l. from c. 300 populations from all parts of Europe were analysed.

### *Ploidy level determination*

In two of the studies (Papers II-III) the relative ploidy level of each sampled plant was assessed by flow cytometry. The analyses were performed by Gerard Geenen, Plant Cytometry Services (Schijndel, The Netherlands).

### *Molecular methods*

Two categories of molecular markers were used: plastid DNA and ITS alleles from the nuclear genome. Both marker systems are supposed to be selectively neutral and are standard tools for population genetic investigations. They have previously been used in studies of *Dactylorhiza* (see background). Plastid markers are generally maternally inherited in angiosperms and are particularly useful in phylogeographic studies since they are expected to provide a more simplified reflection of migration patterns than biparentally inherited markers (Ferris et al., 1999). In this thesis, plastid markers were used in all the studies. In the first study (Paper I) we found appropriate variation at seven microsatellite loci and three loci with indel variation. These ten loci were then used in the following studies. ITS markers (six different alleles) were used in three of the studies (Papers II-III).

### *Morphometry*

Morphological data were used in two studies (Papers II-III). In these studies a total of 35 quantitative and qualitative characters were measured on all plants. Both floral and vegetative characters were taken into account. The methodology was slightly modified from Bateman and Denholm (1985).

### *Ecological data*

Ecological data was used in one study (Paper II). In this study the niche of each sampled plant was characterized by data of presence and cover of associated plant species (herbaceous plants, dwarf shrubs and saplings) in a 40 x 40 cm quadrat centred on the target *Dactylorhiza* individual (cf. Du Rietz, 1921). Eight environmental variables were recorded in each quadrat in addition to the vegetation data: (1) shading, (2) cover of exposed soil, (3) cover of litter, (4) cover of *Sphagnum*, (5) cover of mosses other than *Sphagnum*, (6) pH on the top 10 cm of soil, (7) moisture and (8) grass sward density.

### *Data analysis*

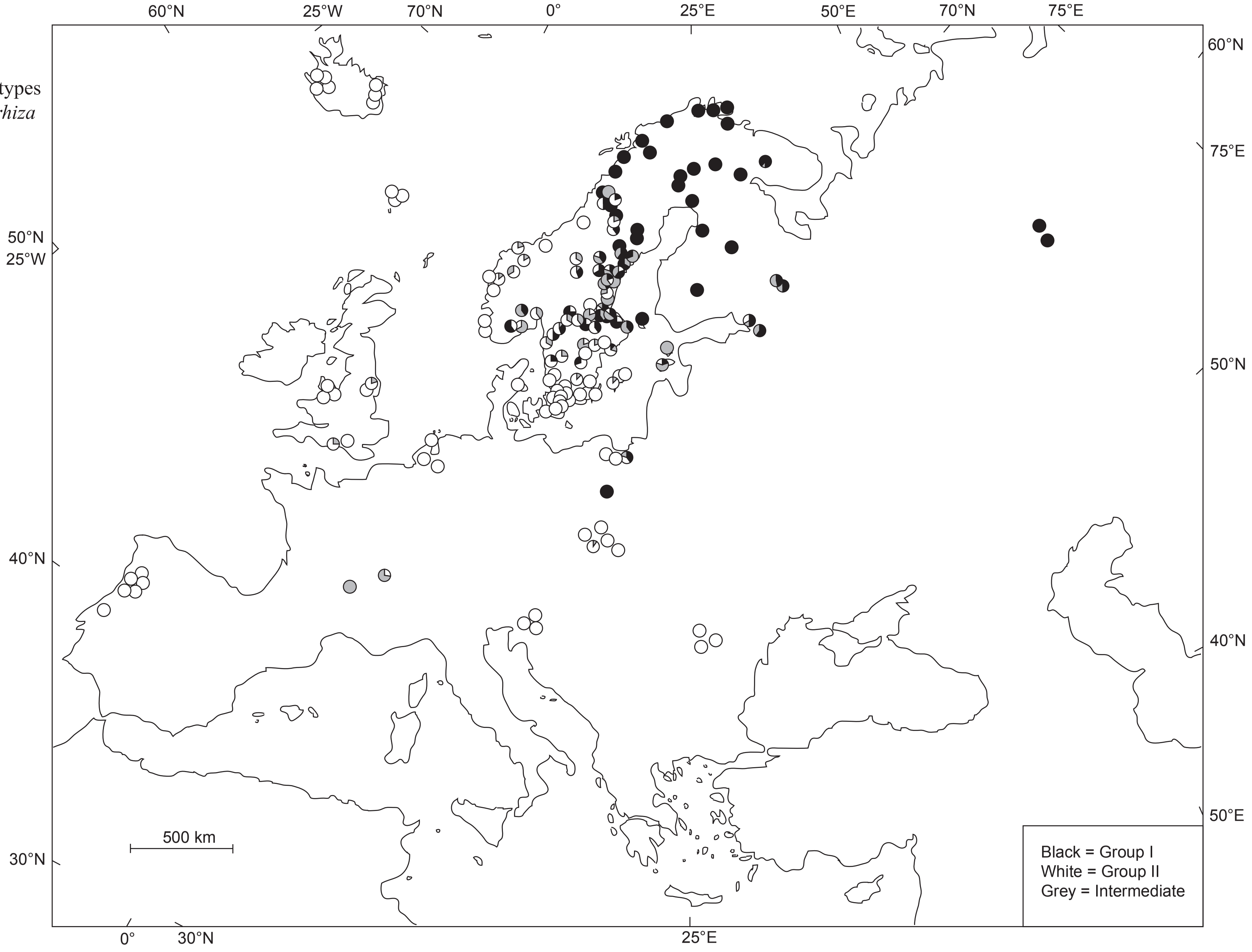
Variation patterns in different data sets were summarized by means of appropriate multivariate techniques (see Papers I-IV for details): canonical correspondence analysis (CCA), canonical variates analysis (CVA), detrended correspondence analysis (DCA), discriminant analysis, multidimensional scaling (MDS), principal coordinates analysis (PCO) and principal components analysis (PCA). Relationships between plastid haplotypes were visualized in minimum spanning networks. Analysis of molecular variance (AMOVA) was used to describe the partitioning of genetic diversity among various spatial levels. Mantel tests were performed to unravel large-scale geographic patterns. Geographic patterns were also described by means of distribution maps of plastid haplotypes and ITS alleles. Genetic diversity at various spatial levels was calculated according to the indices of gene diversity ($H$) and average gene diversity over loci ($\pi$) (Excoffier et al., 2005).

## **Results and conclusions**

### *Plastid haplotypes* (Papers I-IV)

We identified almost 300 plastid haplotypes in the total material of *D. maculata* s.l. and allotetraploid taxa that was investigated. The haplotypes were differentiated into three distinct groups. Group I haplotypes were dominating in populations of ssp. *fuchsii* (including ssp. *saccifera*) and in populations of ssp. *maculata* from northern and northeastern Europe (“northern/eastern ssp. *maculata*”). Group II haplotypes were dominating in populations of ssp. *maculata* from southern, central and western Europe (“southern/western ssp. *maculata*”). Intermediate haplotypes were found in contact zones between the two lineages of ssp. *maculata*. The geographic distribution of haplotypes in populations of ssp. *maculata* is shown in Figure 2. Both haplotype groups were represented in allotetraploid taxa in Scandinavia. The third group of haplotypes was rare and almost completely restricted to allotetraploid material, but must have originated from *D. incarnata* s.l. (see Introduction).

**Figure 2.** Distribution of Group I, Group II and intermediate plastid haplotypes in populations of *Dactylorhiza maculata* ssp. *maculata*.



### *Origin of allotetraploids* (Paper I, complemented by data from Paper IV)

We found several distinct plastid haplotypes in widespread allotetraploid taxa (entities belonging to the *Dactylorhiza majalis* complex), which shows that the allotetraploid group must have multiple origins. Similar (mostly identical) haplotypes were found in both *D. majalis* s.l. and in *D. maculata* s.l. (the maternal lineage). However, some haplotypes are common in *D. majalis* s.l. but rare in *D. maculata* s.l., which suggests a pre-Holocene origin of most allotetraploid lineages. Populations of *D. majalis* s.l. in Scandinavia are invariably associated with widespread allotetraploid lineages and do not seem to have arisen via recent polyploidization events *in situ*. Morphologically distinct populations should be considered as segregates of already existing allotetraploid lineages. Our data indicate further that back-crossing between allotetraploids and their parental lineages is relatively common, and this may be an alternative pathway for the formation of new allopolyploid lineages. Hybridization between independently derived allotetraploids may also occur.

### *Taxonomy of the* Dactylorhiza maculata *complex* (Papers II-IV)

Both plastid and nuclear markers support the current view of four major taxa in the *Dactylorhiza maculata* complex: *D. maculata* ssp. *fuchsii*, *D. maculata* ssp. *saccifera*, *D. maculata* ssp. *maculata* and *D. foliosa* (cf. Devos et al., 2005, 2006a). It should however be observed that ssp. *fuchsii* and ssp. *saccifera* grade into each other. In Scandinavia, we found that ssp. *fuchsii* and ssp. *maculata* have partly overlapping morphological variation patterns, but that the two subspecies could be kept separate if morphology is considered together with habitat features. Based on ITS allele frequencies and morphology, populations of ssp. *maculata* from northernmost Europe can be recognized as var. *kolaënsis*. Morphologically more or less distinct groups of populations from other parts of Europe are sometimes recognized as independent taxonomic entities as well (e.g. Delforge, 1995). However, apart from var. *kolaënsis*, other taxa separated at subspecies or variety level are not supported by molecular markers. Furthermore, some taxa previously recognized on basis of ecology and distribution, including "*elodes*", "*ericetorum*" and "*psychrophila*", were also poorly separated in morphology, based on Scandinavian material.

### *Niche differentiation between diploids and autotetraploids* (Paper III, complemented by data from Paper IV)

Mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. in Scandinavia represent secondary contact zones between diploid ssp. *fuchsii* and tetraploid ssp. *maculata*. I found no patterns of recent and local (*in situ)* autopolyploidization. Based on both molecular markers and morphology it must be concluded that diploids and tetraploids from mixed populations are no less differentiated than diploids and tetraploids from cytologically homogeneous populations. Furthermore, diploid ssp. *fuchsii* and tetraploid ssp. *maculata* are separated on a microhabitat level in mixed populations. Both taxa appear to have wider ecological amplitude in pure populations. The ecological constraints may thus be strengthened in mixed populations, which should contribute to the maintenance of hybrid zones. I found unexpectedly few triploid hybrids. Most of them grew in intermediate habitats between diploids and tetraploids. Introgressive gene flow between ploidy levels was also limited, especially from tetraploid to diploid level (cf. Stebbins, 1971). However, I observed that hybridization and introgression seem to be slightly more common in the Scandinavian mountains than in the lowland, which may be related to differences in disturbance regimes (cf. Anderson, 1948; Arnold, 1997).

The general conclusion that introgression is restricted between ssp. *fuchsii* and ssp. *maculata* is strongly supported by data from particularly southern/western Europe. Of several hundred investigated individuals of ssp. *maculata* from this area, none contained ssp. *fuchsii* plastid haplotypes. However, a local influence of introgression (past or present) was observed in Central Europe, where most populations of ssp. *fuchsii* consist of tetraploid plants. Reproductive barriers between taxa on the same ploidy level should be less efficient than barriers between taxa on different ploidy levels (Grant, 1981).

### *Polyploid evolution* (Paper IV, supported by data from Paper II)

A model of the evolutionary history of the *Dactylorhiza maculata* complex is given in Figure 3. Based on both plastid and nuclear markers we identified three distinct autotetraploid lineages: a southern/western lineage of ssp. *maculata*, a northern/eastern lineage of ssp. *maculata* and a Central European lineage of ssp. *fuchsii*. Given the level of differentiation between major plastid haplotype groups, and the present-day distribution of haplotypes belonging to these groups, we conclude that both the southern/western and northern/eastern lineages of ssp. *maculata* must have arisen before the Holocene. The tetraploid lineage of ssp. *fuchsii* is genetically and morphologically indistinguishable from diploid ssp. *fuchsii* and is most likely of postglacial origin (cf. references in Introduction). The southern/western lineage of ssp. *maculata* has probably arisen from diploid ancestors common to this lineage and to *D. foliosa*. The northern/eastern lineage of ssp. *maculata* has probably arisen from diploid ancestors common to this lineage and to ssp. *fuchsii*. It should be observed that populations belonging to ssp. *maculata* are found in two different genetically defined lineages, and in each of these ssp. *maculata* is connected to a diploid taxon that is different in morphology. This pattern indicates restricted morphological evolution in the tetraploid lineages, which may thus have preserved some characters that have been modified in the diploids. We found that southern/western populations of ssp. *maculata* are morphologically somewhat different from northern/eastern populations, but there is no distinct morphological limit between the two lineages.

### *Glacial refugia and postglacial recolonization* (Paper IV)

The separation of *Dactylorhiza maculata* ssp. *maculata* in two distinct lineages evidently indicates postglacial recolonization from two separate refugial areas. However, the phylogeographic signal within each lineage is weak. Both lineages are dominated by a few widespread plastid haplotypes and by a large number of rare and geographically restricted haplotypes. The weak phylogeographic signal within the two ssp. *maculata* lineages may reflect effective seed dispersal and a propensity for long distance gene flow, as have been suggested for other plant taxa that exhibit similar patterns of plastid haplotype distribution (e.g. *Betula*; Palmé et al, 2003). Orchid seeds are dust-like and wind-borne, which implies a potential for long-distance dispersal. Based on the present-day distribution of genetic diversity, we suggest that source areas for postglacial migration of ssp. *maculata* may have been Central Europe and parts of central Russia located between the Fennoscandian ice sheet and the Urals. Populations of ssp. *fuchsii* were also characterized by a few widespread plastid haplotypes and by many local haplotypes. During the LGM, areas of sheltered topography in Central Europe may have provided suitable habitats for ssp. *fuchsii*, which is a more thermophilous taxon than ssp. *maculata*. The Mediterranean region and the Caucasus have not contributed to northward migration, neither for ssp. *fuchsii*, nor for ssp. *maculata*.

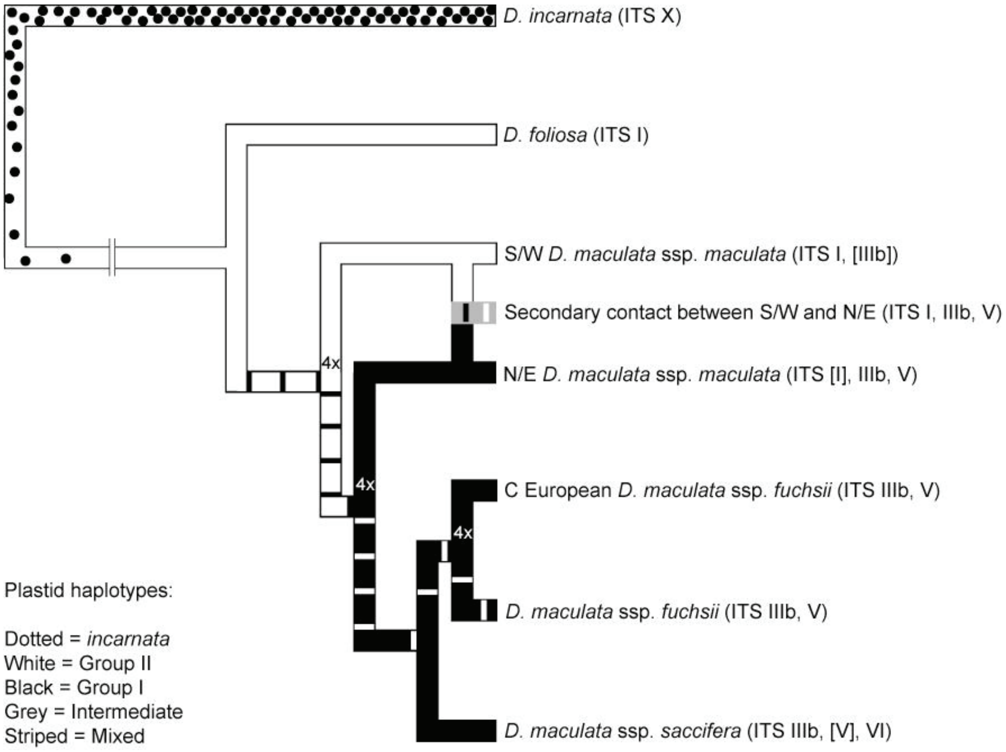


**Figure 3.** Evolutionary history of the *Dactylorhiza maculata* polyploid complex as indicated by plastid and nrDNA markers. Autopolyploidization events are marked by "4x.". Note changes in relative frequencies of Group II and Group I haplotypes. ITS alleles that occur in low frequencies are placed between square brackets.

*Secondary contact between northern/eastern and southern/western lineages* (Paper IV)

Both plastid and nuclear markers conclusively show that the northern/eastern and southern/western lineages of ssp. *maculata* meet in central Scandinavia (Fig. 2). The main route of immigration for the northern/eastern lineage is via northern Finland, but it can also be assumed that some immigration has taken place via the Åland Archipelago in the Baltic Sea. A second contact zone involving the same two lineages seems to occur in the eastern European lowland, between Poland and Lake Ladoga. For Scandinavia, contrasting immigration routes from the northeast and the south has previously been suggested for e.g. brown bear (*Ursus arctos*; Taberlet and Bouvet, 1994), field vole (*Microtus agrestis*; Jaarola and Tegelström, 1995), common shrew (*Sorex araneus*; Fredga, 1996) and some vascular plants (*Festuca ovina*, Bengtsson et al., 1995; *Viola rupestris*, Nordal and Jonsell, 1998; *Cerastium alpinum*; Nyberg-Berglund and Westerbergh (2001); *Silene dioica*, Malm and Prentice, 2005; *Arabidopsis thaliana*, Jakobsson et al., 2007).

We found that the contact zone between the northern/eastern and southern/western lineages of ssp. *maculata* has an extensive distribution in central Scandinavia. The centre is located to the provinces of Hälsingland, Medelpad and Ångermanland along the Bothnian Sea. Jaarola and Tegelström (1995) and Fredga (1996) localized hybrid zones for field vole and common shrew to the same Bothnian region. Nyberg-Berglund and Westerbergh (2001) also suggested that northeastern and southern lineages of *Cerastium alpinum* form a contact zone in this area. Such a pattern of coinciding contact zones could be explained by the deglaciation history of the Weichselian ice sheet. The centre of the ice sheet during the LGM was located to the Ångermanland area, and the deglaciation of southern Ångermanland took place only c. 9300 BP (c. 10 500 cal. yrs. BP) when southern and northeastern Fennoscandia was already ice free (Berglund, 2004). Many species of plants and animals may thus have accumulated in the bordering areas left by the retreating ice. When the ice had finally melted away, the Bothnian region may have become quickly colonized from both the north and the south, which should explain the coincidence of contact zones.

For plastid data, we observed that the genetic diversity is markedly higher in the contact zone in central Scandinavia than in adjacent areas to the north and the south. This is reflected by all measures of genetic diversity. The average gene diversity over loci, which considers divergence between haplotypes, reveals that populations in central Scandinavia, together with populations from the putative contact zone in the eastern European lowland, are more diverse than any other European populations of ssp. *maculata*.

### *Plastid DNA recombination* (Paper IV)

Intermediate plastid haplotypes between northern/eastern Group I haplotypes and southern/western Group II haplotypes are conspicuously common in the contact zone in central Scandinavia (Fig. 2). A quarter of the individuals of ssp. *maculata* in central Scandinavia have intermediate haplotypes. We also observed a high frequency of intermediate haplotypes in the putative contact zone in the eastern European lowland. These remarkable results strongly suggest that recombination takes place in the plastid genome. So far, only a few cases of suspected plastid DNA recombination have been reported (viz. *Pinus contorta*, Marshall et al., 2001; *Cycas taitungensis*, Huang et al., 2001). Our study is the first to provide clear phylogeographic evidence. As a comparison, there is an increasing amount of evidence for mitochondrial genome recombination (e.g. Bergthorsson et al., 2003; Barr et al., 2005; Tsaousis et al., 2005). Plastid DNA markers are standard tools for population genetic and phylogenetic analysis. It is obvious that recombination can be problematic for phylogenetic inference at the species level.

## Main conclusions and perspectives

This thesis has resulted in several interesting and important findings:

- Gene flow between diploid and autotetraploid cytotypes of *Dactylorhiza maculata* s.l. is restricted. In hybrid zones between diploid ssp. *fuchsii* and tetraploid ssp. *maculata*, the differentiation between taxa is reinforced by niche separation at the microhabitat level.

- For *Dactylorhiza maculata* s.l., and many other temperate organisms, postglacial recolonization from source populations at intermediate latitudes in Central and Eastern Europe has played a much more important role than previously thought. In contrast, populations of *D. maculata* s.l. in traditional refugial areas in southernmost Europe have not contributed to northward migration.

- Hybrid zones between southern and northern/eastern immigrant lineages may be a common phenomenon in central Scandinavia, which should have profound consequences for the structuring of genetic diversity.

- The paradigm of the non-recombinant plastid genome is questioned.

**Table 1.** Chromosome counts and ploidy level determinations of *Dactylorhiza maculata* s.l. *N* is number of populations. Taxon refers to taxa listed in the Introduction.

| Country, region(s) | *N* | Taxon | 2n | Comments | Reference |
|---|---|---|---|---|---|
| Austria: All parts | 10 | *fuchsii* | 40 | | Vöth and Greilhuber, 1980 |
| Austria: Kärnten | 1 | *fuchsii* | 60 | Together with 2x and 4x. | Vöth and Greilhuber, 1980 |
| Austria: All parts | 53 | *fuchsii* | 80 | | Vöth and Greilhuber, 1980 |
| Austria: Niederösterreich | 1 | *fuchsii* | 40 | | Groll, 1965 |
| Austria: Niederösterreich | 1 | *fuchsii* | 40 | | Titz, 1965 |
| Austria: Niederösterreich | 1 | *fuchsii* | 40 | | Vöth, 1978 |
| Austria: Niederösterreich | 1 | *fuchsii* | 60 | Together with 2x and 4x. | Groll, 1965 |
| Austria: Niederösterreich | 15 | *fuchsii* | 80 | | Groll, 1965 |
| Austria: Niederösterreich | 1 | *fuchsii* | 80 | | Titz, 1965 |
| Austria: Niederösterreich | 1 | *fuchsii* | 80 | | Vöth, 1978 |
| Austria: Tirol | n.d. | *fuchsii* | 80 | | Vermeulen, 1968 |
| Belarus: Minsk Area | 5 | *fuchsii* | 40 | | Semerenko,1989 |
| Belarus: Minsk Area | 2 | *maculata* | 80 | | Semerenko,1989 |
| Belgium: All parts | 5 | *maculata* | 80 | | Gathoye and Tyteca, 1989 |
| Belgium: Luxembourg | 1 | *fuchsii* × *maculata* | 60 | Together with 2x and 4x. | Gathoye and Tyteca, 1989 |
| Belgium: Wallonia | 6 | *fuchsii* | 40 | | Gathoye and Tyteca, 1989 |
| Czech Republik: Sudeten Mts. | 1 | *fuchsii* | 80 | | Krahulcová, 2003 |
| Denmark: Jylland | n.d. | *maculata* | 80 | | Hagerup, 1944 |
| Denmark: Læsø | n.d. | *maculata* | 80 | | Holmen and Kaad, 1956 |
| Denmark: Møn | n.d. | *fuchsii* | 40 | | Hagerup, 1944 |
| England (?) | n.d. | *maculata* | 79 | | M. M. Richardson unpubl. in Maude, 1939 |
| England (?) | ≥ 2 | *maculata* | 80 | | M. M. Richardson unpubl. in Maude, 1939 |
| England: Durham | 1 | *fuchsii* | 40 | | Lord and Richards, 1977 |
| England: Hampshire | 1 | *maculata* | 80 | | Heslop-Harrison, 1951 |
| England: Hampshire, Durham | 2 | *fuchsii* | 40 | | Heslop-Harrison, 1951 |
| England: Hampshire, Kent | 2 | *fuchsii* | 40 | | Heslop-Harrison, 1953 |
| England: Yorkshire | 1 | *fuchsii* | 40 | | Montgomery et al., 1997 |
| Estonia: Saaremaa | 2 | *maculata* | 80 | | Jagiełło et al., 1989 |
| Estonia: Saaremaa, W Mainland | 4 | *fuchsii* | 40 | | Jagiełło et al., 1989 |
| Faeroe Islands | n.d. | *maculata* | 80 | | Hagerup, 1944 |
| Finland: Lappland | n.d. | *fuchsii* | 40 | | Heslop-Harrison, 1951 |

**Table 1.** Continued.

| Country, region(s) | *N* | Taxon | 2n | Comments | Reference |
|---|---|---|---|---|---|
| Finland: Uusimaa | 1 | *maculata* | 80 | | Sorsa, 1962 |
| France: Ardennes, Gard | 2 | *maculata* | 80 | | Gathoye and Tyteca, 1989 |
| France: Corsica | 3 | *saccifera* | 40 | | Gathoye and Tyteca, 1989 |
| France: Corsica | n.d. | *saccifera* | 80 | | Vermeulen, 1947 |
| France: E Pyrenees | 1 | *fuchsii* | 40 | | Cauwet-Marc and Balayer, 1984 |
| France: E Pyrenees | 1 | *fuchsii* | 80 | | Cauwet-Marc and Balayer, 1984 |
| France: E Pyrenees | 3 | *maculata* | 80 | | Cauwet-Marc and Balayer, 1984 |
| France: E Pyrenees | 1 | *saccifera* | 40 | | Cauwet-Marc and Balayer, 1984 |
| France: Hautes-Alpes | 1 | *fuchsii* | 60 | Together with 2x. | Gathoye and Tyteca, 1989 |
| France: Indre, Averyon, Hautes-Alpes | 3 | *fuchsii* | 40 | | Gathoye and Tyteca, 1989 |
| France: Pas-de-Calais | n.d. | *fuchsii* | 40 | | Van Loon and de Jong, 1978 |
| France: Poitou-Charentes | 2 | *fuchsii* | 40 | | Vermeulen, 1947 |
| Georgia: E Tbilisi | 3 | *maculata* | 40[1] | | D. Ståhlberg, unpublished data |
| Germany: Brandenburg | 16 | *fuchsii* | 80 | | Scharfenberg, 1977 |
| Germany: Mecklenburg-Vorpommern | 1 | *maculata* | 80 | | Wegener, 1966 |
| Germany: Mecklenburg-Vorpommern, Sachsen-Anhalt | 2 | *fuchsii* | 40 | | Wegener, 1966 |
| Germany: Sachsen-Anhalt | 1 | *fuchsii* | 80 | | Wegener, 1966 |
| Germany: Thüringen | ≥ 3 | *fuchsii* | 80 | | Reinhardt, 1988 |
| Greece: Thessalia | 1 | *saccifera* | 42 | | Van Loon and Oudemans, 1982 |
| Iceland | n.d. | *maculata* | 80 | | Hagerup, 1944 |
| Iceland | ≥ 2 | *maculata* | 80 | | Löve and Löve, 1956 |
| Iceland: SW | n.d. | *fuchsii* | 40 | | Löve and Löve, 1956 |
| Ireland | n.d. | *fuchsii* | 40 | | Vermeulen, 1947 |
| Ireland: Clare | 1 | *fuchsii* | 40 | | Heslop-Harrison, 1951 |
| Ireland: Donegal | 1 | *maculata* | 80 | | Heslop-Harrison, 1951 |
| Ireland: Mayo, Wexford | 2 | *fuchsii* | 40 | | Heslop-Harrison, 1953 |
| Italy: Foggia, Salerno, Sicily | 3 | *saccifera* | 40 | | D´Emerico et al., 2002 |
| Italy: Roma, Umbria/Marche | 2 | *maculata* | 40 | | Del Prete et al., 1980 |
| Italy: Sicily | 4 | *saccifera* | 40 | | Mazolla et al., 1981 |
| Italy: Toscana | 1 | *fuchsii* | 40 | | Vöth and Greilhuber, 1980 |
| Italy: Toscana | 1 | *fuchsii* | 60 | Together with 2x. | Vöth and Greilhuber, 1980 |

**Table 1.** Continued.

| Country, region(s) | *N* | Taxon | 2n | Comments | Reference |
|---|---|---|---|---|---|
| Italy: Toscana | 6 | *fuchsii* | 80 | | Vöth and Greilhuber, 1980 |
| Italy: Toscana | 3 | *maculata* | 80 | | Bertolini et al., 2000 |
| Italy: Toscana | n.d. | *saccifera* | 80 | | Vermeulen, 1947 |
| Italy: Toscana, Campania | 2 | *saccifera* | 40 | | Gathoye and Tyteca, 1989 |
| Italy: Toscana, Emilia-Romagna | ca 5 | *fuchsii* | 40 | | Bertolini et al., 2000 |
| Italy: Toscana, Emilia-Romagna | ca 10 | *fuchsii* | 80 | | Bertolini et al., 2000 |
| Italy: Toscana/Emilia-Romagna | 1 | *maculata* | 40 | | Bertolini et al., 2000 |
| Netherlands | n.d. | *fuchsii* | 40 | | Kliphuis, 1963 |
| Netherlands | n.d. | *maculata* | 80 | | Kliphuis, 1963 |
| Netherlands (?) | n.d. | *fuchsii* | 40 | Cult. | Vermeulen, 1938 |
| Netherlands: Limburg | n.d. | *fuchsii* | 40 | | Vermeulen, 1947 |
| Norway: Akershus, Nordland | 2 | *fuchsii* | 40 | | Knaben and Engelskjön, 1967 |
| Norway: Finnmark | 1 | *fuchsii* | 40[1] | | D. Ståhlberg and M. Hedrén, in prep. |
| Norway: Sør-Trøndelag | 1 | *fuchsii* | 40[1] | | Aagaard et al. 2005 |
| Norway: Sør-Trøndelag | 1 | *maculata* | 80[1] | | Aagaard et al. 2005 |
| Norway: Troms, Finnmark | 2 | *maculata* | 80[1] | | D. Ståhlberg and M. Hedrén, in prep. |
| Poland: All parts | 10 | *fuchsii* | 40 | | Jagiełło and Lankosz-Mróz, 1986-1987 |
| Poland: All parts | 11 | *maculata* | 80 | | Jagiełło and Lankosz-Mróz, 1986-1987 |
| Poland: Central Area, Sudeten Mts. | 13 | *fuchsii* | 80 | | Jagiełło and Lankosz-Mróz, 1986-1987 |
| Poland: NE | 1 | *fuchsii* | 60 | Together with 2x. | Jagiełło and Lankosz-Mróz, 1986-1987 |
| Poland: Tatra Mts.-W Carpathians | 4 | *fuchsii* | 40 | | Pogan and Wcisło, 1957 |
| Portugal: Madeira | n.d. | *foliosa* | 40 | Cult. | Vermeulen, 1938 |
| Portugal: Madeira | n.d. | *foliosa* | 40 | | Vermeulen, 1947 |
| Romania: Transylvania | 1 | *saccifera* | 80[1] | | D. Ståhlberg, unpublished data[1] |
| Russia: Baikal Area | 2 | *fuchsii* | 40 | | Belaeva and Siplivinsky, 1975 |
| Russia: Komi | 8 | *fuchsii* | 40 | | Averyanov et al., 1982b |
| Russia: Komi | 2 | *fuchsii* | 41 | Together with 2x. | Averyanov et al., 1982b |
| Russia: Komi | 2 | *fuchsii* (?) | 60 | Together with 2x. | Averyanov et al., 1982b |
| Russia: Komi | 3 | *maculata* | 80 | | Averyanov et al., 1982b |
| Russia: Krasnoyarsk Area | 3 | *fuchsii* | 20 | | Stepanov, 1994 |
| Russia: Krasnoyarsk Area | 2 | *fuchsii* | 40 | | Stepanov, 1994 |

**Table 1.** Continued.

| Country, region(s) | *N* | Taxon | 2n | Comments | Reference |
|---|---|---|---|---|---|
| Russia: N Ural Mts. | 5 | *fuchsii* | 40 |  | Averyanov et al., 1982a |
| Russia: N Ural Mts. | 1 | *fuchsii* | 60 | Together with 2x. | Averyanov et al., 1982a |
| Russia: N Ural Mts. | 1 | *maculata* | 78 | Together with 4x. | Averyanov et al., 1982a |
| Russia: N Ural Mts. | 3 | *maculata* | 80 |  | Averyanov et al., 1982a |
| Russia: Novgorod Area | 2 | *maculata* (?) | 60 | Together with 4x. | Averyanov, 1979 |
| Russia: St. Petersburg Area | 1 | *maculata* (?) | 100 | Together with 4x. | Averyanov, 1979 |
| Russia: St. Petersburg Area, Novgorod Area, Karelia | 8 | *maculata* | 80 |  | Averyanov, 1979 |
| Russia: St. Petersburg Area, Novgorod Arera | 3 | *fuchsii* | 40 |  | Averyanov, 1979 |
| Russia: St. Petersburg Area, Vologda Area | 5 | *fuchsii* | 40 |  | Averyanov, 1977 |
| Russia: St. Petersburg Area, Vologda Area | 11 | *maculata* | 80 |  | Averyanov, 1977 |
| Russia: Tomsk Area | 1 | *fuchsii* | 40 |  | Kartashova et al., 1974 |
| Russia: Tyman | 6 | *fuchsii* | 40 |  | Averyanov et al., 1980 |
| Russia: Tyman | 3 | *maculata* | 80 |  | Averyanov et al., 1980 |
| Russia: Vologda Area | 1 | *fuchsii* × *maculata* | 60 | Together with 2x and 4x. | Averyanov, 1977 |
| Scotland: Inner Hebrides | n.d. | *maculata* | 40 |  | Heslop-Harrison, 1948 |
| Scotland: Outer Hebrides | 1 | *maculata* | 80 |  | Heslop-Harrison, 1951 |
| Slovakia: Bratislava Area | 1 | *maculata* | 80 |  | Uhríková, 1976 |
| Slovakia: Tatra Mts. | 1 | *fuchsii* | 40 |  | Uhríková, 1978 |
| Slovenia: Bevke | 1 | *fuchsii* | 40 |  | Lovka et al., 1971 |
| Spain: Ávila | 1 | *maculata* | 80 |  | Bernardos et al., 2004 |
| Spain: Castilla-La Mancha | 1 | *maculata* | 40 |  | Fernándes Casas et al., 1979 |
| Sweden: All parts | 7 | *fuchsii* | 40[1] |  | D. Ståhlberg and M. Hedrén, in prep. |
| Sweden: Jämtland, Småland, Åsele lappmark | 5 | *maculata* | 80 |  | Heslop-Harrison, 1951 |
| Sweden: Skåne | 1 | *maculata* | 120[1] | Together with 4x. | D. Ståhlberg and M. Hedrén, in prep. |
| Sweden: Skåne, Uppland | 4 | *maculata* | 80[1] |  | D. Ståhlberg and M. Hedrén, in prep. |
| Sweden: Södermanland, Härjedalen | 2 | *fuchsii* × *maculata* | 60[1] | Together with 2x and 4x. | D. Ståhlberg, in prep. |
| Sweden: Torne lappmark | 1 | *fuchsii* | 40 |  | Löve and Löve, 1944 |
| Sweden: Uppland | 1 | *fuchsii* | 40 |  | Afzelius, 1958 |
| Sweden: Östergötland, Jämtland, Torne lappmark | 3 | *fuchsii* | 40 |  | Heslop-Harrison, 1951 |
| Switzerland: Neuchâtel | 1 | *fuchsii* | 60 |  | Vaucher, 1966 |
| Switzerland: Neuchâtel, Bern | 13 | *fuchsii* | 80 |  | Vaucher, 1966 |

**Table 1.** Continued.

| Country, region(s) | *N* | Taxon | 2n | Comments | Reference |
|---|---|---|---|---|---|
| Switzerland: Vaud, Bern | 2 | *fuchsii* | 40 | | Vaucher, 1966 |
| Switzerland: Zürich | 1 | *fuchsii* | 40 | | Heusser, 1938 |
| Switzerland: Zürich, Graubünden, Tessin | 7 | *fuchsii* | 80 | | Heusser, 1938 |

[1] Assessed by flow cytometry.

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