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AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS (AFLP) REVEAL DETAILS OF POLYPLOID EVOLUTION IN *DACTYLORHIZA* (ORCHIDACEAE)[1]

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The utility of the PCR-based AFLP technique (polymerase chain reaction; amplified fragment length polymorphisms) was explored in elucidating details of polyploid evolution in the Eurasian orchid genus *Dactylorhiza.* We emphasized Swedish taxa but also included some material from the British Isles and elsewhere in Europe. Three different sets of primers, amplifying different subsets of restriction fragments, independently revealed similar patterns for relationships among the *Dactylorhiza* samples investigated. The AFLP data support the general picture of polyploid evolution in *Dactylorhiza,* i.e., that allotetraploid derivatives have arisen repeatedly as a result of hybridization beween the two parental groups *D. incarnata* s.l. (sensu lato; diploid marsh orchids) and the *D. maculata* group (spotted orchids). Within the *incarnata* s.l. group, morphologically defined varieties were interdigitated. The *D. maculata* group consisted of two distinct subgroups, one containing autotetraploid *D. maculata* subsp. *maculata* and the other containing diploid *D. maculata* subsp. *fuchsii.* Allotetraploids showed a high degree of additivity for the putative parental genomes, and relationships among them were partly correlated to morphologically based entities, but also to geographic distribution. Thus, allotetraploid taxa from the British Isles clustered together, rather than with morphologically similar plants from other areas.

Key words: AFLP; *Dactylorhiza*; Orchidaceae; phylogeny; polyploid evolution; systematics.

Recent studies of polyploid complexes have shown that such groups are much more dynamic systems than believed some decades ago (Thompson and Lumaret, 1992; Soltis and Soltis, 1993). For instance, it appears to be a general pattern that polyploid taxa have evolved repeatedly from progenitors of lower ploidy, which has resulted in higher levels of genetic diversity at the polyploid level than would otherwise be expected. Some studies also indicated that gene flow and introgression may occur across ploidy levels (Lord and Richards, 1977; Brochmann, Stedje, and Borgen, 1992; Menken, Smit, and Den Nijs, 1995). Furthermore, restructuring of the hybrid genomes in allopolyploids may have resulted in new adaptively valuable combinations of parental characters; thus, polyploids are not evolutionary dead ends (Soltis and Soltis, 1993) and may evolve independently of their parental taxa. Finally, the polyploid genome may later be diploidized, and over a longer timescale the whole cycle could be repeated (Stebbins, 1971; Grant, 1981; Leitch and Bennett, 1997; Soltis and Soltis, 1999).

In the orchid genus *Dactylorhiza* Nevski, allotetraploids have evolved on several occasions due to repeated hybridization (Hedrén, 1996a) between two main groups of parental taxa, one consisting of the diploid marsh orchids, *D. incarnata* sensu lato (s.l.), and the other consisting of the diploid *D. maculata* subsp. *fuchsii* and the autotetraploid *D. maculata* subsp. *maculata.* Based on morphological patterns, the allotetraploid derivatives have been described as a large number of taxonomic species, but it is not known to what degree the various regional and local populations of these species are indeed of common ancestry or if similar morphological types have evolved on several occasions in separate areas.

These problems have profound implications for the formulation of conservation strategies that should be applied to the complex. We know that many constituent members of the complex, particularly allotetraploids, decreased in numbers during the latter part of the 20th century due to destruction of habitat. Most of these taxa grow in calcareous fens, which are often drained, both intentionally and accidentally.

If gene flow from the diploid to the tetraploid level is extensive, due to allotetraploids evolving repeatedly from the parental groups, then the allotetraploid taxa recognized on a morphological basis may contain unrelated populations with local or regional distributions. In that case, conservation efforts could be concentrated on the parental groups because they may be regarded as the basis for further evolution in the complex; from them, allotetraploids, if lost, could be easily regenerated, although the existence of certain local allotetraploids with adaptations to specific habitats and unique combinations of characters should not be disregarded. On the other hand, if allotetraploids evolve on rare occasions from the parental groups, then allotetraploid taxa present today may be coherent evolutionary units over large areas, and the morphologically defined taxonomic units are likely to reflect the evolutionary pattern of the complex. In that case, more conservation effort may be expended on the tetraploids.

One of us has previously used allozymes to describe evolutionary patterns in the complex (Hedrén, 1996a, b, c, d). However, although allozymes may give valuable insights into the general patterns of evolution, allozyme loci are not variable enough to reveal many patterns of detailed relationships that are of interest both in evolutionary and conservation contexts. In this paper, we investigate the degree to which the recently developed amplified fragment length polymorphisms technique (AFLP; Vos et al., 1995) can provide such information. We include some more distantly related members of

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Dactylorhiza, as well as members of related genera, to understand if AFLP data can be used as well to study such patterns.

MATERIALS AND METHODS

Taxa and sampling—The taxa investigated in this study are primarily those members of *Dactylorhiza* that occur in northwestern Europe and for which the general pattern of relationships has previously been described using allozyme markers. This was specifically done to determine the degree of congruence between these two categories of markers. Some additional taxa from other parts of Europe have also been included, particularly those investigated for nuclear internal transcribed spacers (ITS) ribosomal DNA sequence variation by Pridgeon et al. (1997). The diploid taxa *D. incarnata* vars. *incarnata, ochroleuca, cruenta, borealis,* and subspp. *coccinea* and *pulchella* have been regarded as members of *D. incarnata* s.l. The diploid *D. maculata* subsp. *fuchsii,* the autotetraploid *D. maculata* subsp. *maculata,* and the diploid *D. foliosa* (Sundermann and Watke, 1973) were treated as the *D. maculata* group. Allozyme data indicated that the following taxa are allotetraploids with origins in taxa with a high degree of similarity to members of *D. incarnata* s.l. and *D. maculata/fuchsii*: *D. majalis* subspp. *majalis, lapponica, traunsteineri* (Hedrén, 1996a), *praetermissa* (Hedrén, 1996b), *purpurella* (Hedrén, 1996c), *alpestris* (Hedrén, unpublished data), *D. sphagnicola* (Hedrén, 1996a), and *D. elata* (Hedrén, unpublished data). In addition, based on chromosome numbers and morphology (Bateman and Denholm, 1983; Delforge, 1995), the following taxa are also regarded as allotetraploids with a similar origin: *D. majalis* subspp. *scotica, traunsteineroides, cordigera,* and *cambrensis.* We also examined one sample each of *D. iberica, D. romana,* and *D. sambucina,* all diploid taxa. These species are not generally thought to be parental taxa in the polyploid complex described above, although Bateman, Pridgeon, and Chase (1997) noted the possibility that *D. sambucina* is an alternative parent to at least some of the allotetraploids and so should be more carefully examined. Finally, we also included one sample each of three genera closely related to *Dactylorhiza*: *Gymnadenia conopsea, Pseudorchis albida,* and *Coeloglossum viride,* the latter included in *Dactylorhiza* by Bateman, Pridgeon, and Chase (1997).

Most of the material analyzed in this study was collected in Sweden. Some additional samples were included from other areas in Europe, particularly from the UK. A large portion of the British material was studied by Pridgeon et al. (1997), and DNA extracts from these plants were taken from the DNA Bank at the Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, UK. Some samples were collected by colleagues in other parts of Europe. The geographic origin of the material studied is given in Table 1, which also includes authors of scientific names.

All material collected in the wild was dried by the silica-gel method described by Chase and Hills (1991). When available, we collected flowers. Orchid flowers have a thinner cuticle than the vegetative parts, thereby permitting more rapid desiccation. For *Dactylorhiza* taxa growing in mixed populations, we avoided flowers that had been pollinated and thus potentially contaminated with foreign DNA (i.e., pollen from other taxa).

DNA extraction—DNA was extracted from ∼10–50 mg dry mass (1–5 flowers), either purified by using a cesium chloride-ethidium bromide gradient or QIAquick columns according to the manufacturer's protocols (QIAGEN, Crawley, West Sussex, UK). The gradient-purified samples are included in the general collection of DNA samples kept at the Jodrell Laboratory, as indicated in Table 1.

AFLP—We followed the general protocol described by Applied Biosystems (Warrington, Cheshire, UK) that takes advantage of an automated sequencer and computer analysis of fragment length variation. Sample DNA was restricted with the endonucleases EcoRI and MseI and ligated to appropriate double-stranded adapters according to the manufacturer's protocols. Two steps of amplification followed, a preselective amplification in which we used primers with a 1 base pair (bp) extension, and a second amplification in which primers with 3 bp extensions were used, thereby further reducing the number of fragments. For the second amplification, we initially tried nine different primer combinations, including combinations that were found to give good amplification and suitable numbers of fragments (typically 50–150 per accession) in *Orchis simia* (Qamaruz-Zaman et al., 1998). From these, we selected three combinations, -ACT/-CTT, -AGG/-CAA, and -ACC/-CAC, for the extensions to the EcoRI and MseI sites, respectively.

Data analysis—The fragment data generated by the automated sequencer were analyzed by the computer programs GeneScan and Genotyper 2.0.1 (Applied Biosystems, Warrington, Cheshire, UK). In the latter program the band patterns were visualized as fingerprint traces that could be further inspected by eye. We used fragments in the range of 50–500 bp, and the computer-based system was set to consistently discard bands with a weak signal less than a threshold value recommended by the manufacturer. The data were extracted as a table in presence/absence format and subsequently carefully compared to the table generated automatically by the Genotyper program, whereby certain corrections were made. The use of internal size standards in each lane permitted exact calibration of different individuals against each other and made possible separation of nonhomologous fragments that were nearly equal in length. We scored some additional bands in samples that were not automatically scored by Genotyper in which the presence of fragments was obvious as distinct shoulders of more intense bands of an adjoining size class. We also recognized some additional bands in individual samples with more generally weak signals.

The presence/absence data were subjected to parsimony analysis and various phenetic analyses. The parsimony analysis was performed to investigate higher level patterns of relationship (if present) and included representatives of *Pseudorchis, Gymnadenia, Coeloglossum,* and some members of *Dactylorhiza* that were thought not to be components of the polyploid complex (i.e., *D. sambucina, D. romana,* and *D. iberica*) but excluded allopolyploid taxa as we knew a priori that they are the result of reticulate evolution. The computer-program PAUP 3.1.1 (Swofford, 1993) was used to produce Wagner trees based on presence/absence data. A heuristic search strategy was implemented with TBR (tree bisection/reconnection) branch swapping. The ''steepest descent'' option was applied with MULPARS on (saving multiple parsimonious trees at each step). Group support was evaluated by means of bootstrap analysis (Felsenstein, 1985). We used 5000 replicates and assigned equal weights to the characters. *Gymnadenia* and *Pseudorchis* were used as outgroups. Pridgeon et al. (1997) found that these taxa were related to the *Dactylorhiza* clade (including *Coeloglossum*) in analyses of ITS rDNA data.

Phenetic analyses were performed on the members of *Dactylorhiza* that form the polyploid complex. Pairwise comparisons of all possible pairs of individuals by means of Jaccard coefficients (Jaccard, 1908) generated triangular similarity matrices that were used for principal coordinates analyses (PCO; Gower, 1966) and cluster analyses (unweighted pair-group method using arithmetic averages [UPGMA]; Sneath and Sokal, 1973). Separate analyses were made for the whole polyploid complex, *D. incarnata* s.l. and the group of allotetraploid taxa. For the polyploid complex, we also calculated separate similarity matrices for the three different AFLP primer data sets analyzed separately and then compared pairwise by Mantel tests. All phenetic analyses were performed in NTSYS-pc 1.80 (Rohlf, 1994).

RESULTS

AFLP data—We found the AFLP method to produce characters that were highly reproducible between different reactions and different rounds of PCR. Certain samples were rerun for various reasons, and we found that not only were the same bands reproducibly amplified but the relative intensity of different bands was also reproducible. Comparisons of the three independently derived primer data sets also showed a high degree of correspondence, with the matrix correlation ranging between 0.90 and 0.94 (Table 2) and the resulting patterns of ordinations looking virtually the same (not shown).

Higher relationships in Dactylorhiza—The parsimony analysis generated 13 equally most-parsimonious trees of tree length 2818, consistency index 0.31, and retention index 0.69.

TABLE 1. Origin of the material used for the present study. ''Code'' refers to code number used in Figs. 1–5. Voucher specimens, when available, use the herbarium acronym.

Code	Taxon	Locality	Collector/voucher
*Cvir*576	*Coeloglossum viride* (L.) Hartm.		Chase O-576^{ab} K
*Gcon*574	*Gymnadenia conopsea* (L.) R.Br. subsp. *conopsea*		Chase 574^{ab} K
*Palb*992	*Pseudorchis albida* (L.) Á. & D. Löve	Scotland, East Inverness-shire, SE Aviemore	Bateman 63^{ab}
*alp*963	*D. majalis* (Rchb.) P.F. Hunt & Summerh. subsp. *alpestris* (Pugsley) Senghas	Andorra, Pyrennees	Bateman 48/E1978/0625^{ab}
*bor*309	*D. incarnata* var. *borealis* (Neuman) Hyl.	Sweden, Lycksele Lappmark, Tärna II	Hedrén 97309
*cam*987	*D. majalis* subsp. *cambrensis* (R.H. Roberts) R.H. Roberts	Scotland, Caithness, Thurso East	Bateman 51^{b}
*coc*965	*D. incarnata* (L.) Soó subsp. *coccinea* (Pugsley) Soó	Scotland, East Lothian, Aberlady Bay LNR	Bateman 45^{ab}
*cor*316	*D. majalis* subsp. *cordigera* (Fr.) H. Sund	NE Greece, Rhodope Mts., Elatia	Cronberg s.n.
*cor*318	*D. majalis* subsp. *cordigera*	NE Greece, Rhodope Mts., Ulu Yala	Cronberg s.n.
*cru*078	*D. incarnata* (L.) Soó var. *cruenta* (O.F. Müll.) Hyl.	Sweden, Gotland, Hall	Hedrén 97078^{b}
*cru*091	*D. incarnata* var. *cruenta*	Sweden, Gotland, Gerum	Hedrén 97091
*cru*112	*D. incarnata* var. *cruenta*	Sweden, Gotland, Lärbro I	Hedrén 97112
*cru*123	*D. incarnata* var. *cruenta*	Sweden, Gotland, Rute	Hedrén 97123
*cru*136	*D. incarnata* var. *cruenta*	Sweden, Gotland, Lärbro II	Hedrén 97136
*cru*145	*D. incarnata* var. *cruenta*	Sweden, Gotland, Gothem I	Hedrén 97145
*cru*147	*D. incarnata* var. *cruenta*, pale form	Sweden, Gotland, Gothem I	Hedrén 97147
*cru*162	*D. incarnata* var. *cruenta*	Sweden, Västergötland, Rådane	Hedrén 97162
*cru*178	*D. incarnata* var. *cruenta*	Sweden, Östergötland, Kärna	Hedrén 97178^{b}
*cru*193	*D. incarnata* var. *cruenta*	Sweden, Östergötland, Kaga	Hedrén 97193
*cru*200	*D. incarnata* var. *cruenta*	Sweden, Östergötland, Slaka	Hedrén 97200
*ela*962	*D. elata* (Poiret) Soó		Bateman 49 (E1969/4078)^{ab}
*fol*537	*D. foliosa* (Lowe) Soó	Madeira	Chase O-537^{ab}
*fuc*037	*D. maculata* subsp. *fuchsii* (Druce) Hyl.	Sweden, Skåne, Hällestad	Hedrén 97037^{b}
*fuc*096	*D. maculata* subsp. *fuchsii*	Sweden, Gotland, Gerum	Hedrén 97096^{b}
*fuc*102	*D. maculata* subsp. *fuchsii*	Sweden, Gotland, Gothem II	Hedrén 97102
*fuc*108	*D. maculata* subsp. *fuchsii*	Sweden, Gotland, Boge	Hedrén 97108^{b}
*fuc*148	*D. maculata* subsp. *fuchsii*	Sweden, Gotland, Gothem I	Hedrén 97148
*fuc*174	*D. maculata* subsp. *fuchsii*	Sweden, Västergötland, Gudhem	Hedrén 97174
*fuc*186	*D. maculata* subsp. *fuchsii*	Sweden, Östergötland, Kärna	Hedrén 97186^{b}
*fuc*221	*D. maculata* subsp. *fuchsii*	Sweden, Södermanland, Svärta	Hedrén 97221^{b}
*fuc*266	*D. maculata* subsp. *fuchsii*?	Sweden, Härjedalen, Ljusnedal I	Hedrén 97266
*fuc*279	*D. maculata* subsp. *fuchsii*	Sweden, Jämtland, Hammerdal	Hedrén 97279
*fuc*282	*D. maculata* subsp. *fuchsii*	Sweden, Jämtland, Hammerdal	Hedrén 97282^{b}
*fuc*1123	*D. maculata* subsp. *fuchsii*		Chase O-1123 (Bateman)^{ab}
*ibe*960	*D. iberica* (Willd.) Soó		Chase O-960^{ab}
*inc*003	*D. incarnata* (L.) Soó var. *incarnata*	Sweden, Skåne, Börringe	Hedrén 97003
*inc*016	*D. incarnata* var. *incarnata*	Sweden, Skåne, Örup	Hedrén 97016
*inc*019	*D. incarnata* var. *incarnata*	Sweden, Skåne, Lyngsjö	Hedrén 97019
*inc*029	*D. incarnata* var. *incarnata*	Sweden, Skåne, Saxtorp	Hedrén 97029
*inc*034	*D. incarnata* var. *incarnata*	Sweden, Skåne, Trolle-Ljungby	Hedrén 97034^{b}
*inc*052	*D. incarnata* var. *incarnata*	Sweden, Skåne, Hällestad	Hedrén 97052
*inc*082	*D. incarnata* var. *incarnata*	Sweden, Gotland, Hall	Hedrén 97082^{b}
*inc*087	*D. incarnata* var. *incarnata*	Sweden, Gotland, Gerum	Hedrén 97087
*inc*100	*D. incarnata* var. *incarnata*	Sweden, Gotland, Viklau	Hedrén 97100
*inc*103	*D. incarnata* var. *incarnata*	Sweden, Gotland, Gothem II	Hedrén 97103
*inc*106	*D. incarnata* var. *incarnata*	Sweden, Gotland, Boge	Hedrén 97106
*inc*126	*D. incarnata* var. *incarnata*	Sweden, Gotland, Rute	Hedrén 97126
*inc*139	*D. incarnata* var. *incarnata*	Sweden, Gotland, Lärbro II	Hedrén 97139
*inc*142	*D. incarnata* var. *incarnata*	Sweden, Gotland, Hörsne	Hedrén 97142
*inc*163	*D. incarnata* var. *incarnata*	Sweden, Västergötland, Rådane	Hedrén 97163
*inc*164	*D. incarnata* var. *incarnata*	Sweden, Västergötland, Rådane	Hedrén 97164^{b}
*inc*194	*D. incarnata* var. *incarnata*	Sweden, Östergötland, Slaka	Hedrén 97194^{b}
*inc*213	*D. incarnata* var. *incarnata*	Sweden, Södermanland, Svärta	Hedrén 97213^{b}

TABLE 1. Continued.

Code	Taxon	Locality	Collector/voucher
*inc*230	*D. incarnata* var. *incarnata*	Sweden, Uppland, Bladåker I	Hedrén 97230
*inc*232	*D. incarnata* var. *incarnata*	Sweden, Uppland, Bladåker I	Hedrén 97232[b]
*inc*240	*D. incarnata* var. *incarnata*	Sweden, Uppland, Bladåker II	Hedrén 97240
*inc*253	*D. incarnata* var. *incarnata*	Sweden, Uppland, Ed	Hedrén 97253
*lap*269	*D. majalis* subsp. *lapponica* (Hartm.) H. Sudn.	Sweden, Härjedalen, Ljusnedal I	Hedrén 97269[h]
*lap*276	*D. majalis* subsp. *lapponica*	Sweden, Jämtland, Åsarna	Hedrén 97276
*lap*278	*D. majalis* subsp. *lapponica*	Sweden, Jämtland, Ström	Hedrén 97278
*lap*295	*D. majalis* subsp. *lapponica*	Sweden, Åsele Lappmark, Vilhelmina	Hedrén 97295
*lap*279	*D. majalis* subsp. *lapponica*	Sweden, Jämtland, Ström	Hedrén 97297[b]
*lap*298	*D. majalis* subsp. *lapponica*	Sweden, Lycksele Lappmark, Tärna I	Hedrén 97298
*lap*305	*D. majalis* subsp. *lapponica*	Sweden, Lycksele Lappmark, Tärna III	Hedrén 97305[b]
*lap*989	*D. majalis* subsp. *lapponica*	Scotland, E. Skye, Raasay	Bateman 54[ab]
*mac*032	*D. maculata* (L.) Soó subsp. *maculata*	Sweden, Skåne, Trolle-Ljungby	Hedrén 97032
*mac*095	*D. maculata* subsp. *maculata*	Sweden, Gotland, Gerum	Hedrén 97095
*mac*131	*D. maculata* subsp. *maculata*	Sweden, Gotland, Rute	Hedrén 97131
*mac*183	*D. maculata* subsp. *maculata*	Sweden, Östergötland, Kärna	Hedrén 97183
*mac*198	*D. maculata* subsp. *maculata*	Sweden, Östergötland, Slaka	Hedrén 97198
*mac*208	*D. maculata* subsp. *maculata*	Sweden, Södermanland, Kila	Hedrén 97208
*mac*214	*D. maculata* subsp. *maculata*	Sweden, Södermanland, Svärta	Hedrén 97214[b]
*mac*235	*D. maculata* subsp. *maculata*	Sweden, Uppland, Bladåker II	Hedrén 97235
*mac*249	*D. maculata* subsp. *maculata*	Sweden, Uppland, Ed	Hedrén 97249
*mac*263	*D. maculata* subsp. *maculata*	Sweden, Småland, Madesjö	Hedrén 97263
*mac*274	*D. maculata* subsp. *maculata*	Sweden, Härjedalen, Ljusnedal II	Hedrén 97274
*mac*293	*D. maculata* subsp. *maculata*	Sweden, Jämtland, Gäddede	Hedrén 97293
*maj*010	*D. majalis* (Rchb.) P.F. Hunt & Summerh. subsp. *majalis*	Sweden, Skåne, Örup	Hedrén 97010
*maj*028	*D. majalis* subsp. *majalis*	Sweden, Skåne, Saxtorp	Hedrén 97028[b]
*och*075	*D. incarnata* (L.) Soó var. *ochroleuca* (Boll) Hyl.	Sweden, Gotland, Hall	Hedrén 97075[b]
*och*089	*D. incarnata* var. *ochroleuca*	Sweden, Gotland, Gerum	Hedrén 97089
*och*114	*D. incarnata* var. *ochroleuca*	Sweden, Gotland, Lärbro I	Hedrén 97114
*och*120	*D. incarnata* var. *ochroleuca*	Sweden, Gotland, Rute	Hedrén 97120
*och*133	*D. incarnata* var. *ochroleuca*	Sweden, Gotland, Lärbro II	Hedrén 97133
*och*143	*D. incarnata* var. *ochroleuca*	Sweden, Gotland, Gothem I	Hedrén 97143
*och*159	*D. incarnata* var. *ochroleuca*	Sweden, Västergötland, Rådane	Hedrén 97159
*och*179	*D. incarnata* var. *ochroleuca*	Sweden, Östergötland, Kärna	Hedrén 97179
*och*192	*D. incarnata* var. *ochroleuca*	Sweden, Östergötland, Kaga	Hedrén 97192[b]
*pral*124	*D. majalis* subsp. *praetermissa* (Druce) D.M. Moore & Soó		Chase O-936[ab]
*pul*988	*D. incarnata* subsp. *pulchella* (Drue) Soó	Scotland, West Roos, E Poolewe, Loch Kernsary	Bateman 56[ab]
*pur*315	*D. majalis subsp. purpurella* (T. & T.A. Stephenson) D.M. Moore & Soó	The Faeroes, Sandoy, Skálvik	Uppsala Botanical Garden 1988-2360
*pur*964	*D. majalis* subsp. *purpurella*	Scotland, East Lothian, Aberlady Bay LNR	Bateman 46[b]
*rom*760	*D. romana* (Sebast.) Soó	Italy	Rossi s.n.[ab]
*sam*1373	*D. sambucina* (L.) Soó	Sweden, Gotland, Vamlingbo	Chase O-1363[b]
*sco*994	*D. majalis* ssp. *scotica* E. Nelson	Scotland, Outer Hebrides, North Uist	Bateman 55 (E1995?)[b]
ssp098	undescribed allotetraploid *Dactylorhiza*	Sweden, Gotland, Viklau	Hedrén 97098[b]
ssp105	undescribed allotetraploid *Dactylorhiza*	Sweden, Gotland, Boge	Hedrén 97105
*sph*149	*D. sphagnicola* (Höppner) Soó	Sweden, Småland, S. Ljuna	Hedrén 97149
*sph*152	*D. sphagnicola*	Sweden, Småland, S. Ljuna	Hedrén 97152[b]
*sph*204	*D. sphagnicola*	Sweden, Södermanland, Kila	Hedrén 97204[b]
*sph*226	*D. sphagnicola*	Sweden, Uppland, Frötuna	Hedrén 97226[b]
*sph*244	*D. sphagnicola*	Sweden, Uppland, Bladåker II	Hedrén 97244
*sph*260	*D. sphagnicola*	Sweden, Småland, Madesjö	Hedrén 97260[b]
*tra*074	*D. majalis* subsp. *traunsteineri* (Rchb.) H. Sund.	Sweden, Gotland, Hall	Hedrén 97074[b]
*tra*093	*D. majalis* subsp. *traunsteineri*	Sweden, Gotland, Gerum	Hedrén 97093
*tra*119	*D. majalis* subsp. *traunsteineri*	Sweden, Gotland, Lärbro I	Hedrén 97119

TABLE 1. Continued.

Code	Taxon	Locality	Collector/voucher
*tra*130	*D. majalis* subsp. *traunsteineri*	Sweden, Gotland, Rute	Hedrén 97130
*tra*156	*D. majalis* subsp. *traunsteineri*	Sweden, Småland, Kärda	Hedrén 97156
*tra*165	*D. majalis* subsp. *traunsteineri*	Sweden, Västergötland, Rådane	Hedrén 97165[b]
*tra*170	*D. majalis* subsp. *traunsteineri*	Sweden, Västergötland, Gudhem	Hedrén 97170
*tra*189	*D. majalis* subsp. *traunsteineri*	Sweden, Östergötland, Kärna	Hedrén 97189[b]
*tra*210	*D. majalis* subsp. *traunsteineri*	Sweden, Södermanland, Svärta	Hedrén 97210[b]
*tra*227	*D. majalis* subsp. *traunsteineri*	Sweden, Uppland, Bladåker I	Hedrén 97227
*tra*250	*D. majalis* subsp. *traunsteineri*	Sweden, Uppland, Ed	Hedrén 97250[b]
*trs*320	*D. majalis* (Rchb.) P.F. Hunt & Summerh. ssp. *traunsteinerioides* (Pugsley) Soó	Ireland, Westmeath, Mullingar	Klein s.n.
*trs*990	*D. majalis* ssp. *traunsteineroides*	Scotland, West Ross, E. Poolewe, Loch Kernsary	Bateman 53[ab]

[a] The material was used in the study of Pridgeon et al. 1997.

[b] DNA extracts available from the DNA Bank at the Jodrell Laboratory, Royal Botanic Gardens, Kew, UK.

In the consensus tree (Fig. 1) there was a basal polytomy of five groups: (1) *D. romana* and *D. sambucina,* (2) *D. iberica* alone, (3) the *D. maculata* group, (4) *Dactylorhiza (Coeloglossum) viride,* and (5) *D. incarnata* s.l. *Dactylorhiza maculata* subsp. *fuchsii* was distinct from *D. maculata* sensu stricto (s.s.) in the *maculata/fuchsii* clade, and there was weak bootstrap support (52%) for *D. foliosa* to group with *D. maculata* s.s. rather than with subsp. *fuchsii.* The *D. incarnata* clade formed a large polytomy with little resolution. In the bootstrap analysis, only a few pairs of *D. incarnata* s.s. individuals were supported.

The polyploid complex—The PCO analysis produced three main clusters (Fig. 2): one dense cluster including members of *D. incarnata* s.l. to the right, a somewhat less distinct cluster including the allotetraploid taxa in the center, and a loose cluster to the left with three distinct subunits. The first was composed of all samples of *D. maculata* subsp. *fuchsii,* the second all samples of *D. maculata* s.s., and the third was the single specimen of *D. foliosa.*

In the UPGMA phenogram (Fig. 3), *D. fuchsii, D. maculata* s.s., and *D. foliosa* were included in one cluster, and *D. incarnata* s.l. and the allotetraploids were included in the other one. *Dactylorhiza incarnata* s.l. formed a distinct group.

Comparisons between the allotetraploids and *D. incarnata* s.l. resulted in similarity coefficients in the range 0.34–0.50 (Table 3), whereas comparisons with *D. maculata* subsp. *maculata/D. maculata* subsp. *fuchsii* resulted in similarity coefficients in the range 0.21–0.38. Comparisons between allotetraploids and taxa from these two putative parental groups consistently resulted in higher similarity coefficients than those for other taxa (Table 3).

Allotetraploids—The phenogram (Fig. 3) revealed a pattern of relationships among the allotetraploids that was partly related to the taxonomic delimitation of the various allotetraploid taxa and partly related to the geographic origin of the material investigated. The single specimen of *D. majalis* subsp. *alpestris* was relatively distinct from the bulk of the other investigated allotetraploids, but the two samples of *D. majalis* subsp. *cordigera* and the single sample of *D. elata* were also isolated. The specimens of *D. sphagnicola* from southern Sweden (Småland and Södermanland) formed a cluster that grouped with *D. incarnata.* The remaining allotetraploids formed two subgroups that were similar to each other. One of these subgroups included all investigated allotetraploids from the British Isles and the Faeroes plus two specimens of *D. majalis* subsp. *traunsteineri* from Sweden. In the other subgroup, two samples of *D. majalis* subsp. *majalis* came out together, two specimens of an unnamed tetraploid taxon from Gotland formed another group, and finally the remaining samples of *D. majalis* subsp. *traunsteineri, D. sphagnicola,* and *D. majalis* subsp. *lapponica* (all from Sweden) were interdigitated and did not form discrete groups.

The same general pattern expressed in the phenogram was also revealed by PCO (Fig. 4) with the slight difference that the two specimens of *D. majalis* subsp. *majalis* here appeared to be more similar to each other and, likewise, that the two samples of *D. majalis* subsp. *cordigera* appeared closer to each other than indicated by the phenogram.

Comparisons of samples within allotetraploids consistently resulted in higher similarity coefficients (range 0.49–0.68; Table 3) than comparisons between different taxa (range 0.33–

TABLE 2. Mantel tests testing for associations between pairs similarity matrices derived from the three different sets of selective primers used to generate AFLP data. In each comparison, 9999 permutations were made.

Primers	Numbers of samples included	Matrix correlation (= normalized Mantel statistic Z)	Approximate Mantel *t* test	*P* [random Z ≥ obs. Z]
EcoRI-ACC/MseI-CAC vs. EcoRI-AGG/MseI-CAA	76	0.89617	21.369	0.0001
EcoRI-ACC/MseI-CAC vs. EcoRI-ACT/MseI-CTT	74	0.93930	18.641	0.0001
EcoRI-AGG/MseI-CAA vs. EcoRI-ACT/MseI-CTT	44	0.90348	19.366	0.0001

0.63), except for *D. majalis* subsp. *traunsteineroides*, for which comparisons with other western taxa gave higher similarities than the single comparison between the two samples of this taxon.

The D. maculata group—The lower part of the phenogram (Fig. 3) contains three subgroups, which corresponded to the three taxa included in this group (cf. the PCO; Fig. 2). The *D. maculata* subsp. *fuchsii* subgroup joined the *D. maculata* s.s. subgroup at a similarity level of 0.4 in the phenogram. The *D. foliosa* subgroup was more dissimilar and joined the other two subgroups at a similarity level of 0.3.

Mean similarity coefficients within *D. maculata* subsp. *fuchsii* and *D. maculata* s.s. were relatively similar, 0.57 and 0.52, respectively, whereas comparisons between the two taxa gave a mean similarity of 0.40; *D. foliosa* was somewhat more different from both.

Differentiation within D. incarnata s.l.—Within the *D. incarnata* s.l. group shown in the phenogram (Fig. 3), the samples of *D. incarnata* s.s. were spread out over the subtree. Most samples of the characteristic color variety *ochroleuca* were concentrated on a shorter branch within the *incarnata* cluster, and most samples of var. *cruenta* grouped in a cluster that excluded the majority of *ochroleuca* samples, but were more interspersed with *incarnata* s.s. The same structure was evident from the separate PCO ordination including *D. incarnata* s.l. only; in the resulting plot of this ordination (Fig. 5), var. *cruenta* was concentrated to the lower left, var. *ochroleuca* was concentrated to the upper left, and *D. incarnata* s.s. was more evenly spread. In the phenogram, there was also some structure due to geographic origin of the samples. For instance, most specimens of *D. incarnata* s.s. from the province of Skåne (inc003–inc052) formed a cluster distinct from that containing the majority of *D. incarnata* s.s. from Gotland (inc103–inc142), but the differentiation was far from perfect, with several specimens of *D. incarnata* s.s. from these provinces falling in other parts of the *D. incarnata* s.l. cluster.

In the phenogram (Fig. 3), the single specimen of *D. incarnata* var. *borealis* was somewhat more different from those of *D. incarnata* vars. *incarnata*, *ochroleuca*, and *cruenta*. The two representatives of the western European taxa *D. incarnata* subspp. *coccinea* and *pulchella* came out together and were more different yet. This pattern was also seen in the PCO plot (Fig. 5), in which *D. incarnata* var. *borealis*, subsp. *coccinea*, and subsp. *pulchella* were positioned far up along the vertical axis, i.e., they had high values for the third principal coordinate.

Comparisons of samples within *D. incarnata* vars. *cruenta* and *ochroleuca* resulted in slightly higher similarity coefficients (0.87 and 0.79, respectively; Table 3) than comparisons between these taxa and other members of *D. incarnata* s.l. (range 0.63–0.75). Comparisons between *D. incarnata* s.s. and other members of *D. incarnata* s.l. sometimes resulted in mean similarity coefficients equally as high as comparisons within *D. incarnata* s.s. All comparisons between members of *D. incarnata* s.l. gave higher similarity coefficients than comparisons between other taxa.

DISCUSSION

AFLPs as data in the study of polyploid evolution—The study of polyploid evolution involves several types of questions: (1) identifying the parentage of polyploid derivatives and determining their origin in time and area; (2) describing the genetics of duplicated loci in their polyploid genome, i.e., to find out whether the plants should be described as allo- or autopolyploids; and (3) comparing variation patterns at several loci spread out over the genome with each other, i.e., studying the processes of genome repatterning and diploidization of the duplicated genome.

The AFLP data will be most useful in studying the first of these questions; AFLP fragments have been shown to be spread over the entire nuclear genome (e.g., Nilsson et al., 1999), as expected from the distribution of sites for these restriction enzymes. Different primer combinations reveal independent subsets of this variation. Provided that the numbers of fragments studied are large enough, different primers should give similar patterns of differentiation among the samples studied. We used three different primer combinations, and a comparison of the pairwise similarity coefficients among individuals by means of Mantel tests (Table 2) revealed little difference between the primer data sets. Thus, the AFLP method generates data sets that reliably reflect differentiation between entire individual genomes.

Patterns of hybridization and polyploid speciation are usually described by means of phenetic methods such as the ones used here. For analysis of small data sets, e.g., data sets generated by allozyme studies in which the variation at each locus could be interpreted independently (Brochmann, Soltis, and Soltis, 1992; Hedrén, 1996a), most-parsimonious interpretations of the data may be possible by comparing relatively few alternative hypotheses. For large data sets generated by fingerprint methods (which would allow for a higher degree of resolution), computer-based methods must be used, but parsimony programs designed to study reticulate evolution are not available.

Higher relationships—Under the assumption that the fragments amplified by the AFLP method are distributed randomly and evenly over the size range investigated, the number of bands shared by two individuals by pure coincidence would approximately be given by $a = n^2/S$, where n is the number of bands of different size classes found in each of the two individuals and S is the maximum possible number of size classes that could be distinguished. As already mentioned, the computer-based analysis of the output from an automated sequencer allows for a separation of fragments that are equal in length but

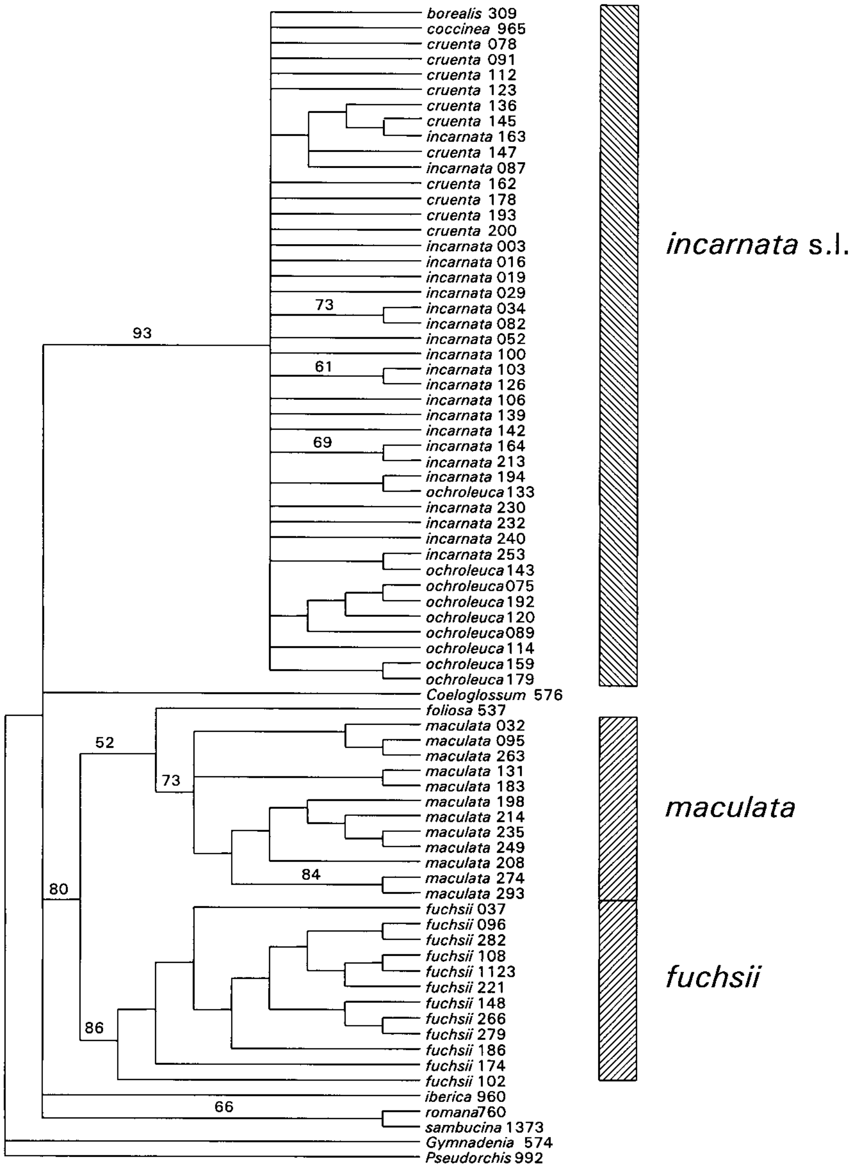

Fig. 1. Strict consensus tree of 13 equally most-parsimonious trees found. Bootstrap support values are given above branches. The analysis excluded allotetraploid members of *Dactylorhiza.*

differ in sequence by a (pseudo)difference of 0.5 bp or less. The range of fragments here studied was 50–500 bp, and a conservative estimate of the number of size classes would be 900 over this range relative to the degree of resolution discussed above. If two completely unrelated samples each have 70 recognized bands for one primer combination (a fairly typical number), then 5–6 bands would nevertheless be shared, equalling a Jaccard similarity coefficient of $J = 0.037$–0.045. Furthermore, the great majority of bands are concentrated in the lower size classes (50–250 bp). Two unrelated samples with 50 bands each in this range would accordingly share 6–7 bands for nonhomologous reasons (a Jaccard coefficient of $J = 0.064$–0.075), and the total number of shared bands over the entire range would be somewhat higher still. The number of bands shared by coincidence would be higher if the fragments are unequally distributed over the range studied.

We find in our data that comparisons between genera indeed give similarity coefficients close to these values (Table 3), and it seems possible that the regions of the genome analyzed by the AFLP procedure are not conserved enough to reveal relationships among these genera. However, it is still possible that a fraction of variation seen in AFLP is phylogenetically accurate, and that this information would be possible to extract using parsimony methods. It also seems likely that AFLP data represent coding as well as noncoding regions that would thus evolve at different rates. Although a large portion of the shared bands found in comparisons of taxa belonging to different genera are possibly false homologies stemming from rapidly evolving parts of the genome, some bands may still be true homologies that could be used in reconstructing phylogenies. Parsimony methods should be able to differentiate between these types of data if true homologies outnumber randomly coincident bands. Obviously, one way to increase the probability of finding accurate trees would be to enlarge the total

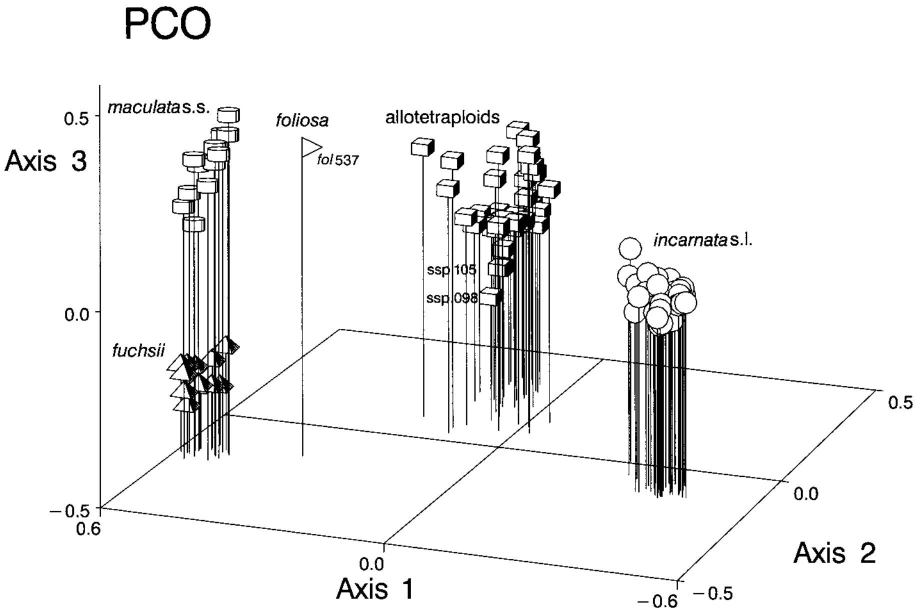

Fig. 2. Plot of the first three axes from a principal coordinates analysis, including all polyploid material of *Dactylorhiza* and the putative parental groups. The proportion of total variance along the first three axes was 23.2, 6.0, and 4.2%, respectively. Symbols denote taxa defined by genome composition or chromosome number (see text).

number of characters used by parsimony by increasing the numbers of primers used to generate the AFLP data set.

Our tree contained less resolution than those presented by Pridgeon et al. (1997), which were based on ITS sequences. They also found that *D. foliosa* and *D. maculata* subsp. *maculata* formed a clade with subsp. *fuchsii* as sister. Other findings that are not supported, but which are also not contradicted by our results, include a close relationship of *D. iberica* to *D. maculata* s.s. and *D. foliosa,* a distant position of *D. incarnata* s.l. from *D. maculata* s.l. within the genus, and *Coeloglossum* embedded in *Dactylorhiza. Dactylorhiza sambucina* was not included in the ITS tree, but the closely related *D. romana* was more related to *D. maculata* s.l. than to *D. incarnata* s.l.

The polyploid complex and the allotetraploids—In the PCO (Fig. 2), the group of allotetraploids has a more or less intermediate position between the *incarnata* s.l. cluster and the *maculata* cluster, which is expected if the allotetraploids originated as hybrids between members of these groups. However, the allotetraploid group is somewhat displaced towards the back (i.e., they have higher values than expected for the second principal coordinate). This displacement may be an indication that the allotetraploids either have an origin in taxa slightly different from the present-day representatives of the putative parental group or that the tetraploid genomes have evolved further after the origin of the various allotetraploid taxa, perhaps by recombination between parental genomes. Both these possibilities indicate that the allotetraploids are relatively old and/or that they have their origin in areas where the parental groups have different genotypes from those in northwestern Europe. This same displacement for allopolyploid taxa is observed in *Calopogon* (Goldman, M. W. Chase, and M. F. Fay, unpublished data).

The allotetraploids are apparently more similar to the *incarnata* s.l. group than to the *maculata/fuchsii* group (Table 3), which is why they also cluster together with *incarnata* s.l. in the phenogram (Fig. 3). However, it is also evident from Table 3 that similarities within taxa in *incarnata* s.l. are much higher than in *fuchsii* or in *maculata.* It is likely that this difference in similarity coefficients is due to a higher degree of homozygosity in members of *incarnata* s.l. than in *fuchsii* or *maculata,* a difference that is clearly seen in single-locus data provided by allozyme markers (Hedrén, 1996a). Thus, every comparison between an allotetraploid and a sample of *incarnata* s.l. will show a higher degree of similarity than comparisons with *maculata/fuchsii,* because the *incarnata* sample is likely to share more alleles with the *incarnata* s.l. parent that gave rise to the allotetraploid than is the *maculata/fuchsii* sample with the *maculata/fuchsii* parent.

Because the allotetraploid samples from the British Isles form a rather coherent group in the phenogram (Fig. 3), it may be speculated that these allotetraploids would be more closely related to samples of *incarnata* s.l. from the British Isles than to *incarnata* from other areas. We find no support for this hypothesis from our similarity data (Table 3). However, we studied only two British samples of *incarnata* s.l., and because these samples yielded slightly fewer bands than did *incarnata* s.l. on the average, we cannot reject the hypothesis of a close relationship.

The Dactylorhiza maculata group—Allozyme data indicate that *D. maculata* subsp. *maculata* is an autotetraploid (Hedrén, 1996a) and that there is a close correspondence of *D. maculata* subsp. *maculata* with *D. maculata* subsp. *fuchsii* in both allele composition and allele frequencies. Accordingly, it may be hypothesized that *D. maculata* subsp. *maculata* has an origin in a diploid taxon closely related to present-day *D. maculata* subsp. *fuchsii* and that gene transfer from diploids to tetraploids may still be occurring. Therefore, it is surprising to find that *D. maculata* subsp. *fuchsii* and *D. maculata* s.s. come out as distinct groups in the analyses presented here. There is a possibility that some of the differences found are due to the fact that tetraploids are compared to diploids (as discussed above), but from inspection of the electropherograms it appears that a high proportion of the differences found are indeed due to presence of distinct fragments with good amplification, and that the separation of the two taxa is real. The clear separation of *D. maculata* s.s. and *D. maculata* subsp. *fuchsii* in Sweden could indicate that Swedish *D. maculata* s.s. arose from diploid stocks elsewhere (perhaps now extinct) and migrated independently of *D. maculata* subsp. *fuchsii* after the last glaciation.

The *maculata* s.s. cluster, as well as the majority of allotetraploids investigated, attain high values along the third (vertical) principal coordinate (Fig. 2). Assuming intermediacy of the allotetraploids between the parental groups, it seems likely that material similar to *maculata* s.s. rather than *fuchsii* gave rise to most allotetraploids. Considering the model for stepwise evolution of allopolyploids by means of unreduced gametes (Müntzing, 1930a, b; deWet, 1980; Ramsey and Schemske, 1998), it is possible that allotetraploid *Dactylorhiza* taxa may have evolved by hybridization between *D. maculata* s.s. (4×, genome FFFF) and *D. incarnata* (2×, genome II). First, a triploid hybrid with genome constitution FFI would be formed, followed by amalgamation of an unreduced gamete from this hybrid with a normal gamete from *D. incarnata*, resulting in an allotetraploid plant (FFII; Hedrén, 1996a). However, it is not necessary to postulate that the tetraploid *maculata* gave rise to the allopolyploids directly; it is also possible that the allotetraploids originated from the same group of diploids that gave rise to the autotetraploid *maculata*. As indicated by the plot, and as evident from the phylogenetic trees (Fig. 1), the Madeiran endemic *D. foliosa* is such a diploid species, although its limited occurrence today indicates that it may not have been directly involved. It is clear that diploids from the entire distribution area need to be investigated. In contrast to the other allotetraploids, the two specimens of the undescribed allotetraploid from Gotland (ssp098 and ssp105) have low values for the third principal coordinate, and it could be speculated that the non-*incarnata* parent was indeed the diploid *fuchsii*. This assumption also agrees with the fact that *fuchsii* is much more abundant on Gotland than *maculata* s.s. and that *fuchsii* is now found close to these allotetraploid populations today. Thus, these allotetraploids may have had a recent and local origin.

The PCO (Fig. 2) indicates that *foliosa* is more similar to *maculata* s.s. than to *fuchsii*. However, the phenogram (Fig. 3) reveals that *maculata* s.s. and *fuchsii* are more similar to each other than to *foliosa*. Apparently, *foliosa* differs from the two other taxa by other characters than those expressed along the first three axes in the PCO ordination. These three axes summarize the main differentiation pattern in the entire polyploid complex within which *incarnata* s.l. and *maculata/fuchsii* are the most differentiated groups.

Differentiation within Dactylorhiza incarnata s.l.—Within the *incarnata* s.l. group the varieties *cruenta*, *ochroleuca*, and *incarnata* s.s. showed a complex pattern of relationships in which the variation was partly correlated with geographic origin. The single *borealis* sample appeared to be slightly more different. However, as this sample came from a more northern location in Sweden, genomic differentiation correlated with geographic origin and correlated to morphological characters cannot be separated. The western European subspecies *coccinea* and *pulchella* came out together and were somewhat more different from the remainder of *incarnata* s.l., but again the effect of geographic origin and general differentiation affecting morphology cannot be distinguished.

The relatively low degree of differentiation within *incarnata* s.l. as compared to *D. maculata/fuchsii* agrees with findings from allozyme analyses. Whereas Swedish samples of *D. incarnata* s.l. were fixed for single alleles at seven loci used to describe the general pattern of relationships within the allopolyploid complex, *D. maculata* subsp. *fuchsii* and *D. maculata* subsp. *maculata* each showed variation at all of these loci (Hedrén, 1996a).

Taxonomic conclusions—We included several samples of *D. incarnata* var. *incarnata*, var. *cruenta*, and var. *ochroleuca* in our study. These taxa may be strikingly different from each other in corolla color, leaf spotting or leaf curvature. Because the taxa appear distinct, they are often treated as subspecies or even species in orchid floras (e.g., Delforge, 1995). The three taxa also differ in ecology; whereas var. *incarnata* grows in a variety of rich fens, var. *cruenta* and var. *ochroleuca* are restricted to calcareous fens in which the latter, on average, seems to prefer slightly wetter and shadier subsites.

In our analyses, the three taxa are interdigitated, and the differences in external morphology are apparently not correlated with general differentiation of the genomes (Figs. 1, 3, 5). The separating characters may thus be due to just a few genes, but the taxa are probably fixed for different alleles at these loci. Intermediate plants are sometimes found in mixed populations. Accordingly, crosses between the three taxa may take place, and it seems likely that plants belonging to a given taxon could give rise to variable offspring approaching the other taxa.

An explanation for this type of differentiation pattern may be given by ecotype formation (Turesson, 1922). We hypothesize that the forms recognized as *ochroleuca* and *cruenta* may occur in the progeny of typical *incarnata* plants and are adapted to particular habitats. If similar selection pressures occur at different places, each of these forms may be independently derived from *incarnata* on more than one occasion. Such a scenario is consistent with our data. The value of naming these forms may be questioned because they contain populations that are not more related to each other than to populations of the other taxa. Still, there may be a need for ecologists and conservationists to separate the various forms because the names bear information on the habitat in which the plants were found. We suggest that these forms are best treated as varieties (implying that the concept of subspecies should be restricted to situations in which the taxa are more coherent, with constituent populations being more closely related to each other than to populations of other subspecies; Jonsell, 2000).

We analyzed only a few specimens of *D. incarnata* s.l. from the British Isles. However, extended sampling from this area may show that they are more genetically distinct and that it

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Fig. 3. UPGMA phenogram including all polyploid material of *Dactylorhiza* and the putative parental groups. The phenogram is based on the same similarity matrix as the one used to produce the PCO ordination given in Fig. 2. The cophenetic correlation coefficient (correspondence between the original values in the matrix of similarity data and the similarity values given in the tree) was 0.95396.

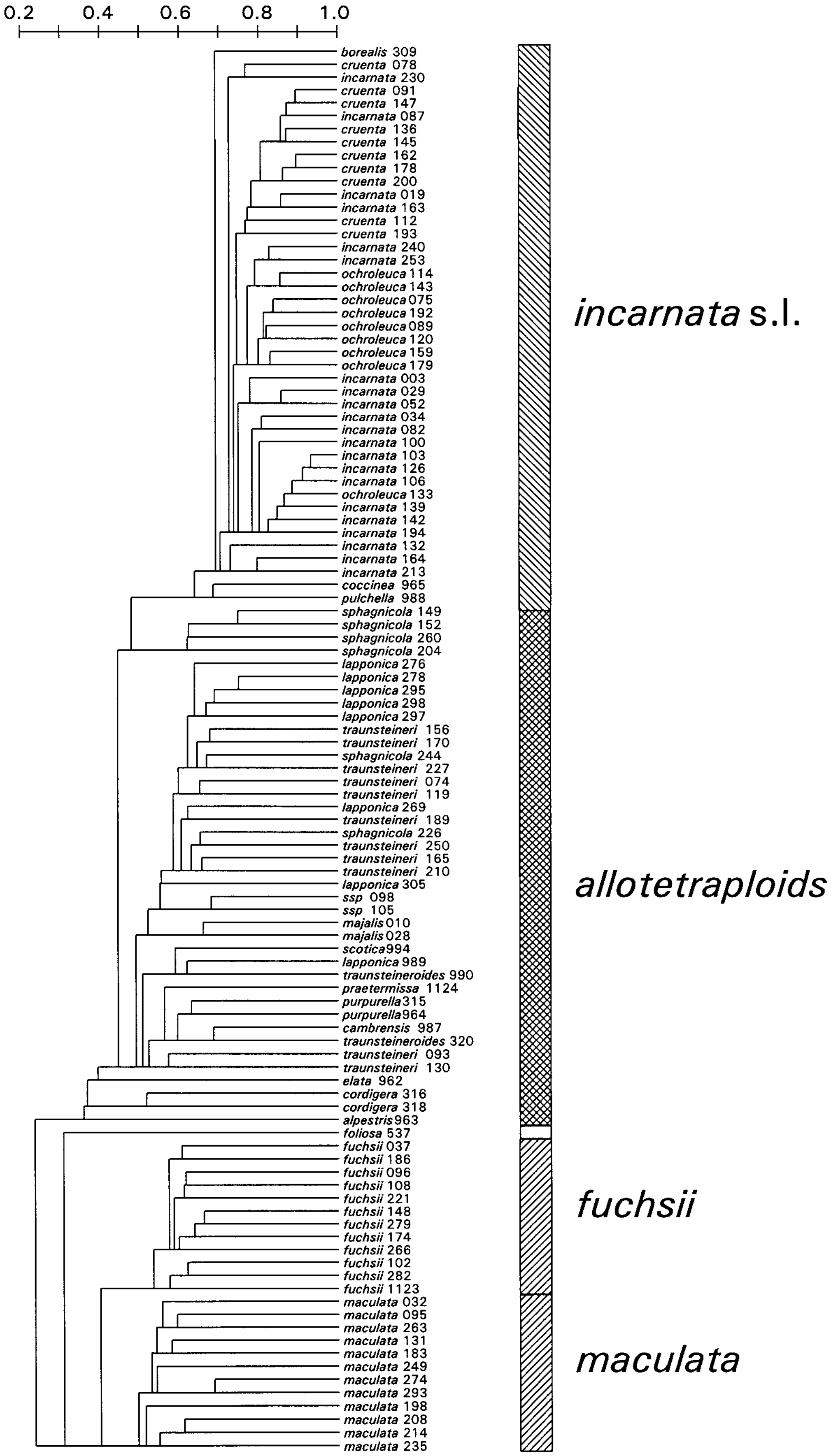
0.2
0.4
0.6
0.8
1.0
borealis 309
cruenta 078
incarnata 230
cruenta 091
cruenta 147
incarnata 087
cruenta 136
cruenta 145
cruenta 162
cruenta 178
cruenta 200
incarnata 019
incarnata 163
cruenta 112
cruenta 193
incarnata 240
incarnata 253
ochroleuca 114
ochroleuca 143
ochroleuca 075
ochroleuca 192
ochroleuca 089
ochroleuca 120
ochroleuca 159
ochroleuca 179
incarnata 003
incarnata 029
incarnata 052
incarnata 034
incarnata 082
incarnata 100
incarnata 103
incarnata 126
incarnata 106
ochroleuca 133
incarnata 139
incarnata 142
incarnata 194
incarnata 132
incarnata 164
incarnata 213
coccinea 965
pulchella 988
sphagnicola 149
sphagnicola 152
sphagnicola 260
sphagnicola 204
lapponica 276
lapponica 278
lapponica 295
lapponica 298
lapponica 297
traunsteineri 156
traunsteineri 170
sphagnicola 244
traunsteineri 227
traunsteineri 074
traunsteineri 119
lapponica 269
traunsteineri 189
sphagnicola 226
traunsteineri 250
traunsteineri 165
traunsteineri 210
lapponica 305
ssp 098
ssp 105
majalis 010
majalis 028
scotica 994
lapponica 989
traunsteineroides 990
praetermissa 1124
purpurella 315
purpurella 964
cambrensis 987
traunsteineroides 320
traunsteineri 093
traunsteineri 130
elata 962
cordigera 316
cordigera 318
alpestris 963
foliosa 537
fuchsii 037
fuchsii 186
fuchsii 096
fuchsii 108
fuchsii 221
fuchsii 148
fuchsii 279
fuchsii 174
fuchsii 266
fuchsii 102
fuchsii 282
fuchsii 1123
maculata 032
maculata 095
maculata 263
maculata 131
maculata 183
maculata 249
maculata 274
maculata 293
maculata 198
maculata 208
maculata 214
maculata 235
incarnata s.l.
allotetraploids
fuchsii
maculata

TABLE 3. Mean Jaccard similarity coefficients (percent) within and between the taxa investigated. Within-individual comparisons have been excluded from within-taxon values. ** = only one sample available for calculation; no between-individual comparisons possible.

							Allotetraploids													*incarnata* s.l.					*fuchsii/maculata*		
	Palb	*Cvir*	*Gcon*	*rom*	*sam*	*ibe*	*alp*	*cor*	*sco*	*ela*	*lap*	*maj*	*cam*	*pra*	*pur*	*ssp*	*sph*	*tra*	*trs*	*bor*	*coc*	*cru*	*inc*	*och*	*fol*	*fuc*	*mac*
Palb	**																										
Cvir	5	**																									
Gcon	14	11	**																								
rom	7	13	12	**																							
sam	10	8	15	27	**																						
ibe	9	10	16	16	12	**																					
alp	15	13	18	16	20	17	**																				
cor	11	16	16	25	22	19	30	52																			
sco	13	14	20	24	21	20	47	40	**																		
ela	11	12	20	21	20	18	36	39	47	**																	
lap	13	13	20	22	23	22	39	38	49	44	59																
maj	16	14	21	17	21	19	42	35	48	40	50	66															
cam	13	14	20	24	24	19	35	40	51	51	51	47	**														
pra	13	16	18	22	23	18	39	40	51	48	51	43	57	**													
pur	13	15	24	28	26	26	36	39	48	49	53	45	63	57	**												
ssp	13	15	21	21	23	23	36	36	44	40	53	52	53	53	52	68											
sph	14	13	20	16	20	22	33	34	46	42	48	48	45	43	44	51	58										
tra	13	15	20	21	22	21	36	38	47	43	57	51	50	49	50	54	50	58									
trs	13	15	20	24	23	18	41	40	53	46	50	46	59	53	54	45	43	49	49								
bor	13	17	16	23	20	21	34	41	51	38	46	41	46	48	45	48	46	46	42	**							
coc	11	16	16	21	18	20	34	34	53	39	42	41	48	44	44	43	43	43	42	61	**						
cru	11	16	17	21	20	23	35	37	53	37	46	44	47	48	45	50	49	48	46	69	65	87					
inc	12	15	17	20	19	22	35	35	50	35	45	42	45	46	43	48	47	47	44	70	65	75	74				
och	13	15	17	20	19	21	35	36	49	35	46	43	47	46	44	50	47	48	44	69	63	75	73	79			
fol	11	8	16	19	19	13	21	27	27	37	26	24	25	24	26	24	28	26	25	20	17	20	19	20	**		
fuc	13	13	20	17	21	15	23	24	30	23	33	32	33	31	34	38	25	32	30	18	16	17	16	17	26	57	
mac	13	10	20	19	20	14	23	28	25	36	31	31	30	27	30	31	31	32	30	18	16	17	17	17	35	40	52

would be appropriate to treat them as subspecies (cf. Bateman and Denholm, 1985).

In the *D. maculata* group, the northern European representatives *D. maculata* subsp. *maculata* and subsp. *fuchsii* were clearly differentiated from each other, and they can be viewed as monophyletic sister taxa. However, they are morphologically ill-defined and not always distinguishable, as pointed out by several continental authors (e.g., Hylander, 1966; cf. also discussion in Bateman and Denholm, 1988), for which reason we treat them as subspecies rather than species. The exact

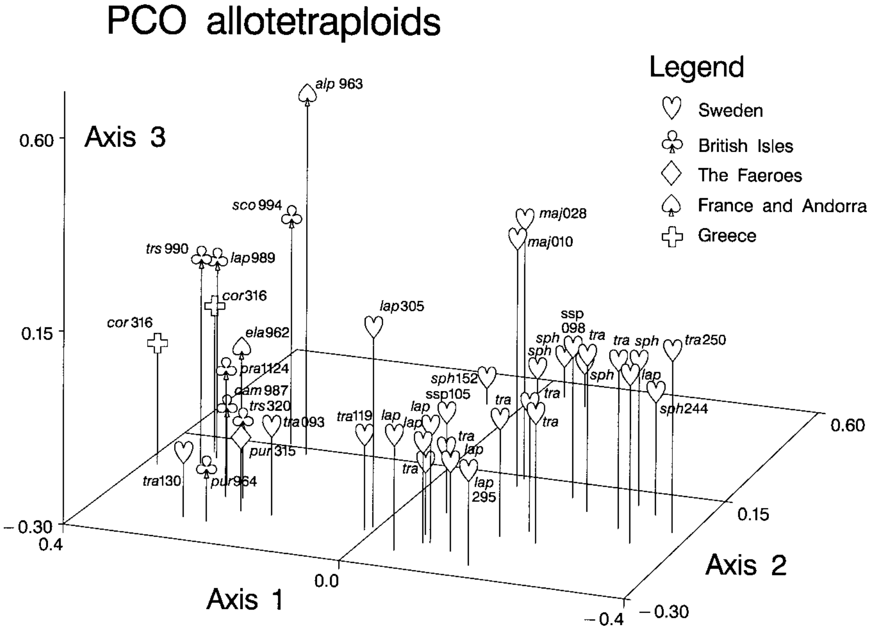

Fig. 4. Plot of the first three axes from a principal coordinates analysis, including all allotetraploid material of *Dactylorhiza*. The proportion of total variance along the first three axes was 9.4, 7.8, and 5.4%, respectively.

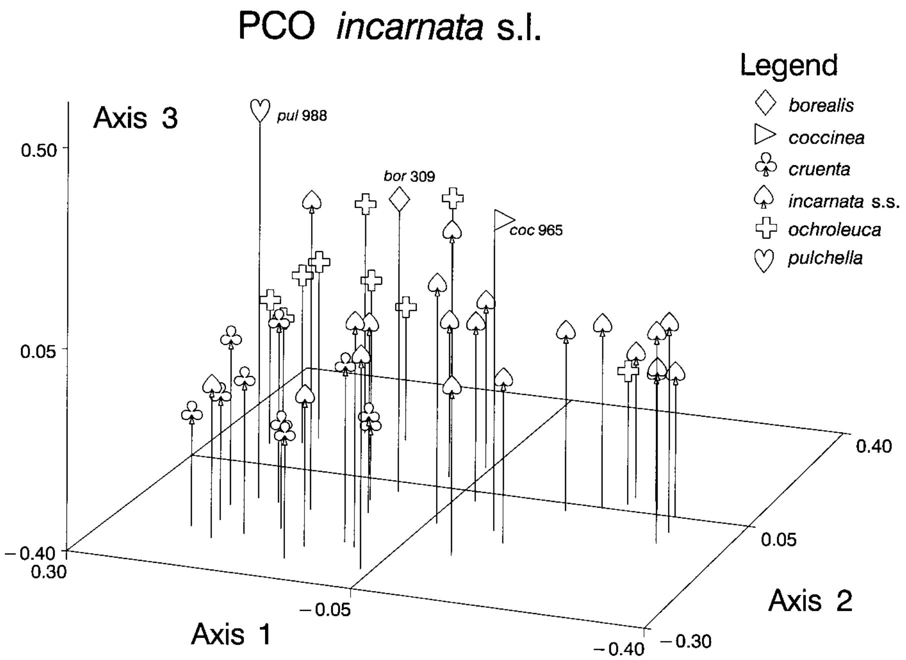

Fig. 5. Plot of the first three axes from a principal coordinates analysis, including all *Dactylorhiza incarnata* s.l. The proportion of total variance along the first three axes was 10.7, 8.7, and 7.2%, respectively. Symbols denote different taxa within *D. incarnata* s.l.

position of *D. foliosa* remains unclear. It appears to be morphologically distinct, and in the absence of any conclusive evidence regarding its phylogenetic position, we continue to treat it as a separate species.

The allotetraploid members of *Dactylorhiza* have apparently evolved on repeated occasions from the same set of broadly defined parental species. Although the allotetraploids have similar origins, they may be more or less differentiated in morphology and habitat preferences, and they are often found in geographically separate areas. It is implied that each recognized allotetraploid either has a single origin or several origins from the same subset of the parental groups; in any case, they each contain a unique combination of characters from the parental genomes and a subset of the variation found in each parental group. Although not monophyletic in a strict sense (as they have originated by reticulate evolution), they have unique origins and could be viewed as evolutionary units evolving relatively independently of other such units. On the other hand, there are a fairly large number of cases in which different allotetraploids are difficult to separate from each other. First, allotetraploids in separate geographic areas may approach each other in morphological characters, although they have different origins; examples of this are *D. majalis* subsp. *alpestris* in the Alps and in the Pyrennees, subsp. *cordigera* on the Balkans, subsp. *majalis* in north-central Europe, and subsp. *cambrensis* in the British Isles. Secondly, at sites where they coincide, different, otherwise morphologically well-defined allotetraploids may be connected to each other by intermediate plants, indicating that hybridization and backcrossing occur, leading to gene flow between the allotetraploids. Examples of this may be *D. majalis* subsp. *traunsteineri* and *D. sphagnicola* at several Swedish sites. We propose that the various allotetraploids should be recognized as subspecies of one species separate from the parental species, which is in accordance with the treatment presented by Bateman and Denholm (1983). This solution would take into account that the various subspecies are connected to each other by intermediate forms and may be difficult to separate from each other. At the same time we recognize that they are still relatively independent from each other and have unique origins. Treating the allotetraploids as subspecies would decrease the naming of new taxa and would make the taxonomic treatment of *Dactylorhiza* comparable to that used in most other plant groups. However, subspecies should be delimited as carefully as possible to reflect the evolutionary patterns in the complex, and much work remains to investigate the subspecies that have already been described. Hybrids between the allotetraploids and the diploid parents have lower fertility (Stace, 1975); thus, we are motivated to treat the allotetraploid lineages as a separate species.

Taxonomic note—Many allotetraploid taxa have already been treated as subspecies of *D. majalis* and we follow this practice here if names at subspecies level are available. The southwestern European *D. elata* is also an allotetraploid, and an amalgamation of this species with *D. majalis* should be considered as well. The name *D. elata* would have priority over *D. majalis*, which would require numerous combinations to be made. However, we require better knowledge of the pattern of variation in *D. elata* before we can eventually decide upon the taxonomy of this complex, and we refrain from proposing new combinations until more data are available. We also treat *D. sphagnicola* as a separate species until this question has been settled.

Conservation—Conservation strategies for polyploid complexes containing rare species should consider among other factors the rate at which new polyploid derivatives are formed from the stock of parental taxa, the relationships of different polyploid derivatives, the area of origin of polyploids, and the size of their distribution areas. They should also evaluate the status of the parental species to ensure that future evolution of the complexes are not restricted by human activity. These factors are rarely well known for polyploid complexes, although the use of molecular markers has contributed considerably to the knowledge of polyploid evolution in recent years (e.g., Cook et al., 1998).

Our data indicate that new polyploid derivatives arise at a moderate rate in *Dactylorhiza.* The allotetraploids occurring in the British Isles cluster together in our analyses, indicating that these allotetraploids have a regional origin different from those occurring in Scandinavia. This finding contrasts with some taxonomic treatments of certain allopolyploids, in which both British and Scandinavian populations are accommodated in *D. majalis* subsp. *lapponica,* subsp. *majalis,* or subsp. *traunsteineri.* On the other hand, there are indications that local populations in larger regions have the same origins (e.g., *D. sphagnicola* in southern Sweden, or *D. majalis* subsp. *purpurella* in northwestern Europe), which indicates that some allotetraploids may have spread over fairly extensive areas after formation.

It appears that *Dactylorhiza* contains a moderately large number of independently evolved allotetraploids. We propose that each of the allotetraploids should be given equal value in a conservation program. For effective conservation, we need a taxonomy that reflects as closely as possible their independent origins. In the circumscription of these allotetraploids, morphological data alone does not appear to be sufficient (which is also why we prefer to treat them as subspecies rather than as species) and should be supplemented by genetic, ecological, and geographical data.

Our data also indicate that present-day gene transfer from the diploid *D. maculata* subsp. *fuchsii* to the autotetraploid subsp. *maculata* is restricted and that new autotetraploid populations do not evolve often. It is suggested that in a conservation context these two subspecies should also be considered as independent entities of equal importance.

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