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Polymorphic populations of *Dactylorhiza incarnata s.l.* (Orchidaceae) on the Baltic island of Gotland: morphology, habitat preference and genetic differentiation

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• *Background and Aims* Organisms may be polymorphic within natural populations, but often the significance and genetic background to such polymorphism is not known. To understand the colour polymorphism expressed in the diploid marsh-orchids *Dactylorhiza incarnata*, morphological, habitat and genetic differentiation was studied in mixed populations on the island of Gotland, supplemented with genetic marker data from adjacent areas.
• *Methods* A total of 398 accessions was investigated for plastid haplotype and three nuclear microsatellites. Morphometric data and vegetation data were obtained from a subset of 104 plants.
• *Key Results* No clear pattern of habitat differentiation was found among the colour morphs. Within sites, the yellow-flowered morph (*ochroleuca*) was slightly larger than the others in some flower characters, whereas the purple-flowered morph with spotted leaves (*cruenta*) was on average smaller. However, populations of the same colour morph differed considerably between sites, and there was also considerable overlap between morphs. Morphs were often genetically differentiated but imperfectly separated within sites. Most populations were characterized by significant levels of inbreeding. The *ochroleuca* morph constitutes a coherent, highly homozygous sublineage, although introgression from purple-flowered morphs occurs at some sites. The *cruenta* morph was genetically variable, although Gotland populations formed a coherent group. Purple-flowered plants with unspotted leaves (*incarnata* in the strict sense) were even more variable and spanned the entire genetic diversity seen in the other morphs.
• *Conclusions* Colour polymorphism in *D. incarnata* is maintained by inbreeding, but possibly also by other ecological factors. The yellow-flowered morph may best be recognized as a variety of *D. incarnata*, var. *ochroleuca*, and the lack of anthocyanins is probably due to a particular recessive allele in homozygous form. Presence of spotted leaves is an uncertain taxonomic character, and genetic differentiation within *D. incarnata* would be better described by other morphological characters such as leaf shape and stature and size and shape of lip and spur.

Key words: *Dactylorhiza incarnata*, *cruenta*, ecology, genetic differentiation, Gotland, microsatellites, *ochroleuca*, plastid DNA, polymorphism.

INTRODUCTION

> '*Orchis cruenta* often grows together with typical *incarnata*, even the waxen variety *ochroleuca*, of approximately the same morphology. Thus, an intricate problem is presented which must remain unsolved until further notice.'
>
> Rosvall and Petterson, *Gotlands orkidéer*, 1951

Polymorphism is common in angiosperms and is often expressed in the flower. For instance, in heterostylous species such as *Primula veris* and *Lythrum salicaria*, male and female parts are located at different levels in different morphs (Richards, 1997), and in dioecious species such as *Valeriana dioica* male flowers can be larger than female flowers (Mossberg and Stenberg, 2003). In these cases, phenotypic differences are obviously linked to the reproductive system, and it is clear that the different morphs belong to the same species. In other plant groups the link to reproductive biology is more obscure, and it may be debated whether forms belong to the same taxon or should be separated as different biological species if they represent reproductively isolated lineages.

Here, polymorphism is studied within the diploid marsh-orchids *Dactylorhiza incarnata sensu lato* (*s.l.*). The complex extends from westernmost Europe to central Asia and from southern Europe and Asia Minor to northern Scandinavia (Hultén and Fries, 1986). It is highly variable in characters such as plant stature, flowering period, leaf markings, lip shape and pattern and tepal colour (e.g. Rosvall and Pettersson, 1951; Heslop-Harrison, 1954; Hylander, 1966; Nelson, 1976; Landwehr, 1977; Bateman and Denholm, 1985; Buttler, 1991; Mossberg and Lundqvist, 1994). The complex has been subdivided into many taxa, but there is little consensus on how they should be circumscribed and at which taxonomic levels they are best treated (for a critical discussion, see Haggar, 2003*a*, *b*, 2004*a*, *b*, 2005*a*, *b*). Forms can occur sympatrically and create mixed populations. However, they may still have different overall distributions and partly

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different ecological requirements (Hylander, 1966; Mossberg and Nilsson, 1977).

In the analyses, two easily identified morphs were separated from the remainder of *D. incarnata* and contrasted the resulting three groups with each other. The *cruenta* morph (*D. cruenta*, *D. incarnata* subsp./var. *cruenta)* is characterized by large, often confluent spots on both sides of leaves and bracts and purplish flowers with distinct markings on the lip. The *ochroleuca* morph (*Dactylorhiza ochroleuca*, *D. incarnata* subsp./var. *ochroleuca*) is characterized by yellow flowers totally devoid of any red/purple pigmentation. It is usually a tall plant with long erect leaves, and the lip typically has a protruding midlobe and notched sidelobes. The remainder of *D. incarnata* constitutes a variable complex. All plants with unspotted leaves and flowers ranging from almost completely white to purple, but not pure yellow were assigned to this morph, *incarnata*. There are different opinions on delimitation of these taxa, and *D. incarnata* is sometimes subdivided into several additional taxa (Hylander, 1966; Mossberg and Lundqvist, 1994). This question is revisited in the Discussion.

Various hypotheses have been put forward to explain the sympatric occurrence of distinct morphs of *D. incarnata s.l.* at single sites. Some authors have argued that these morphs represent distinct lineages with only occasional gene flow between them, and it is sometimes argued that they should be treated as separate species (e.g. Buttler, 1991; Mossberg and Lundqvist, 1994; Delforge, 2001). However, as it has been shown that artificial crosses are possible between morphs (Roberts, 1975; Malmgren, 1992; Vallius *et al.*, 2008; L. Lövgren, Department of Systematic Botany, Uppsala University, Sweden, unpubl. res.) isolating mechanisms other than genetic incompatibility must be operating if such morphs represent distinct lineages. One suggested mechanism is habitat specialization. Regarding *ochroleuca*, several authors (Hylander, 1966; Mossberg and Stenberg, 2003; Vallius *et al.*, 2004) have pointed out that it is limited to calcareous fens. As fens were investigated where *ochroleuca* grows in sympatry with other taxa, there must also be habitat specialization within sites if this form of isolation mechanism is operating, and such differentiation has indeed been suggested by some authors (Ekstam *et al.*, 1988; Foley, 2000). Also common in calcareous fens is the *cruenta* morph, where it may occupy specific habitats (Ekstam *et al.*, 1988; Ingmansson and Johansson, 2005), but it is also found in medium-rich fens outside the calcareous regions, for instance along the Swedish western coast or Scandinavian mountain chain (Hylander, 1966; Mossberg and Nilsson, 1977). Secondly, Vallius *et al.* (2004) indicated that morphs of *incarnata* may be pollinated by different pollinators and suggested that constancy of these pollinators to a particular morph may in fact lead to genetic isolation. They also found that sympatric morphs differed in characters other than flower colour at some of their study sites. This pattern was interpreted as ongoing local differentiation that could possibly result in sympatric speciation (Vallius *et al.*, 2004). Thirdly, studies of allozyme variation in *D. incarnata* (Pedersen, 1998; Hedrén, 2001*a*) have shown that populations of *D. incarnata* are characterized by high levels of inbreeding (Hedrén, 2001*a*). It is possible therefore that morphs of *D. incarnata* represent inbred lines, and gene flow across varieties is rare for this reason. Inbreeding may explain rarity of intermediate morphs, but it may not necessarily be taken as an argument for recognizing morphs as separate species.

In contrast, it can also be argued that these colour morphs all belong to the same species and that colour polymorphism is adaptive as such. With the exception of *Dactylorhiza viridis* (Devos *et al.*, 2006), all members of the genus offer no reward to pollinators (Nilsson, 1980). In the polymorphic congener *D. sambucina*, which often forms mixed populations of yellow- and and purple-flowered individuals, colour variants in populations may prolong the time required for pollinating bumblebees to learn to avoid the species, thereby resulting in increased numbers of visits, higher pollination efficiency (Nilsson, 1980; Gigord *et al.*, 2001) and selection for the rare colour variant (Gigord *et al.*, 2001). This explanation may apply to *D. incarnata* as well, but it is incompatible with the hypothesis presented by Vallius *et al.* (2003) that different morphs are visited by different insects.

Further hypotheses can also be formulated that are paralleled in other groups of *Dactylorhiza*. For instance, pigmentation may be of direct adaptive significance in situations where plants are exposed to high levels of direct sunlight. In the allotetraploid marsh-orchid *D. majalis* subsp. *lapponica*, strong anthocyanin pigmentation of flowers, bracts and stem is a typical feature for plants growing in open fens at high elevation, but this pattern is much less prominent in plants in more shaded situations in the lowlands. Genetic markers have shown that these forms are part of the same taxon (Nordström and Hedrén, 2008) and accordingly that pigmentation is a variable character within this taxon. Applying these results to *D. incarnata* it could be hypothesized that *ochroleuca* would grow in more shaded habitats than the purple-flowered morphs and that *cruenta* would grow in more exposed situations. Such differentiation should appear from analysis of vegetation data. Alternatively, flower colour may be linked to other characters of adaptive significance, for instance, physiological traits connected with habitat specialization and, if so, other patterns of habitat separation may be found.

In this study, morphometric, ecological and genetic data are combined in an attempt to obtain a better understanding of mechanisms maintaining polymorphism in the *D. incarnata* complex. The study was performed on the Baltic island of Gotland where these morphs often occur in mixed populations (Rosvall and Pettersson, 1951; Ekstam *et al.*, 1988; Hedrén, 2001*b*; Petersson and Ingmansson, 2007). To put the results into a more general context, reference material from elsewhere in the Baltic region was also included, but analysis of this material was restricted to just genetic variation.

MATERIALS AND METHODS

Plant material

Material from six sites on Gotland were studied for habitat, morphology and genetic markers (Fig. 1; Table S1 in Supplementary data, available online). All morphometric and vegetation data were collected between 11 June and 24 June 2002. At Hoburgsmyr, Lojsthajd and Storsund all three morphs were examined, at Harudden and Lillmyr *incarnata* and *ochroleuca* were examined, and at Agbod only *incarnata*

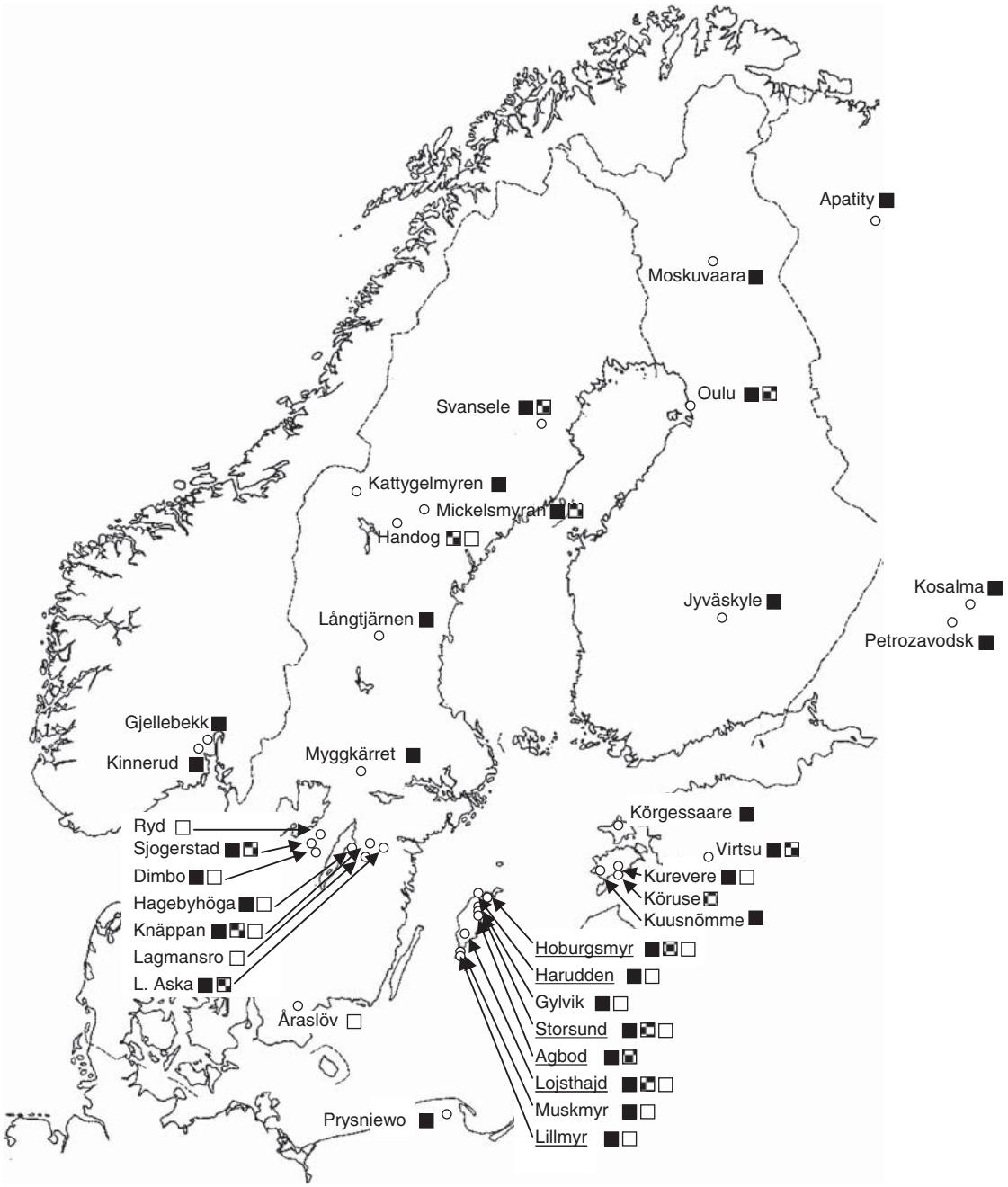


FIG. 1. Map of the Baltic area showing sampling sites of *D. incarnata s.l.* The three morphs in this study are represented by boxes with different shading, as indicated. The sites from which morphometric data and vegetation data were collected have been underlined. Exact location of sites and sample sizes in the data sets are given in Table S1 in Supplementary data, available online.

was examined. A single sample of *cruenta* found at Agbod was studied for genetic markers only. All sites were fens overlying calcareous bedrock, populated by between one and six of the following indicator species typical of calcareous fens: *Carex lepidocarpa*, *Epipactis palustris*, *Eriophorum latifolium*, *Primula farinosa*, *Schoenus ferrugineus* and *S. nigricans*. In addition to these six sites, two more sites from Gotland and 28 sites from the areas surrounding the Baltic were investigated for molecular markers only (Fig. 1; Table S1 in Supplementary data). This material was sorted into the same morphs as the Gotland material, and some of these sites also contained a mixture of taxa: *incarnata* was present on 25 sites, *cruenta* at nine sites and *ochroleuca* at ten sites. Different varieties from any site were treated as separate populations in the analyses of data. At two sites, Agbod and Kattygelmyren, the sampled material was collected from different parts of the site, so these materials could be used to describe differentiation beween subsites.

Although identification of most material was straightforward, a few intermediates were also recognized. Material

from Kurevere, Estonia, contained samples intermediate between *incarnata* and *ochrouleuca* in having lips with pink margins combined with a yellow centre. Material from Oulu, Finland, contained some samples intermediate between *incarnata* and *cruenta* in having leaves with pale spots. These two intermediate morphs were treated as separate groups when they were compared with co-occurring morphs.

Morphometry

Morphological measurements were taken from all parts of the plant, including stem, leaves and flowers (Table 1). Twenty-eight characters were measured. Most characters were taken from Bateman and Denholm (1985), but leaf size and position were described somewhat differently by using composite characters (characters 24–28 in Table 1). However, all characters describing flower pigmentation, patterning and leaf spotting were excluded as these characters were used *a priori* to sort the material into contrasting morphs.

The morphometric data were subjected to a canonical variates analysis (CVA), in which population was used as grouping variable. Calculations were performed in NTSYS-pc (Rohlf, 2005).

Vegetation analysis

Vegetation data (relevées) were collected for each individual measured for morphological characters. All species occurring in a circle 40 cm in diameter surrounding each individual were recorded. The full data set was subsequently subjected to principal component analyses (PCA). Ordination plots were accompanied by plots of factor loadings, showing the influence of recorded species on the separation of relevées. Calculations were performed in NTSYS-pc (Rohlf, 2005).

Molecular methods

DNA was extracted from silica gel-dried flowers (Chase and Hills, 1991) or from fresh leaf material by the 2× CTAB procedure (Doyle and Doyle, 1987).

Two size-variable sites were studied in the plastid genome, and the combined pattern was recognized as haplotypes. The two selected marker sites were developed in Hedrén *et al.* (2008) for general studies of plastid DNA variation in *Dactylorhiza* and have been found to be the most polymorphic sites in *D. incarnata s.l.* in the Baltic area. Site 10b is a polyA–polyTA–polyT repeat located in the *psbA-trnK* spacer just upstream the *trnK* exon I, whereas site 11b is polyA repeat located in the *rpl16* intron (Jordan *et al.*, 1996). Haplotypes were denoted by two-digit numbers where the digits report differences in base pairs from the shortest fragment found at sites 10b and 11b, respectively.

Variation in the nuclear genome was described by three nuclear microsatellites developed for *Dactylorhiza* by Nordström and Hedrén (2007). The three selected loci, ms3, ms8 and ms11, consist of trinucleotide repeats and were found to produce easily interpreted banding patterns with one or two peaks in any individual sample of *D. incarnata*.

Size-variable fragments in the plastid genome were amplified by means of specific primers and PCR conditions reported in Hedrén *et al.* (2008), whereas nuclear microsatellites were amplified according to conditions reported in Nordström and Hedrén (2007). The PCR product from each reaction was mixed with 20 μL formamide and appropriate size standards to enable exact size determination of the amplified fragments on the ALFexpress II automated sequencer.

Plastid haplotype data were used to calculate between-population differentiation and genetic diversity statistics. The overall pattern of differentiation was described by first calculating the diagonal matrix of average number of pairwise differences between populations (Nei and Li, 1979) and then subjecting this matrix to a principal co-ordinates analysis (PCO). Populations within sites (different morphs within sites) were compared by pairwise F_{ST} based on number of alleles. Genetic diversity within populations and within varieties was estimated as average gene diversity over loci. Nuclear microsatellite data were used to calculate between-population differentiation, genetic diversity statistics and inbreeding coefficients. Analyses were performed as for plastid haplotype data. All analyses were performed in Arlequin 3·01 (Excoffier *et al.*, 2005) except PCO for which NTSYSpc 2·2 (Rohlf, 2005) was used.

RESULTS

Morphometry

Figure 2 shows the individual samples and population mean values plotted against the first two canonical variates from the CVA. No clear conclusions regarding differentiation of individual samples could be drawn; there was a high degree of overlap between populations. However, two patterns could be seen regarding the position of population mean scores. First, morphs from the same site are positioned relatively close to each other in the plot: populations from Storsund, Hoburgsmyr and Lojsthajd to the left, Harudden to the right, Agbod to the centre, and Lillmyr to the lower middle. Second, *ochroleuca* is placed to the right of the other varieties from the same site whereas *cruenta* is placed below the other morphs. Pairwise *t*-tests performed on the entire data set (Table 1) reveal that morphs differ significantly from each other in position of maximum labellum width (6); *ochroleuca* has the maximum width on the lower part of the lip and *cruenta* on the upper part of the lip. For spur width at its entrance (10), *ochroleuca* is wider than *incarnata*, and *incarnata* is wider than *cruenta*. The other two morphs differ from *incarnata* in having smaller maximum leaf length (26), *cruenta* in having shorter labellum (1), less-divided lateral labellum lobes (8) and fewer flowers (19), and it is smaller in several size characters (12, 14, 15, 17, 20, 21, 24, 27); *ochroleuca* has a wider labellum (5) and a wider spur halfway along its length (11). These characters often also separated morphs within sites; other characters also contributed to such differentiation, but the set of significant separating characters was not consistent among sites (data not shown).

Vegetation analysis

The PCA of habitat data for all investigated sites is provided in Fig. 3. Relevées from Agbod are dispersed over the central

TABLE 1. *Morphometric characters studied*

Character no.	Character	*incarnata* (mean ± s.d.)	*cruenta* (mean ± s.d.)	*ochroleuca* (mean ± s.d.)	*P*-values (pairwise *t*-tests)		
					incarnata vs. *cruenta*	*incarnata* vs. *ochroleuca*	*cruenta* vs. *ochroleuca*
1 (1)	Labellum length from spur entrance to apex of central lobe (mm)	6·71 ± 0·73	6·13 ± 0·35	6·75 ± 0·57	**0·00**	0·81	**0·00**
2 (2)	Presence or absence of sinuses separating central and lateral lobes	0·73 ± 0·41	0·42 ± 0·51	0·68 ± 0·46	0·07	0·60	0·13
3 (3)	Length from spur entrance to base of sinus (mm)	5·37 ± 0·72	5·30 ± 0·44	5·27 ± 0·61	0·69	0·49	0·85
4 (4)	Length from base of spur entrance to apex of right lateral lobe (mm)	5·75 ± 0·69	5·49 ± 0·35	5·70 ± 0·61	0·08	0·74	0·14
5 (5)	Maximum width of labellum (mm)	6·98 ± 0·98	6·68 ± 0·83	7·44 ± 0·92	0·30	**0·02**	**0·01**
6 (6)	Position of maximum width in relation to axis of maximum length	1·88 ± 0·53	2·33 ± 0·65	1·61 ± 0·65	**0·04**	**0·04**	**0·00**
7 (7)	Amount of reflexion of lateral lobes	4·71 ± 0·92	4·50 ± 0·90	4·50 ± 0·90	0·49	0·28	1·00
8 (14)	Indentations on right lateral lobe	0·95 ± 0·83	0·50 ± 0·52	1·16 ± 0·77	**0·03**	0·21	**0·00**
9 (15)	Spur length from entrance to apex (mm)	8·25 ± 0·91	7·73 ± 0·81	8·29 ± 0·83	0·07	0·85	0·05
10 (16)	Spur width at entrance (mm)	2·45 ± 0·37	2·10 ± 0·21	2·64 ± 0·38	**0·00**	**0·02**	**0·00**
11 (17)	Spur width halfway along length (mm)	1·82 ± 0·22	1·78 ± 0·34	2·02 ± 0·29	0·67	**0·00**	**0·04**
12 (18)	Spur curvature	3·90 ± 0·42	4·33 ± 0·49	3·95 ± 0·30	**0·01**	0·44	**0·02**
13 (19)	Position of lateral outer perianth segments	30·6 ± 3·03	30·0 ± 0·00	30·1 ± 2·75	0·16	0·40	0·79
14 (22)	Length of basal bracts (mm)	26·2 ± 6·18	21·5 ± 4·46	26·8 ± 5·89	**0·01**	0·64	**0·00**
15 (23)	Length of bracts halfway up inflorescence (mm)	19·1 ± 3·45	15·7 ± 2·49	18·2 ± 3·25	**0·00**	0·22	**0·01**
16 (28)	Stem stature (mm)	282 ± 82·6	223 ± 78·3	267 ± 71·4	**0·03**	0·35	0·10
17 (29)	Inflorescence length (mm)	54·6 ± 19·3	45·4 ± 10·8	53·5 ± 13·3	**0·04**	0·76	**0·04**
18 (30a)	Ovary length (mm)	11·6 ± 1·62	11·6 ± 0·97	11·4 ± 1·14	0·98	0·68	0·70
19 (31)	Number of flowers	25·7 ± 13·3	16·2 ± 4·24	26·7 ± 9·22	**0·00**	0·67	**0·00**
20 (32)	Stem diameter above lowermost sheathing leaf (mm)	5·67 ± 2·23	4·25 ± 0·97	6·02 ± 1·58	**0·00**	0·38	**0·00**
21 (34)	Number of sheathing leaves	3·23 ± 0·72	2·50 ± 0·67	3·02 ± 0·46	**0·00**	0·10	**0·02**
22 (35)	Number of non-sheathing leaves	1·29 ± 0·74	1·33 ± 0·78	1·16 ± 0·71	0·87	0·38	0·49
23 (43)	Hooding of apex of sheathing leaves	1·92 ± 0·28	1·58 ± 0·51	1·93 ± 0·25	0·05	0·79	**0·04**
24	Total leaf area of all vegetative leaves* (mm^2)	2172 ± 1736	1051 ± 629	1760 ± 907	**0·00**	0·15	**0·00**
25	Position of median leaf area along the stem† (mm)	95·7 ± 39·0	65·1 ± 30·2	87·2 ± 40·2	**0·01**	0·30	0·05
26	Maximum length of any vegetative leaf (mm)	121 ± 34·1	87·2 ± 37·7	103 ± 29·8	**0·01**	**0·01**	0·19
27	Maximum width of any vegetative leaf (mm)	19·4 ± 11·3	13·5 ± 2·20	18·0 ± 3·65	**0·00**	0·42	**0·00**
28	Mean position of maximum width of all vegetative leaves (%)	21·4 ± 4·51	25·1 ± 6·30	20·8 ± 3·69	0·07	0·53	**0·04**
	Relative position of median leaf area along stem (25/16)‡	0·33 ± 0·08	0·28 ± 0·05	0·32 ± 0·08	**0·01**	0·36	0·07
Numbers of samples		48	12	44			

Characters studied by Bateman and Denholm (1985) are given within parentheses.
Significant differences as revealed by *t*-tests are given in bold.
* Leaf area calculated as length × width × 0·65.
† Calculated as $\Sigma DA/\Sigma D$, where D is distance from the stem base of each leaf, and A is the leaf area of each leaf.
‡ Not used in CVA (duplicates data given by characters 16 and 25).

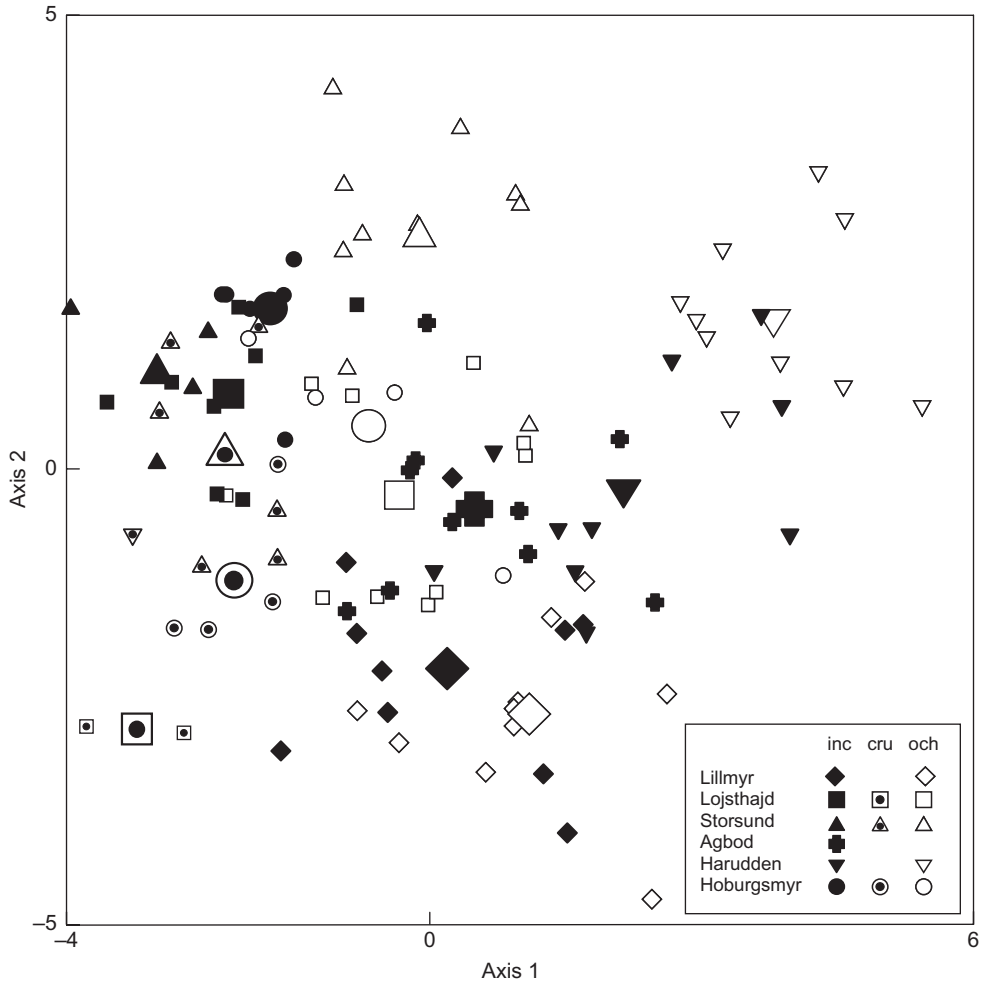


FIG. 2. Canonical variates analysis of morphological data in *D. incarnata*. Large symbols represent population means, whereas small symbols represent individual samples. The first and second canonical axes accounted for 27·6% and 20·0% of the total, respectively.

and left parts of the plot, whereas the remaining material is aggregated to the right. Plants responsible for the separation of the Agbod relevées include several typical seashore plants, but the presence of *Schoenus nigricans* at the locality demonstrates that it is not an ordinary seashore meadow.

Because relevées from localities other than Agbod were so poorly separated in the analysis of all data, a second analysis was performed from which relevées from Agbod were excluded (Fig. 4). Some further separation of localities was evident in this analysis: Lojsthajd to the right, Harudden to the lower left and Lillmyr, Hoburgsmyr and Storsund to the upper left. However, there was still clear overlap of relevées from localities in the central part of the plot, and there was no obvious habitat differentiation between morphs within any site.

Genetic differentiation

Twelve haplotypes were created by combining fragment-length variants at the two plastid marker sites (Table S2 in Supplementary data available online). Haplotypes 01 and 02 dominated *cruenta*, but it also had a few samples with haplotype 23 (Fig. 5). Haplotype 02 was even more dominant in *ochroleuca*, but it also had a few samples with haplotypes 01, 12, 23 and 33. All haplotypes were found in *incarnata*, but haplotypes 01 and 02 were the most common.

Patterns of haplotype differentiation between populations are illustrated in the PCO (Fig. 6). Twenty-five populations fixed for haplotype 02 are located at a single point in the left part of the diagram. This group included nine populations of *ochroleuca*, six populations of *cruenta*, seven population of *incarnata* and the two populations of *incarnata*/*ochroleuca* and *incarnata*/*cruenta* intermediates. Of the remaining populations, four populations of *ochroleuca* were located close to this cluster, five populations of *cruenta* were located in the left part of the diagram and the remaining 23 populations of *incarnata* were found in other parts of the plot.

The four *ochroleuca* populations containing rare haplotypes (i.e. that were not fixed for haplotype 02) were intermixed with *incarnata*, and in three of the sites (e.g. Harudden, Fig. 5) the rare haplotypes were also found in the local *incarnata* population. The exception was the Hoburgsmyr population, but from this site only two individuals of *incarnata* were examined

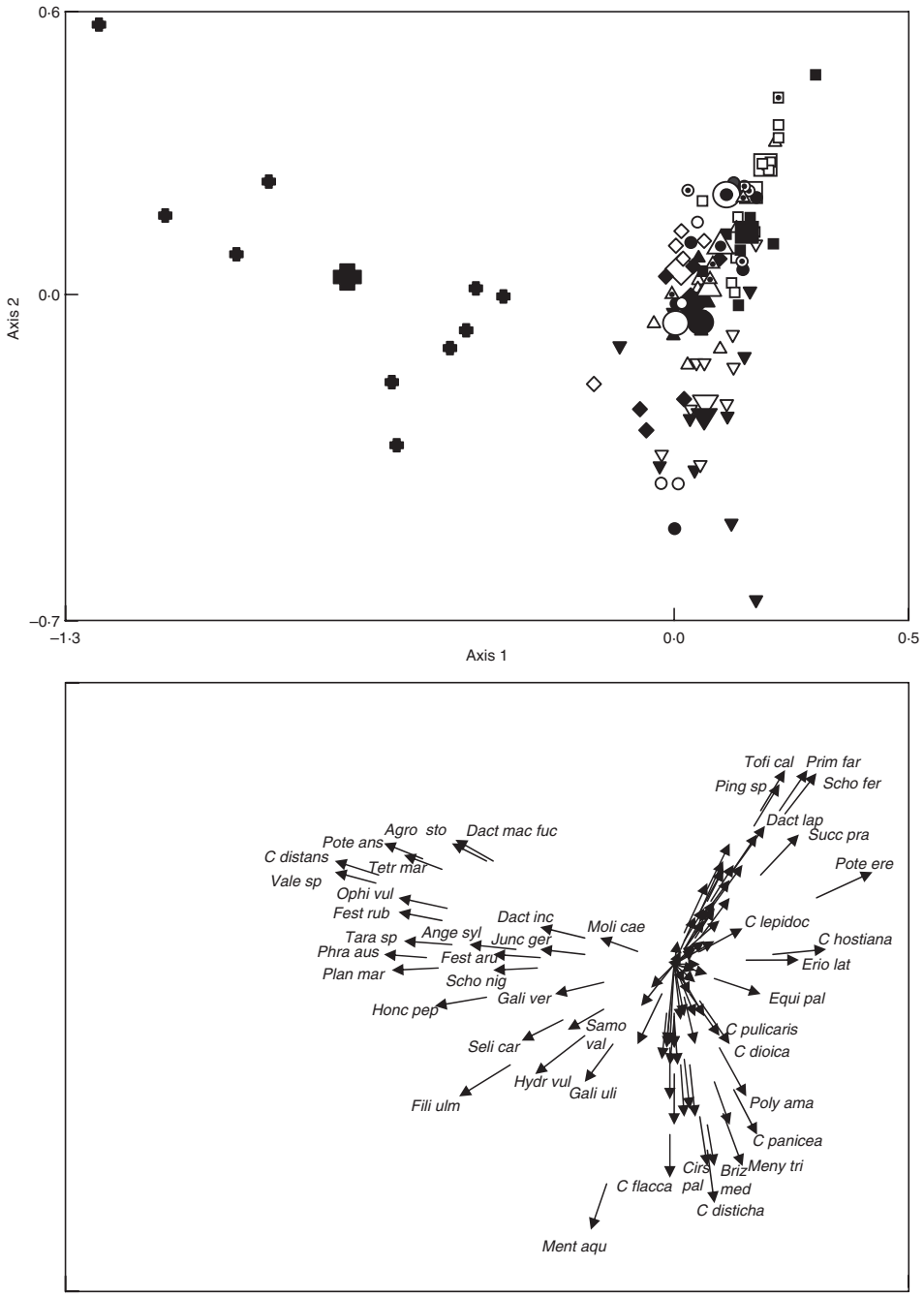


FIG. 3. Principal components analysis of habitat data for plots surrounding individual samples of *D. incarnata*. Symbols as in Fig. 2. The lower plot illustrates factor loadings (species). The first and second principal axes accounted for 6·7% and 4·8% of the total, respectively.

for plastid variation. Populations of *cruenta* growing mixed with *incarnata* usually had the same haplotypes as the local *incarnata* population; in a few cases, *cruenta* included additional haplotypes, most notably, at Knäppan on mainland Sweden, where seven samples of *cruenta* contained haplotype 01, but this haplotype was not found in any of the other varietes collected at this site (Fig. 5). Most populations of *incarnata* carried some proportion of haplotypes 01 and/or 02, but a few populations including Körgessaare, Muskmyr and Kuusnõmme (located to the right of the PCO, Fig. 6) lacked these haplotypes.

Seven, eight and three alleles, respectively, were found at the three nuclear microsatellite loci, (Fig. 5; Table S3 in Supplementary data), and population differentiation is summarized by a PCO (Fig. 7). Most populations of *ochroleuca* were located in one point to the left of the plot; they were fixed for the fragment combination of 162/194/156 bp. Populations containing alleles other than the dominant ones

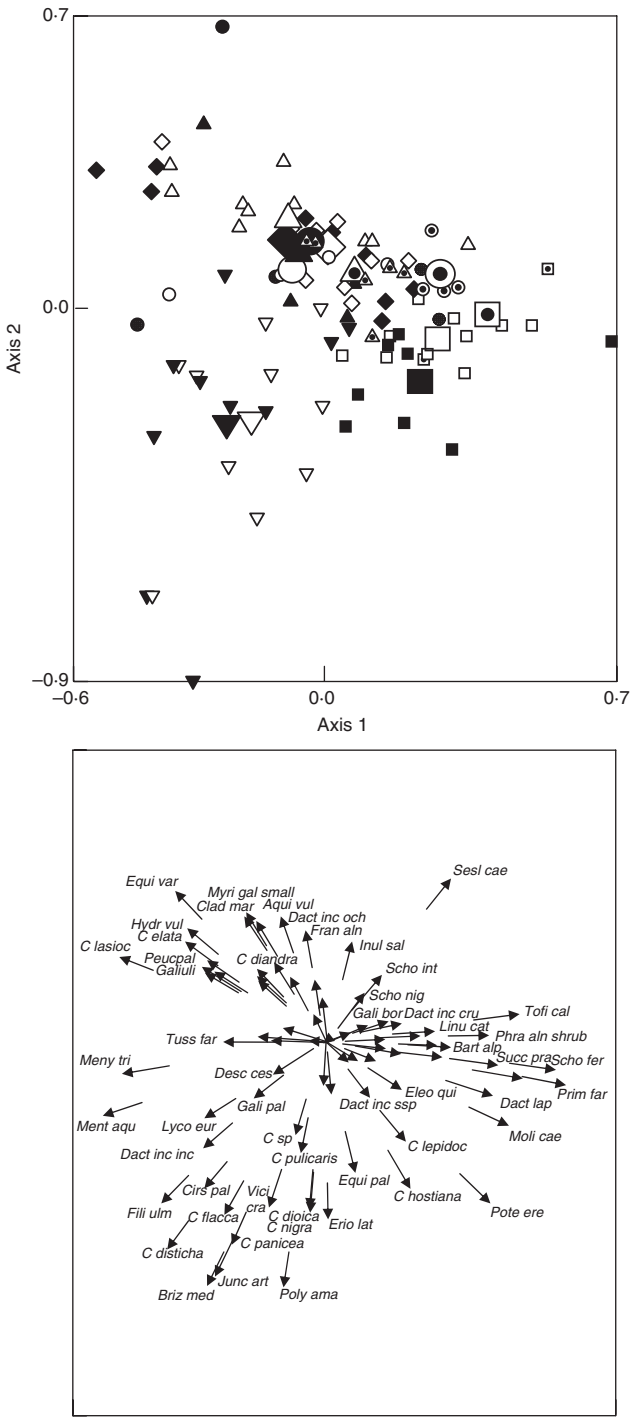


FIG. 4. Principal components analysis of habitat data for all sites excluding Agbod. Symbols as in Fig. 2. The lower plot illustrates factor loadings (species). The first and second principal axes accounted for 6·3% and 5·6% of the total, respectively.

all grew in mixed populations with other morphs, and in most cases these carried atypical alleles (e.g. Harudden, Fig. 5). Again, the Hoburgsmyr population differed from the rest. Populations of *cruenta* from areas around the southern parts of the Baltic Sea were placed in the left half of the PCO, whereas most populations from the north were placed in the right half. Four populations of *cruenta* from Gotland were all fixed for the fragment combination 153/194/156 bp and were placed together at a single point to the lower left. Populations of *incarnata* from the south were spread out over the entire plot, whereas populations from the north were mostly placed in the right half.

Within-site comparisons

At study sites with mixed populations, differentiation between morphs was decribed by pairwise F_{ST} values (Fig. 5; Table S4 in Supplementary data). As inferred from nuclear microsatellite data, differentiation between *incarnata* and *cruenta* was moderate at most of the sites in the south, but less at the two northern sites, Mickelsmyran and Svansele. A similar pattern was found in comparisons based on plastid data, apart from the fact that no differentiation between any morphs was found at Storsund, Gotland. Although *ochroleuca* was genetically well defined, no consistent pattern of differentiation was found in comparisons with *incarnata*, and there was also no consistent pattern when pairwise F_{ST} values derived from plastid and nuclear markers were compared with each other. A similar lack of pattern was seen in pairwise comparisons of *ochroleuca* and *cruenta*. Comparison of subpopulations within Agbod and Kattygelmyren resulted in low to moderate F_{ST} values (Fig. 5; Table S4 in Supplementary data).

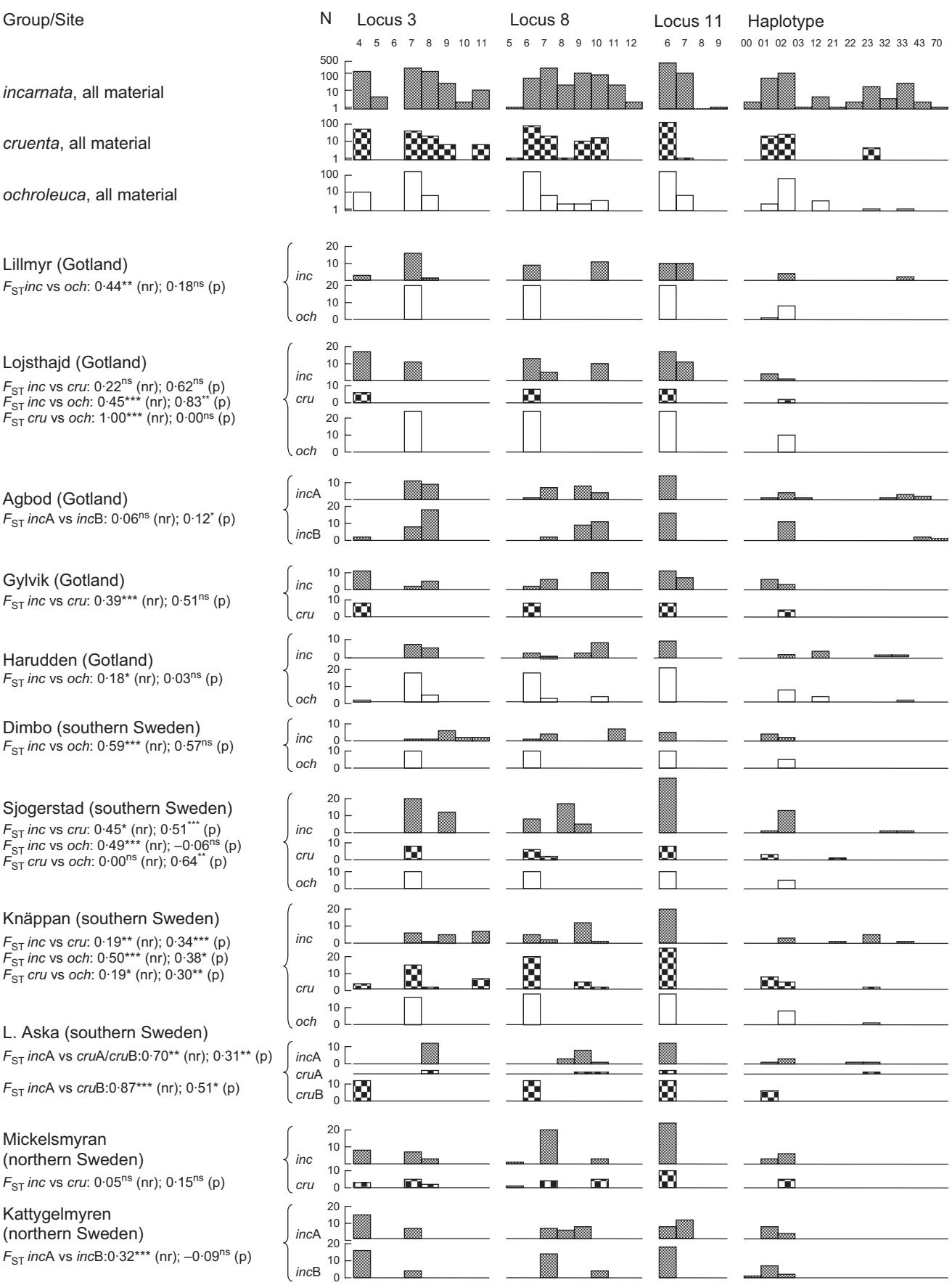
Genetic diversity

Various estimates of genetic diversity revealed that *incarnata* was the most genetically diverse morph, followed by *cruenta* and *ochroleuca* (Fig. 8; Table S5 in Supplementary data). This pattern was repeated at the population level, such that populations of *incarnata* were more genetically diverse than populations of *cruenta*, which were in turn more genetically variable than those of *ochroleuca*.

Inbreeding coefficients

Inbreeding coefficients for morphs and loci are reported in Table 2. Populations of *incarnata* and *ochroleuca* were characterized by inbreeding coefficients around 0·6, although there was fairly large variance around these values. In *cruenta*, different estimates were obtained for different loci, and the weighted mean value over loci was somewhat lower than for

FIG. 5. Summary of genetic marker variation within morphs at representative sites. The shading is the same as in Fig. 1: *incarnata* morph, black; *cruenta* morph, checkerboard patterned; *ochroleuca* morph, white. *N* values are numbers of gene copies, i.e. numbers of alleles at microsatellite loci or numbers of plastid haplotypes. Note, a logarithmic scale is used for morphs, but a linear scale is used for sites. Numbers of trinucleotide repeats are given for each microsatellite allele, cf. Table S3 in Supplementary, data available online. Haplotypes are annotated as in Table S2 in Supplementary data. Differentiation of morphs within sites has been estimated by pairwise F_{ST} calculated separately for nuclear microsatellites (nr) and plastid haplotypes (p); for details, see Table S4 in Supplementary data. At L. Aska and Kattygelmyren different subpopulations have been compared (as indicated by capital letters); at L. Aska partly coinciding with the separation of *incarnata* and *cruenta*. *P*-values: *** <0·001; ** <0·01; * <0·05; ns ≥0·05.



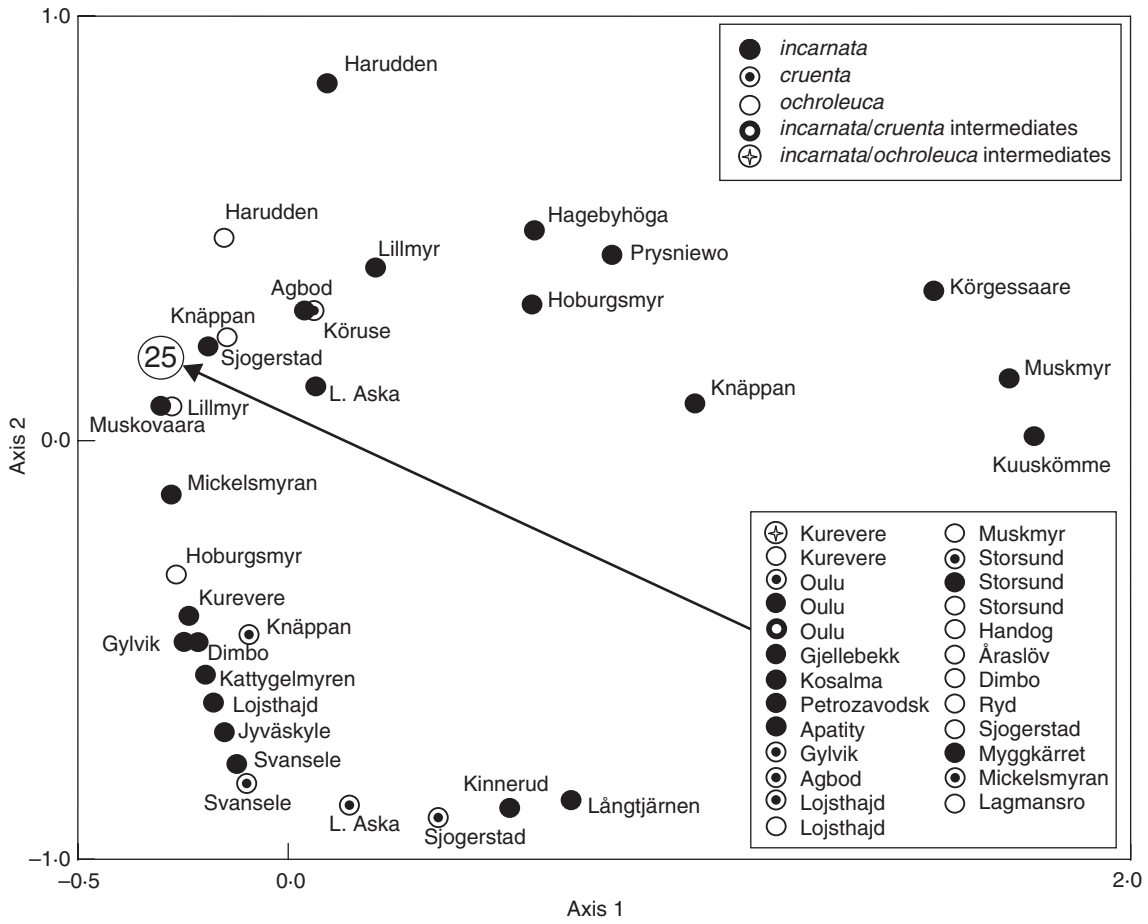


FIG. 6. Principal co-ordinates analysis of plastid haplotype data for populations of *D. incarnata s.l.* The first and second principal co-ordinate axes accounted for 27·1% and 18·2% of the total, respectively.

the other two morphs. Relatively few loci were variable for calculations in populations of *ochroleuca* and *cruenta* because many populations were fixed for a single allele at some loci.

DISCUSSION

ochroleuca

In continental Europe, *D. incarnata* var. *ochroleuca* is sometimes narrowly circumscribed such that only the most morphologically typical plants are included (Delforge, 2001). Atypical plants are then regarded as occasional forms of purple-flowered *incarnata*, forma *ochrantha* (Pedersen, 1998; Delforge, 2001; Haggar, 2005*b*), implying that these forms have no closer relationship with each other than with purple-flowered forms. Similarly, Kreutz (1993) published a report on the orchid flora of Gotland where he claimed that genuine *ochroleuca* is rare on Gotland and that most yellow-flowered populations should instead be regarded as *ochrantha*. Some populations studied here were also discussed by Kreutz. He regarded material from Storsund and Muskmyr as true *ochroleuca*, but material from Harudden as *ochrantha*. In contrast, the present data show that all yellow-flowered populations from Gotland belong to a single coherent subgroup of *D. incarnata* and that this group also occurs in other areas around the Baltic.

Apart from the Baltic area, plants conforming to *ochroleuca* are also known from scattered localities in central Europe (Buttler, 1991; Delforge, 1991; Baumann *et al.*, 2006) and a few localities in England (Bateman and Denholm, 1985; Foley, 2000). A single sample from Market Weston Fen, England, had the same marker combination as Baltic *ochroleuca* (M. Hedrén, S. Nordström and R. M. Bateman, unpubl. res.), supporting the view that English and Baltic *ochroleuca* are related. Without access to any material from central Europe, it appears likely that all populations of the *ochroleuca* morph constitute a relatively distinct sublineage within the *D. incarnata s.l.* complex.

According to Bateman and Denholm (1985), a yellow anthoxanthin pigment is ubiquitous in *D. incarnata*, but because this pigment is masked by anthocyanin pigments in red/purplish flowered plants, the yellow flower colour of *ochroleuca* should be the result of failure of the anthocyanin synthesis pathway (see also Haggar, 2005*b*). Because different populations of *ochroleuca* are typically characterized by the same combination of nuclear microsatellites and the same plastid haplotype, it is likely that all plants carry the same

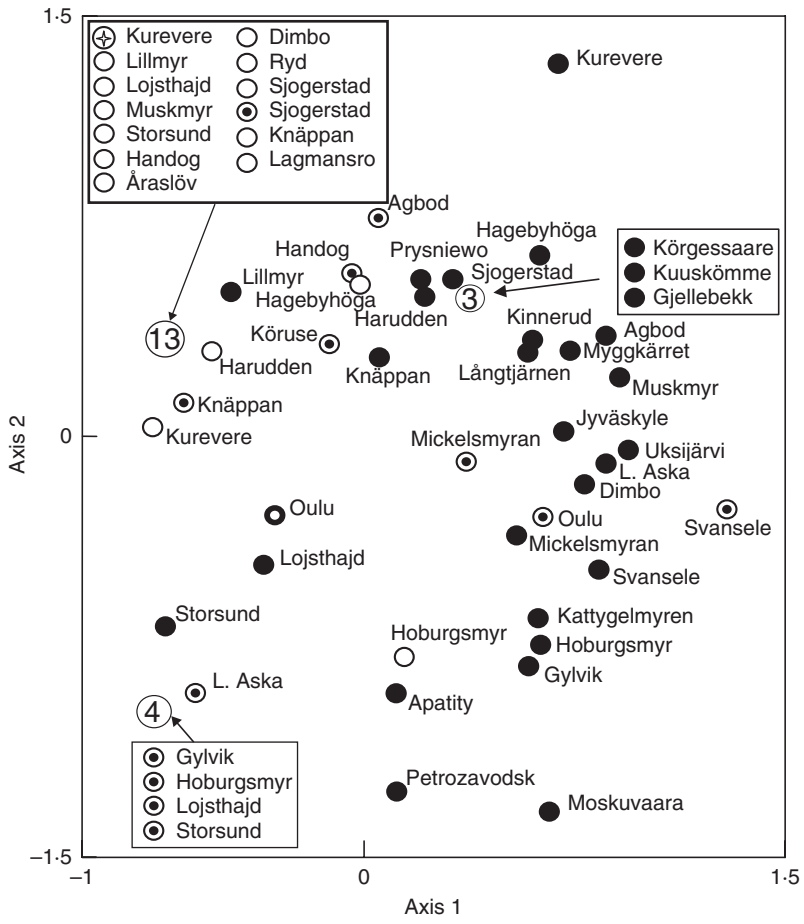


FIG. 7. Principal co-ordinates analysis of nuclear microsatellite data for populations of *D. incarnata s.l.* Symbols as in Fig. 6. The first and second principal co-ordinate axes accounted for 19·2% and 16·8% of the total, respectively.

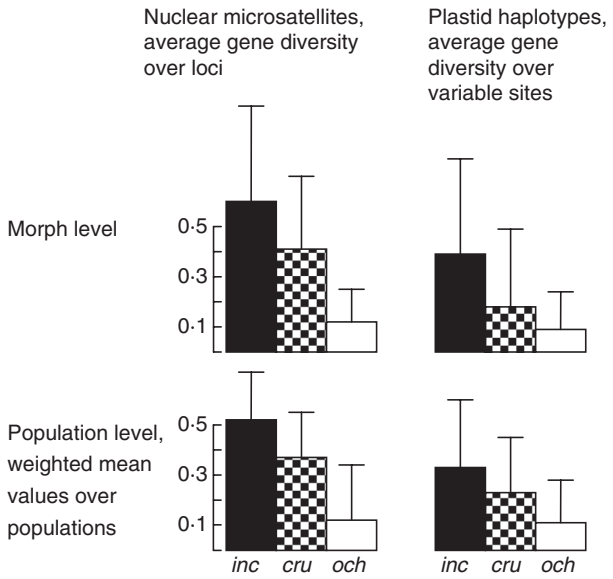


FIG. 8. Gene diversity statistics for morphs of *D. incarnata s.l.* Gene diversity estimates are given + s.d.; for morph level standard deviations are for the sampling and the stochastic processes, for population level standard deviations are for population estimates.

non-functional gene copy in homozygous form. The lack of variation within *ochroleuca* also suggests that the variety went through a genetic bottleneck at its formation, and this may be the reason why the non-functional gene has become fixed.

Elsewhere in Europe, cream or yellow-flowered plants are known in other segregates of *D. incarnata*, for instance the western subsp. *pulchella* (Bateman and Denholm, 1985). Preliminary data from a mixed cream- and purple-flowered population of subsp. *pulchella* in England show that the two colour morphs overlap considerably in molecular markers and that the population bears no resemblence to the *ochroleuca* studied here (M. Hedrén, S. Nordström and R. M. Bateman, unpubl. res.). However, it would be most important to study whether the appearence of anthocyanin-deficient individuals in subsp. *pulchella* is due to the same deficiency in the anthocyanin synthesis pathway as in *ochroleuca* and whether perhaps the same gene is also responsible for the flower-colour polymorphism in the more distantly related *D. sambucina* complex.

cruenta

Apart from spotted leaves, the form of *cruenta* that grows on Gotland and Öland typically differs from other southern

TABLE 2. *Mean inbreeding coefficients,* F_{IS}

Morph	Locus ms3 N_p	ms3 N_S	ms3 F_{IS}	ms8 N_p	ms8 N_S	ms8 F_{IS}	ms11 N_p	ms11 N_S	ms11 F_{IS}	Weighted mean over loci F_{IS}
incarnata	21	182	0·58 ± 0·36	22	191	0·57 ± 0·46	13	129	0·58 ± 0·33	0·58
cruenta	4	28	0·58 ± 0·35	6	34	0·23 ± 0·50	1	3	0·00	0·38
ochroleuca	4	21	0·63 ± 0·35	3	17	0·55 ± 0·40	3	17	0·54 ± 0·41	0·58

N_p, Number of variable populations at locus; N_S, total number of samples available for calculation at a locus.
Inbreeding coefficients for separate loci are given ± s.d.

Scandinavian forms of *D. incarnata s.l.* in being shorter and having relatively flat and short, often twisted leaves that curve outwards (Rosvall and Pettersson; 1951; Ekstam *et al.*, 1988; Ingmansson and Johansson, 2005). Analysis of the present morphometric data reveals that *cruenta* differs in several additional characters and confirms that *cruenta* is morphologically divergent on Gotland (Table 1). Furthermore, most *cruenta* material from Gotland formed a distinct homozygous group within *D. incarnata s.l.* The only exception was a single leaf-spotted sample encountered at Agbod, which, however, corresponded in genetic markers with *incarnata* growing at the same site. Still, as the characteristic markers of Gotland material were also present in populations from the surrounding mainland areas, we cannot argue that the Gotland population is distinct from the rest of *cruenta*. It is probable that the Gotland *cruenta* passed through a genetic bottleneck when it was first established on the island, and this may be the reason why it is also relatively distinct from the other morphs.

Since the *incarnata* morph typically combines red/purple flowers with unspotted leaves, the leaf markings that characterize *cruenta* must apparently be formed independently of the red/purple pigmentation of the flower (Bateman and Denholm, 1985), although it appears likely that the strongly contrasting coloration of the lip that is typical of *cruenta* has the same genetic background as leaf markings. As the character leaf spotting is due to an anthocyanin pigment, spotted plants may be either homozygous or heterozygous for genes synthesizing this pigment (or responsible for the expression of this pigment), meaning that these alleles should be dominant. If there was gene flow from *cruenta* into other morphs at mixed sites, one would expect to find some hybrid offspring that were heterozygous for the nuclear markers diagnostic of these morphs, but they would have the spotted leaves that characterize *cruenta*. Little evidence for such gene flow was found on Gotland, in spite of the fact that the *cruenta* populations were sampled from sites where they were growing mixed with unspotted varieties carrying other alleles. Thus, it appears that *cruenta* on Gotland is homozygous for the genes responsible for leaf spotting. However, this inference is not consistent with the observation that typical *cruenta* plants are sometimes found in mixed populations with unspotted plants of the same general morphology on some sites on Gotland (Hedrén, 2001*b*; Ingmarsson and Johansson, 2005). Further genetic analysis of such populations is required.

Nordic botanists such as Hylander (1966) and Nilsson (1991) commented that the *cruenta* morph in northern Scandinavia differs from that in southern Scandinavia, and a corresponding genetic differentiation has been observed at an esterase locus (Hedrén, 1996). The present data support the hypothesis that northern and southern *cruenta* are not closely related, but this does not mean that they are distinct.

The northern form is often found in mixed populations with unspotted plants of the same general morphology (Hylander, 1966; Nilsson, 1991; Danielsson, 1994). Accordingly, some authors accept a northern taxon *cruenta* (at the level of subspecies or variety) that is restricted to calcareous or rich fens in the mountain chain but polymorphic for leaf spotting (Hylander, 1966; Nilsson, 1991). Others regard the spotted and unspotted morphs as separate taxa (Mascher, 1990).

In the present analyses, the *cruenta* and *incarnata* morphs were distinguished according to the presence of spots, but the results are clearly in agreement with opinions expressed by Hylander and Nilsson. At Mickelsmyran (Fig. 5) and Svansele in northern Sweden where *cruenta* and *incarnata* morphs formed mixed populations, there was virtually no differentiation between morphs and almost complete overlap in both genetic markers and gene frequencies. The presence of leaf spotting in these populations appears to be of no systematic significance, and the character can be interpreted as a simple polymorphism.

The status of spotted forms from other parts of the distribution area is also debated (Bateman and Denholm, 1985; Haggar, 2004*b*). Spotted plants of the *D. incarnata* complex are known from, for example, south-eastern Turkey, the Alps, Scotland and Ireland (Renz and Taubenheim, 1984; Bateman and Denholm, 1985; Buttler, 1991; Haggar, 2004*b*). There have been few genetic data from these areas at present, but it has already been observed that a population of *cruenta* from Switzerland was characterized by a plastid haplotype that is absent from members of the *D. incarnata* complex examined from the Baltic region (Hedrén, 2009), which indicates that spotted plants from different regions of Europe may not be related. In some areas there are also mixed populations of spotted and unspotted plants that appear to be homogeneous in characters other than leaf spotting (Bateman and Denholm, 1985).

It is concluded that leaf spotting is a poor taxonomic character in the *D. incarnata* complex. Populations of spotted plants from different parts of Europe are not necessarily

more closely related to each other than to unspotted populations of the *D. incarnata* complex in general. Leaf spotting may have a systematic value in the circumscription of some regional variants of *D. incarnata s.l.* such as the Gotland populations, but in other regions genetic divergence patterns may be better described by other morphological characters.

incarnata

Clearly, the most variable morph for genetic markers is *incarnata*, and it contained all markers encountered in the other morphs. It was also found that populations of *incarnata* were often divergent from each other and maintained comparatively high levels of diversity at microsatellite loci within populations.

As genetic variation in *incarnata* appeared to be more-or-less continuous, the *incarnata* morph was not subdivided into subgroups. However, material corresponding to *incarnata* is sometimes split into several taxa. Hylander (1966) separated tall, sturdy plants with broad leaves, large bracts and many-flowered inflorescences as var. *latissima* and less robust plants with shorter bracts and fewer flowers as var. *incarnata*. In the present study, some degree of morphological differentiation of the *incarnata* populations that was in agreement with this subdivision was found. Thus, material from Storsund, Lojsthajd and Hoburgsmyr, possibly conforming to var. *incarnata*, was positioned in the left half of the CVA plot based on morphometric data (Fig. 2), whereas material from Agbod, Lillmyr and Harudden, possibly conforming to var. *latissima*, was positioned in the right half. The same separation was seen in the PCO of microsatellite data, in which the former were positioned in the lower part of the plot and the latter in the upper part of the plot (Fig. 7). The resulting patterns from plastid data (Fig. 6) and habitat data (Figs 3 and 4) were more ambiguous, but did not contradict separation into these two varieties. However, additional analyses are needed to confirm the suggested subdivison of *incarnata*. In both morphology and associated vegetation there was overlap between individual samples, few populations were studied and few samples were analysed from each population. The initial patterns may well change with the addition of more samples.

Small, late-flowering forms of *incarnata* with narrow leaves and dark purplish flowers are sometimes separated as var. *serotina* (Mossberg and Nilsson, 1977; Mossberg and Lundqvist, 1994). Such populations appear to be particularly common on the nearby island of Öland (Mossberg and Lundqvist, 1994). The population from Muskmyr, Gotland, clearly fitted this description, but it was only analysed for genetic markers. This population was somewhat differentiated in plastid haplotypes, but less distinctly so in nuclear microsatellites. Additional samples are clearly needed to clarify the status of var. *serotina*. The variety has been compared with the western *D. incarnata* subsp. *pulchella* (Haggar, 2003*a*, *b*), but the latter clearly differs in containing high frequencies of plastid haplotypes unknown in Baltic *D. incarnata s.l.* (Hedrén, 2009).

Populations of *incarnata* from northern Scandinavia can be characterized by relatively short and narrow, blunt leaves that just reach the base of the inflorescence; they have been separated as var. *borealis* (Hylander, 1966; Mossberg and Nilsson, 1977; Nilsson, 1991). This taxon is sometimes circumscribed to include spotted plants, and populations may be polymorphic for this character. However, it is also separated from the northern form of *cruenta* in its slender growth habit and not being restricted to calcareous habitats (Hylander, 1966; Mossberg and Nilsson, 1977; Nilsson, 1991). We are not convinced that these two taxa could be separated and suggest that more detailed analyses should be performed to clarify this issue.

Populations of *incarnata* could be variable in morphology and may express various flower colours ranging from almost white, pale pink, flesh-coloured, purple to deep lilac purple. For instance, several sites contained a mixture of flesh-coloured and purplish plants, which according to some authors may correspond to var. *incarnata* and var. *latissima*, respectively, and some other sites contained a distinct pale pink form sometimes separated as var. *lilacina* (Mossberg and Lundqvist, 1994). In our experience these colour forms could be distinct and constant within populations, which suggests that *incarnata* itself may be a polymorphic taxon composed of different subgroups with little intermixing. However, although inbreeding coefficients were comparable to those of other morphs, most populations contained a mixture of multilocus genotypes that combined alleles in all possible ways. Only at two sites, Myggkärret and Moskuvaara, did the local populations segregate into genotypes fixed for different alleles at all three microsatellite loci. However, sample sizes from these sites were small. Further sampling may disclose additional genotypes that combine genes from the genotypes observed so far. On the other hand, particularly large sample sizes of *incarnata* from the sites polymorphic for flower colour were not available for the present study, and it is still possible that further sampling from such sites may reveal some degree of differentiation between contrasting colour forms.

Factors responsible for maintenance of colour polymorphism in D. incarnata

Except for mixed populations of *incarnata* and *cruenta* in the north, it was found that morphs were genetically isolated from each other at sites where they were growing in sympatry. There was also some differentiation in morphology between morphs within sites, which was repeated between sites. Apparently, some form of isolation mechanism operates within sites to keep the morphs apart. The mechanism may be directly linked to colour, or colour is an easily observable trait linked to other characters that are under selection.

Inbreeding coefficients of approx. 0·6 were found in most morphs and loci, indicating that the morphs are partly inbred. However, it is unlikely that any of the morphs is reproducing by selfing, as *D. incarnata* has all the signs of a chasmogamous species and is known to be pollinated by inexperienced bumble-bee workers (Daumann, 1941; cited in Nilsson, 1980). Accordingly, it is suggested that the species is normally cross-pollinated, but that it is either affected by geitonogamy or pollination within family groups. When pollinaria are detached from flowers by the bumble-bee, the stipes would slowly bend such that the massulae would be deposited

on the stigma of the next flower visited. Bending normally takes longer than the time spent by a bumble-bee on an individual inflorescence, and geitonogamy should thus be avoided. Pollination within family groups seems to be the most plausible explanation for the high inbreeding coefficients. Such pollination may occur if (*a*) pollinators do not move long distances between plants, and (*b*) plant relatedness is correlated with physical proximity. The morphs of *D. incarnata* are often clumped in an apparently non-random fashion within sites (Rosvall and Pettersson, 1951), which supports the latter hypothesis. No signs of habitat specialization were observed that could explain such a clumped distribution pattern (Figs 3 and 4). However, because *D. incarnata* is pollinated by a deceit mechanism, fruit set may be irregular, and it should be investigated whether plants within clumps may be siblings emanating from a single successful pollination event within a local area in a particular year.

Although inbreeding may partly explain the constancy of morphs within populations, inbreeding is incomplete, so other factors must also contribute. Vallius *et al.* (2004) found significant differences in fruit set between morphs in some of their study sites and suggested that morphs could be pollinated by different pollinators. The significant differences in spur width found by us (Table 1) agree with their observations. Flower colour should thus be part of attraction, and differences in size of floral characters could be seen as adaptations to different groups of pollinators. In a more recent study, Vallius *et al.* (2008) showed that pollen is frequently transferred between yellow-flowered *ochroleuca* and purple-flowered *incarnata*, but that *ochroleuca* more often serves as pollen donor than recipient. This observation could help to explain why *ochroleuca* is less variable than *incarnata* because it would not receive foreign alleles. Haggar (2005*b*) reported that crosses between yellow-flowered and purple-flowered morphs invariably result in purple-flowered offspring, which means that offspring from crosses between morphs would be identified as *incarnata*. However, some yellow-flowered individuals would be expected among backcrosses or later-generation individuals originating from such hybrids. These plants would be identified as *ochroleuca*, but carry some atypical alleles at diagnostic loci. It seems likely that a few *ochroleuca* plants studied here have such a background, for instance some of the plants at Harudden (Figs 5–7).

It was assumed that the composition of species surrounding these *D. incarnata* plants should be indicative of as yet unknown ecological factors affecting the distribution of morphs (i.e. habitat preference). However, no clear sign of habitat separation between morphs was observed within sites. It may still be speculated that such factors exist, but that they would need further study to be identified with confidence. For instance, it may be hypothesized that morphs are dependent on particular strains of mycorrhiza-forming fungi that are distributed in a non-random fashion not directly correlated with the vegetation. Also, only species composition near the sampled plants was studied. Such data may primarily reflect soil factors of importance for the distribution of morphs. However, if morphs are pollinated by different groups of pollinators, the distribution of morphs may also be correlated with the distribution of food plants on which pollinators are dependent (magnet species hypothesis, remote habitats hypothesis; cf. Lammi and Kuitunen, 1995). Such plants may grow at distances of several metres from the deceptive orchids, suggesting that potential food plants should be identified and mapped in future studies.

Another possible factor leading to isolation is phenological separation. However, all morphs occurring at a site were studied within the same period of 1–2 d, and there was a high degree of overlap in flowering period between morphs at the study sites. Thus, phenology alone could also not explain constancy of morphs within sites. Our best hypothesis is that a combination of factors is responsible for maintaining reproductive isolation of morphs within sites. The strength of these factors may vary over time independently of site, with the effect that patterns of gene flow could also differ among sites. It is not known for how long morphs could remain distinct from each other, but considering the wide distribution of the apparently coherent var. *ochroleuca*, some morphs could perhaps be traced back at least to the last glaciation, when they could have survived in separate refugia and become fixed for certain combinations of genetic markers.

All three possible intermediates between *incarnata*, *cruenta* and *ochroleuca* morphs are known from natural sites (Mossberg and Lundqvist, 1994). Such intermediates are often interpreted as hybrids, and their relative rarity has been taken as evidence that the typical morphs constitute separate species. However, there may be alternative explanations for the appearence of intermediate morphs. Bateman and Denholm (1985) speculated that yellow anthoxanthin is invariably present in *D. incarnata*, but the pigment may be masked by anthocyanin pigments in red/purplish morphs. Apparently, plants lacking anthocyanins express different intensity of the yellow anthoxanthin pigment, which is part of the reason why the yellow forms have been subdivided into different taxa. Anthocyanin-containing morphs also vary in pigment intensity, which must be at least partially genetically determined, as shown by the fact that colour variants remain distinct in mixed populations. Furthermore, the various floral pigments may be similarly synthesized, which may also be the reason why red/purplish morphs usually express less yellow pigment than the yellow morphs (Haggar, 2003*a*). Plants with strong expression of both red and yellow pigments may then appear on rare occasions and be sometimes found in large populations that appear to be constant and isolated from typical *ochroleuca* and *incarnata* morphs (Mossberg and Lundqvist, 1994). Such populations may have a history of gene flow between morphs of different pigmentation, but they appear as independent entities just like any other population. On the other hand, the intermediates analysed from Kurevere were genetically similar to *ochroleuca*, but different from *incarnata* at the same site, and are best interpreted as an introgressed form of *ochroleuca*. Larger sample sizes would be needed to clarify the variation observed at this site.

Plants with pale spots on the leaves are often interpreted as hybrids between *cruenta* and unspotted morphs (Mossberg and Lundqvist, 1994). A few such plants were examined together with *incarnata* and *cruenta* from Oulu, Finland. However, there was little variation in genetic markers at this site, and it was not possible to confirm that these plants were hybrids between the more typical forms. However, at sites where gene flow could be demonstrated between the *cruenta* and

incarnata morphs, all plants either had intense spots or no spots at all, which shows that hybrids do not need to express an intermediate state of faint spots. Apparently, there must be genes regulating the intensity of leaf spots and no reason can be seen why plants with faint spots must necessarily be interpreted as hybrids. Controlled crosses would be valuable to understand how leaf spotting and intensity are genetically determined.

Taxonomy

The yellow-flowered *ochroleuca* is easily identified in Scandinavia and constitutes a distinct group with little gene flow to other morphs. However, no genetic markers were found that could be used to separate this group, and it obviously constitutes part of the otherwise red/purple-flowered *D. incarnata s.l.* Although it may be larger than the average *D. incarnata s.l.* in some morphological characters, there is still a high degree of overlap with anthocyanin-containing forms (Table 2). Furthermore, the yellow flower is also not a unique character, as plants with creamy yellow flowers may appear within apparently unrelated members of *D. incarnata*, most notably the western European subsp. *pulchella* (Bateman and Denholm, 1985). Genuine *ochroleuca* is confined to calcareous fens, where it usually forms mixed populations with anthocyanin-containing forms. Combining this information, we consider that *ochroleuca* is best recognized as a variety of *D. incarnata s.l.* To be recognized at the level of subspecies, it would be required to appear in areas outside the range of other *D. incarnata s.l.* forms or in habitats where red/purple-flowered forms did not occur (cf. Jonsell, 2004). It would also be expected that it would carry some unique genetic markers. Var. *ochrolueca* should be circumscribed such that yellow-flowered plants belonging to other genetically circumscribed subgroups within the *D. incarnata s.l.* complex are excluded; consequently, further studies should be performed to describe in detail the morphological range of variation and distribution of var. *ochroleuca*.

Although the spotted *cruenta* morph constitutes a separate coherent group that is largely isolated from other forms of *D. incarnata* on the island of Gotland, it is less distinct on the surrounding mainland areas, and it merges completely with unspotted *D. incarnata* in the northern sites. Lumping spotted plants as a single taxon within *D. incarnata* must be regarded as highly provisional. Ultimately, it would be better to subdivide the *D. incarnata* complex according to other characters that better reflect underlying patterns of genetic differentiation and to accept that any proportion of spotted plants may be found in these segregates. If there was still reason to distinguish the spotted morph as a separate taxon, it could not be recognized as anything but a form of *D. incarnata*, forma *cruenta*. The epithet '*cruenta*' is apparently associated with the northern population (Müller, 1782), and it may be necessary to find other names for the southern forms of *D. incarnata s.l.* with spotted leaves.

Conclusions

Sympatric morphs of the diploid marsh-orchid *D. incarnata s.l.* are mostly genetically isolated from each other, but gene flow between morphs is extensive at certain sites. Reproductive isolation is partly explained by high levels of inbreeding. Accordingly, colour polymorphism within *D. incarnata* is not comparable to that in *D. sambucina* and cannot be explained by adaptation to increased pollination efficiency. Sympatric morphs do not appear to differ in habitat preferences within sites.

The yellow-flowered morph distributed in the Baltic area constitutes a genetically well-defined and highly homozygous sublineage within *D. incarnata s.l.* However, it overlaps with other forms of *D. incarnata* in morphological characters, and it contains only a subset of the genetic markers found in purple-flowered morphs. Its inability to produce anthocyanins is probably due to a recessive allele that appears in homozygous form. It is best treated as a variety, *D. incarnata* var. *ochroleuca* (Boll) Hylander.

The '*cruenta*' morph with spotted leaves is genetically heterogeneous in the Baltic area, although it forms a well-defined homozygous group on Gotland. However, gene flow between spotted and other morphs is extensive at many sites in mainland Scandinavia, and there is no detectable genetic differentiation in the north. Leaf spotting is a prominent feature of some plants and populations, but it should be determined whether the *D. incarnata* complex is better subdivided by other morphological characters that describe underlying patterns of genetic differentiation.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following tables. Table S1: Origin of material studied and numbers of samples included in different data sets. Table S2: Haplotype counts for different morphs of *D. incarnata s.l.* Table S3: Microsatellite allele frequencies for different morphs. Table S4: Pairwise F_{ST} values comparing morphs at sites with mixed populations. Table S5: Genetic diversity statistics for different morphs of *D. incarnata s.l.*

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