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Taxonomic complexity, conservation and recurrent origins of self-pollination in *Epipactis* (Orchidaceae)

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ABSTRACT

The recent post-glacial colonisation of Britain has given little time for the evolution of endemic plant species. The few British endemic species that have been recognised tend to occur in taxonomically complex groups that possess mechanisms promoting rapid diversification. Such taxonomic complexity leads to problems for plant conservation because a species has to be circumscribed and recognised before its distribution, status and threats can be established. One classic example of the challenges for conservation in taxonomically complex groups is the British endemic orchid *Epipactis youngiana*. This species is afforded full legal conservation protection but is one of a large number of taxonomically difficult species recently recognised in the genus; it is difficult to distinguish from the more common *E. helleborine,* and its species status has been questioned. We have used a combination of genetic markers (allozymes, chloroplast microsatellites and RFLPs) from a large sample set to establish the taxonomic and conservation status of *E. youngiana* and to place it in the wider context of patterns of breeding system variation and taxon differentiation in the genus. Our data provide evidence that recurrent breeding system transitions between cross-pollination and self-pollination are an important mechanism for diversification in the genus, and there are numerous genetically different taxa that are homozygous and uniform for different subsets of allelic diversity found in allogamous species. *E. youngiana* is the one major exception to this pattern. It has a floral morphology consistent with self-pollination, but has not achieved reproductive isolation from *E. helleborine.* The potential mechanisms underlying the recurrent evolution of self-pollination in *Epipactis* are discussed, as is the need for developing conservation strategies that reflect dynamic diversification in the genus, rather than the current heavily typological (is it distinct or not?) species-based approach.

Keywords: allozymes, cpSSRs, endemic, *Epipactis*, genetic markers, RFLPs, self-pollination, taxonomic complexity.

INTRODUCTION

The British flora has benefited from a long history of floristic study (Clapham *et al.* 1989; Stace 1991, 1997), two major mapping exercises based on over 9 million records (Preston *et al.* 2002), various accounts of species of conservation importance (Stewart *et al.* 1994; Wigginton 1999), detailed biological floras of individual species, a comprehensive evaluation of chromosome number variation, a vice comital flora (Stace *et al.* 2003), and an overview of hybridisation in the flora (Stace 1975). This enormous resource base provides an excellent foundation for taxonomic, ecological, evolutionary and conservation research.

Building on this resource base, the delimitation and identification of many species in the British flora is now considered routine. The recognition and description of clear-cut morphological differences between species has effectively led to a consensus on the most appropriate taxonomic treatment for a large number of genera. There are, however, some persistently troublesome groups of plants that defy attempts to achieve a widely accepted taxonomic treatment. These taxonomically complex groups contain a large proportion of Britain's endemic higher plant species (e.g. *Sorbus*, *Epipactis*, *Euphrasia*, *Ulmus*, *Hieracium*, *Taraxacum*, *Rubus*, *Limonium*).

Taxonomic complexities which affect the most appropriate delimitation of species cause problems for conservation. Making an assessment of the distribution and conservation status of any species first requires that the species can be delimited and recognised. If the unit to be conserved is in a taxonomically complex group, there can be major problems in assessing threats, devising conservation strategies and monitoring their success. As taxonomically complex groups account for almost half of the species on the UK Biodiversity Action Plan 'short list', there are real difficulties in implementing conservation actions for these species (Hollingsworth 2003).

The association between endemism and taxonomic complexity in the British flora is at least partly attributable to recent ice ages. The vast majority of species in the British flora have achieved their current distributions via recent post-glacial colonisation. The limited time period since colonisation (within the last 12,000 years) has given little time for the evolution of endemic species. Those endemic species that are recognised within the British flora typically show a mechanism, or combination of mechanisms that promote rapid biological diversification: these include polyploidy, hybridisation, self-pollination, agamospermy and clonal growth (Stace 1989, 1997). In a recent review of the history of the North Atlantic biota, Brochmann *et al*. (2003) noted that among the 43 hardy vascular plant species accepted as being endemic to the region, there was not a single sexual diploid indicative of long-term evolution. Similarly, mechanisms which promote rapid diversification have been central to the evolution of the endemic element in the British flora; this rapid diversification also results in taxonomic complexity and knock-on problems for conservation.

MECHANISMS UNDERLYING TAXONOMIC COMPLEXITY

One mechanism that promotes rapid diversification and also leads to taxonomic complexity is a change in breeding system. The evolution of self-pollination (autogamy) from outcrossing (allogamy) is one of the most frequent evolutionary transitions in flowering plants (Stebbins 1974). This change can lead to a neospecies achieving rapid reproductive isolation from its progenitor (Levin 2000), and can generate morphological differences between selfers and their allogamous progenitors due to an increased level of homozygosity. Morpho-

logical characters that include phenotypes encoded by recessive alleles can be selected for, as self-pollination can lead to the fixation of advantageous recessive mutations (Charlesworth 1992, Levin 2000). As the age of the self-pollinating lineage increases, novel mutations can result in further morphological differences.

Self-pollinating species typically show higher levels of variation between populations than do outcrossing species (Hamrick & Godt 1996, Nybom 2004). This is attributable to reduced opportunities for gene flow and also potentially an association between self-pollination and short-lived and/or colonist populations, which show rapid differentiation as a consequence of repeated founder events and genetic drift. There is thus the opportunity for morphological divergence between populations within a selfing species. In addition, there is the possibility that outcrossing progenitor species may generate multiple independent selfing lineages of morphologically similar appearance. Both of these scenarios yield sets of populations separated only by subtle morphological differences which can result in difficulties in the allocation of the most appropriate taxonomic rank and affiliation.

TAXONOMIC COMPLEXITY IN *EPIPACTIS*

The genus *Epipactis* is a classic example of a plant group in which active diversification and breeding system transitions appear to have led to taxonomic complexity and subsequent problems for conservation. The genus consists of between 25 and 60 species. They exhibit a predominantly Eurasian distribution with outlying species in North America and North Africa (Bateman *et al.* 2005). The taxonomic complexity in the genus is reflected in the uncertainty regarding species numbers, and for instance the number of European species accepted by Delforge changed from 36 to 58 in seven years (cf. Delforge 1995, 2001), whereas Sundermann (1970) recognised only 14 species.
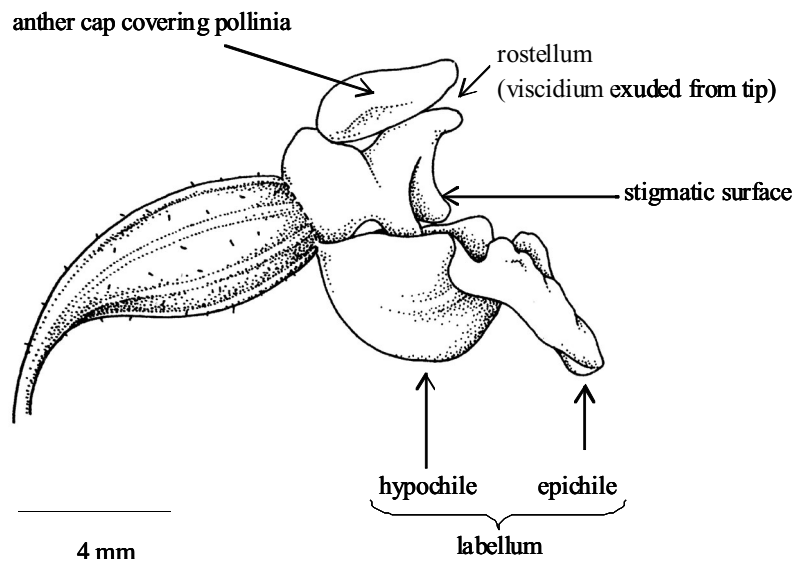

FIGURE 1. Floral morphology of an allogamous *Epipactis* flower. Drawing by Mary Mendum

The floral morphology of species in the genus varies considerably. The two major informal groups in the genus are the '*palustris*' group (rhizomatous clonal species, with an open concave hypochile) and the 'helleborine' group which are typically more vegetatively discrete and possess a more cupped hypochile. The 'helleborine' group (the subject of this paper) encompasses two contrasting floral morphologies. Many species, including the widespread *E. helleborine* have a well-developed rostellum that serves as a barrier between the male and female parts of the flower (Fig. 1). The viscidium is exuded from the tip of the rostellum (Richards 1982) and serves as a glue to bind the pollinia to visiting insects. The vast majority of taxonomic problems in the genus are centred around a large number of named taxa in which the viscidium and rostellum are strongly reduced or are less persistent. Any reduction in the rostellum diminishes the physical barrier between the male and female parts of the flower, while any reduction in the viscidium lowers the likelihood of the pollinia being transferred to flowers on a different plant. Instead, the pollinia can remain *in situ* and fall downward onto the stigmatic surface, resulting in self-pollination. A series of changes such as a reduction in flower size, a more pendulous habit, and reduction in pigmentation have been invoked as secondary modifications which further promote self-pollination (Richards 1982).

Taxa with a floral morphology consistent with self-pollination are typically of restricted distribution, and can show limited variation within populations but subtle and consistent morphological differences between populations. At the heart of taxonomic complexity in *Epipactis* is the extent to which populations with floral morphologies such as these reflect minor mutational variants of single self-pollinating lineages, or whether they reflect independent taxa resulting from iterative allogamy-to-autogamy transitions.

In Britain seven species were recognised by Stace (1997). Four of these have an outcrossing type of floral morphology: *E. palustris, E. atrorubens, E. helleborine* and *E. purpurata* and three have a floral morphology consistent with autogamy: *E. phyllanthes*, *E. leptochila* and *E. youngiana.* Molecular data have shown that within the variation encompassed by *E. leptochila* there are two clearly distinct taxa best treated separately as *E. dunensis* (T. Stephenson & T.A. Stephenson) Godfery and *E. leptochila* s.s. (Godfery) Godfery, and also a third lineage endemic to Lindisfarne (Squirrell *et al.* 2002) which has subsequently been named *E. sancta* (P. Delforge) P. Delforge (Delforge & Gévaudan 2002; Bateman 2006).

EPIPACTIS YOUNGIANA AS A CASE STUDY

One British *Epipactis* species that remains enigmatic is the high conservation profile endemic *E. youngiana* A. J. Richards & D. F. Porter. This species was first described in 1982 and was found growing on some mine spoil heaps in Northumberland (Richards & Porter 1982). It has subsequently been recorded from some similar habitats in Scotland, centred around Glasgow and Falkirk (Dickson *et al*. 2000). It has a floral morphology consistent with self-pollination; in particular its viscidium withers very rapidly. Its presence predominantly in anthropogenic sites has led to suggestions of a recent evolutionary origin stemming from either (a) divergence from *E. helleborine* or (b) a hybrid origin between *E. helleborine* and *E. dunensis*. Self-pollination is considered to have led to rapid reproductive isolation from other *Epipactis* species. As an endemic British orchid species (one of very few endemic plant species recognised in Britain), *E. youngiana* has been afforded full conservation status under Schedule 8 of the Wildlife and Countryside Act (1981). The species also has a Species Action Plan as part of the UK Biodiversity Action plans (UK Biodiversity Group 1995), and associated allocation of conservation resources. However, populations of *E. youngiana* occur

sympatrically with plants of *E. helleborine* (and also at some sites with either *E. phyllanthes* or plants resembling *E. dunensis*). The mixture of taxa growing together and the presence of plants of intermediate morphologies has caused difficulties in identification of material and uncertainty over the taxonomic status of *E. youngiana.* Thus *E. youngiana* receives formal conservation protection, but conservation actions are extremely difficult to implement due to taxonomic uncertainty.

To provide some insights into the taxonomic distinctness of *E. youngiana*, and more generally into the evolutionary processes underlying taxonomic complexity in *Epipactis,* we have carried out large-scale genetic surveys using a combination of sequencing of the internal transcribed spacers (ITS) of nuclear ribosomal DNA and the chloroplast *trn*L intron, allozyme analyses, and screening of RFLP variation and mononucleotide length variation in the *trn*L intron. The full details of this work will be published elsewhere; our goals here are to explore the taxonomic status of *E. youngiana* and to infer the most appropriate conservation treatment of this putative species.

MATERIALS AND METHODS

A total of 2828 individuals of 164 populations of 23 named species in the 'helleborine' group have been examined using a range of genetic markers (Table 1). The species were selected to encompass a range of putative species including several with a floral morphology consistent with self-pollination and several with a floral morphology consistent with outcrossing. The sampling of *E. youngiana* involved two populations from Northumberland and two from Scotland. Sympatric plants of *E. helleborine* (and *E. dunensis* and *E. phyllanthes* where present) were also sampled from these sites.

These samples have been examined using a range of molecular approaches (allozyme electrophoresis, chloroplast and ITS sequencing, chloroplast microsatellites and RFLPs). Between nine and ten allozyme loci were screened, eight of which were polymorphic. At the polymorphic loci there were between 2-4 alleles. The chloroplast data set considered here consists of information from the *trn*L intron. Based on sequences of 86 individuals, two variable markers were selected for widescale screening. Firstly, we screened for the presence or absence of a 10 bp duplication in the *trn*L intron via RFLPs, and secondly we examined length polymorphism in a chloroplast microsatellite that consisted of a mononucleotide poly-A repeat of between $(A)_9$ and $(A)_{13}$ bp. Sequencing of the nuclear ITS regions from 63 individuals revealed phylogenetic resolution at deeper levels within the genus, but within the taxonomically complex *E. helleborine* group little variation was detected. Consequently information from ITS is not discussed further in this paper.

RESULTS AND DISCUSSION

PATTERNS OF ALLOZYME VARIATION

The *Epipactis* species examined here show some marked differences in the amounts and organisation of genetic variation among individuals and populations (Table 2). With the exception of plants from the *E. youngiana* sites (which are discussed later), all of the species examined with a floral morphology consistent with self-pollination consisted of homozygous and genetically uniform lines. No heterozygous individuals were detected in the total dataset

TABLE 1. *EPIPACTIS* TAXA SAMPLED, DISTRIBUTIONS, FLORAL MORPHOLOGY, SAMPLE LOCATIONS, AND SAMPLE SIZES FOR DIFFERENT MOLECULAR ASSAYS. Floral morphology designation and geographical range is based on field observations and Delforge (2001). n = sample size for different assays.

Species	Floral morphology	Distribution	Countries sampled	n pops	n Allo-zymes	n ITS	n trnL	n duplication	n cp SSR
Epipactis albensis	Autogamous	Germany/Austria	Austria	1	2	1	1	1	1
Epipactis atrorubens	Allogamous	Eurasia	England, Scotland, Wales, France, Norway	9	162	1	1	26	48
Epipactis campeadorii	Autogamous	Spain	Spain	1	5	1	1	5	1
Epipactis confusa	Autogamous	Scandinavia/Germany	Denmark	1	20	1	1	18	11
Epipactis distans	Allogamous	European Alpine	France	1	20	2	2	6	16
Epipactis dunensis	Autogamous	UK	England, Scotland, Wales	8	171	15	14	130	108
Epipactis fibri	Autogamous	France	France	1	21	1	1	18	1
Epipactis futakii	Autogamous	Slovakia	Slovakia	1	3	1	1	2	1
Epipactis helleborine	Allogamous	Eurasia (introduced N. America)	England, Scotland, Denmark, France, Belgium, Canada, Switzerland, Germany	47	1117	10	10	865	746
Epipactis leptochila	Autogamous	Europe	England, France, Germany	18	234	5	5	162	131
Epipactis microphylla	Autogamous	Eurocaucasia	France, Switzerland, Germany	7	72	1	1	53	9
Epipactis muelleri	Autogamous	Mid-Europe	France, Germany	7	77	4	4	62	72
Epipactis parviflora	Allogamous	Spain/France	Spain, France	2	10	1	1	8	0
Epipactis peitzii	Autogamous	Germany	Germany	1	3	1	1	6	1
Epipactis phyllanthes	Autogamous	Atlantic Europe	England, France, Switzerland, Germany, Ireland, Spain	27	430	13	13	307	157
Epipactis placentina	Autogamous	Central Europe	France, Slovakia	2	1	2	2	3	17
Epipactis provincialis	Autogamous	France	France	1	22	3	3	22	22
Epipactis pseudopurpurata	Autogamous	Eastern Europe	Slovakia	1	4	0	0	1	0
Epipactis purpurata	Allogamous	Temperate Europe	England, Denmark, France, Germany	18	207	3	3	23	79
Epipactis rhodanensis	Autogamous	France/Switzerland	France, Switzerland	4	96	4	4	67	33
Epipactis sancta	Autogamous	England	England	1	26	4	4	26	16
Epipactis tremolsii	Allogamous	Mediterranean	France	1	16	0	0	16	0
Epipactis youngiana	Autogamous	UK	England, Scotland	4	89	2	2	64	92

for the examined loci. Although this uniformity precludes formal breeding system estimates, it is at the very least strongly congruent with self-pollination leading to homozygosity and uniformity. In contrast, all of the species with a floral morphology consistent with outcrossing exhibited at least some genetic variation and some heterozygosity (Table 2). The inbreeding coefficient in all of these species was not significantly different from zero, and thus was consistent with random mating. The one minor exception was *E. atrorubens*; despite heterozygous individuals being detected, there was a small but significant deficit of heterozygotes compared with Hardy-Weinberg expectations (F_{IS} = 0.180; Table 3). It is not clear why this species should show some slight heterozygote deficit when the other allogamous species do not. Possible explanations are that these populations experience higher levels of geitonogamy or lower pollinator availability than the other species. However, aside from this minor exception, there is a marked correlation between floral morphology and patterns of genetic variation, and these results confirm the importance of minor modifications in floral morphology as determinants of the organisation of genetic variation (Richards 1982).

However, floral morphology is not the only determinant of patterns of allozyme variation in these species. Although there is homogeneity in the variation patterns within the putative self-pollinating taxa (all taxa show zero within-species variation for these loci), the situation is more varied among the allogams (Table 3). Levels of variation ranged from 11% to 56% of allozyme loci being variable within populations, and although all populations of most species showed some variation, in *E. purpurata* only 47% of sampled populations were variable (Table 3). This suggests that other factors, such as population size, plant size, demographic history and pollinator activity are also likely to be important determinants of the amounts and partitioning of genetic variation.

Considering the range of allelic variation between the species with outcrossing and selfing floral morphologies, for the most part the different selfing lineages are fixed for different character combinations that represent subsets of the alleles found in the allogams (Table 2). This pattern is consistent with multiple transitions from outcrossing to selfing, giving rise to a series of genetically and morphologically discrete lineages, rather than the taxonomic complexity in the genus stemming from a single outcrossing-selfing transition followed by mutational divergence. Thus the transition from allogamy to autogamy seems particularly labile in *Epipactis.* Of course, multiple origins of selfing and mutational divergence of lineages are not mutually exclusive and following independent origins there is the possibility for further divergence. Although this aspect of the work is beyond the scope of this paper and will be explored elsewhere, it is worth noting that there is evidence for some alleles present in autogams that have not been found in allogams, and also for between-population divergence in autogamous taxa (Table 2). For example, the inland populations of *E. dunensis* differ genetically from the coastal populations (Table 2; see also Squirrell *et al.* 2002) and there are further differences between populations of *E. microphylla* for the rapidly evolving cpSSRs locus (Table 2).

In summary, genetic variability and heterozygosity are found within putative outcrossing taxa (albeit with differences in absolute levels of diversity). In contrast, the autogamous lineages are united in their allozyme uniformity and complete lack of within-population variation. How does the British endemic *E. youngiana* match these patterns of variability, and does the evidence support the notion that it is a distinct, recently evolved species that has achieved reproductive isolation via the evolution of autogamy?

TABLE 2. THE DISTRIBUTION OF ALLOZYME AND CHLOROPLAST VARIANTS WITHIN EUROPEAN *EPIPACTIS* SPECIES. (See top of following page for details)

Epipactis species	Allozyme loci								Chloroplast (*trn*L)	
Autogams	mdh-1	mdh-2	idh-1	pgm	aat-1	pgi-1	pgd	idh-2	Duplication present	SSR no. repeats
confusa[1]	c	c	a	c	a	b	a	a	No	9
phyllanthes[1]	c	c	a	c	a	b	a	a	No	9
albensis	b	a	a	a	a	c	a		Yes	10
campeadorii[2]	b	c	a	b	a	c	a		No	10
dunensis (w coastal)	b	c	a	b	a	c	a		Yes	11
dunensis (inland)	b	c	a	b	a	c	a		Yes	10
fibri	b	b	a	a	c	a	a		Yes	10
futakii	b	b	a	a	a	c	a		No	9
leptochila	b	b	a	a	a	c	a		No	11
microphylla	a	d	a	a	a	b	a		No	12/13[3]
muelleri	a	b	b	a	b	b	a		No	10
peitzii	a	b	a	a	a	c	a		Yes	12
placentina	a	b	a	a	b	b	a		Yes	9
provincialis	b	b	a	a	a	a	a		No	11
pseudopurpurata	a	b	a	a	b	a	a		No	
rhodanensis	b	c	a	a	a	c	a		No	11
sancta[2]	b	c	a	b	a	c	a		No	10
Allogams										
atrorubens	ab	abc	acd	abc	abc	abc	a		No	9
distans	bc	b	a	a	a	c	a		No	11
helleborine	ab	abc	abc	ab	abcd	abc	ab		No & Yes	9/10/11
parviflora	a	ab	ad	a	a	ab	a		No	
purpurata	ab	bc	ab	ab	abc	abc	a		Yes	10/11
tremolsii	ab	abc	abd	ab	abc	abc	a		No	10
Settlingstones										
helleborine	ab	abc	a	ab	abc	abc	a		No & Yes	10/11
youngiana	ab	abc	a	ab	abc	abc	a		No & Yes	10/11
phyllanthes	a	c	a	c	a	b	a	a	No	9
Bardykes Bing										
helleborine	ab	bc	ab	ab	abc	abc	a		Yes	9/10
youngiana	ab	bc	ab	ab	abc	abc	a		No & Yes	9/10
Bothwell Castle										
helleborine	ab	bc	ab	ab	abc	abc	a		No & Yes	10/11
youngiana	ab	bc	ab	ab	ab	abc	a		No & Yes	9/10/11
dunenesis[4]	ab	bc	ab	ab	a	bc	a		Yes	10/11
Gosforth Park										
helleborine	ab	abc	a	ab	ab	abc	a		No & Yes	10/11
youngiana	ab	abc	a	ab	a	abc	a		No & Yes	10/11

Table 2 cont. The different letters represent the allelic variants found within each taxon. [1]Our genetic data were unable to distinguish between *E. confusa* and *E. phyllanthes*; the taxonomic implications of this will be discussed elsewhere. [2]The data presented here do not distinguish *E. sancta* and *E. campeadorii*[2] but these two species can be distinguished based on their ITS sequences (data not shown). [3]Different populations of *E. microphylla* are fixed for either 12 or 13 repeats at the cpSSR locus. [4]Plants with the morphology of *E. dunensis* at the *E. youngiana* sites in Scotland do not show the classic homozygous and uniform *E. dunensis* allozyme profile.

TABLE 3. WITHIN-POPULATION DIVERSITY MEASURES AND ESTIMATES OF THE INBREEDING COEFFI-CIENT IN ALLOGAMOUS EUROPEAN *EPIPACTIS* SPECIES BASED ON NINE ALLOZYME LOCI

Species	N pops	Mean n	P	A	F_{IS}	H_E	PP
E. atrorubens	9	17.8	29	1.37	0.180*	0.120	100
E. distans	1	19.0	11	1.11	0.390ns	0.038	100
E. parviflora	2	5.0	12	1.12	-0.148ns	0.056	100
E. purpurata	18	11.5	11	1.12	0.033ns	0.024	47
E. tremolsii	1	15.2	56	2.00	0.009ns	0.227	100
E. helleborine	47	23.5	56	1.81	0.003ns	0.231	100

N pops = number of populations; Mean n = mean sample size per population per locus; P = % polymorphic loci; A = mean number of alleles per locus; F_{IS} = global inbreeding coefficient; * = significantly different from zero $p < 0.05$, ns = not significant; H_E = gene diversity; PP = % of populations that are polymorphic.

TABLE 4. WITHIN-POPULATION DIVERSITY MEASURES AND ESTIMATES OF THE INBREEDING COEFFI-CIENT IN FOUR SYMPATRIC POPULATIONS OF *E. YOUNGIANA* AND *E. HELLEBORINE* IN BRITAIN BASED ON NINE ALLOZYME LOCI

Species	Region	Location	n	P	A	F_{IS}
E. helleborine	Northumberland	Settlingstones	30	56	1.89	0.110ns
	Northumberland	Gosforth Park	9	56	1.78	0.139ns
	Glasgow	Bardykes Bing	52	67	1.89	0.035ns
	Glasgow	Bothwell Castle	31	67	1.89	-0.059ns
E. youngiana	Northumberland	Settlingstones	36	56	1.89	0.036ns
	Northumberland	Gosforth Park	8	44	1.67	0.099ns
	Glasgow	Bardykes Bing	21	67	1.89	0.141*
	Glasgow	Bothwell Castle	24	67	1.78	-0.048ns

n = sample size, P = % polymorphic loci, A = mean number of alleles per locus, F_{IS} = inbreeding coefficient, * = significantly different from zero $p < 0.05$.

IS *E. YOUNGIANA* AUTOGAMOUS? (NO)

E. youngiana typically occurs in sympatry with *E. helleborine* and sometimes also with other *Epipactis* species. The mechanism proposed for the development of reproductive isolation and speciation is self-pollination. There is, however, no clear evidence that the plants ascribed to *E. youngiana* are undergoing self-pollination. Both our studies and those of Harris & Abbott (1997) have recovered high levels of heterozygosity in populations of this species. In three of the four populations examined here, the distribution of alleles among individuals is consistent with random sexual mating (Table 4). Only in the Bardykes Bing population was a (just) significant deviation from Hardy-Weinberg equilibrium detected ($p = 0.05$), and this was attributable to a very minor deficit of heterozygosity that might represent a sampling artefact (an allozyme survey of the same population by Harris & Abbott (1997) did not detect any significant deviation from Hardy-Weinberg expectations). If complete self-pollination was occurring, the rate of homozygosity should increase by 50% per generation. Even allowing for non-overlapping generations and a recent origin for the species, if self-pollination had been sufficiently extensive to lead to reproductive isolation from sympatric plants of *E. helleborine,* it would be expected to leave a much stronger signature on the partitioning of allelic variation within and among individuals.

IS *E. YOUNGIANA* DISTINCT? (NO)

If *E. youngiana* is a distinct cohesive species, we should expect allele frequencies in different populations of *E. youngiana* to be more similar to one another than to local populations of *E. helleborine.* However, this is not the pattern recovered from either the allozyme data or the cpDNA data. For both chloroplast and nuclear allozyme data, the allelic diversity in *E. youngiana* and *E. helleborine* shows greater similarities by site than by taxon (Figs 2, 3). Indeed, if one tests for random mating by pooling individuals of *E. helleborine* and *E. youngiana* at each site, at three of the four sites no significant deviation from random mating is detected among individuals *between* 'species' ($F_{IS} = 0.062$ Bardykes Bing, $F_{IS} = -0.036$ Bothwell Castle, $F_{IS} = 0.172$ Gosforth Park; all non-significant). In the fourth population (Settlingstones), pooling individuals between *E. helleborine* and *E. youngiana* does result in a statistically significant departure from random mating, but the deviation from panmixia is again minor ($F_{IS} = 0.091$, $p = 0.043$).

ARE POPULATIONS OF *E. YOUNGIANA* NORMAL POPULATIONS OF *E. HELLEBORINE* THAT HAVE BEEN TAXONOMICALLY OVER-SPLIT? (NO)

Even the briefest of visits to the populations of *E. youngiana* in both Northumberland and Scotland reveals a pattern of morphological diversity outwith the norm. The classic '*youngiana*' morphology is not a phenotype that is present in typical popula-tions of *E. helleborine*. There is undoubtedly something unusual about these popula-tions which contain atypical mixtures of floral morphologies, ranging from individuals whose floral morphology resembles autogamous plants, to those whose floral morphol-ogy resembles outcrossing plants. Given that our data suggests that self-pollination has
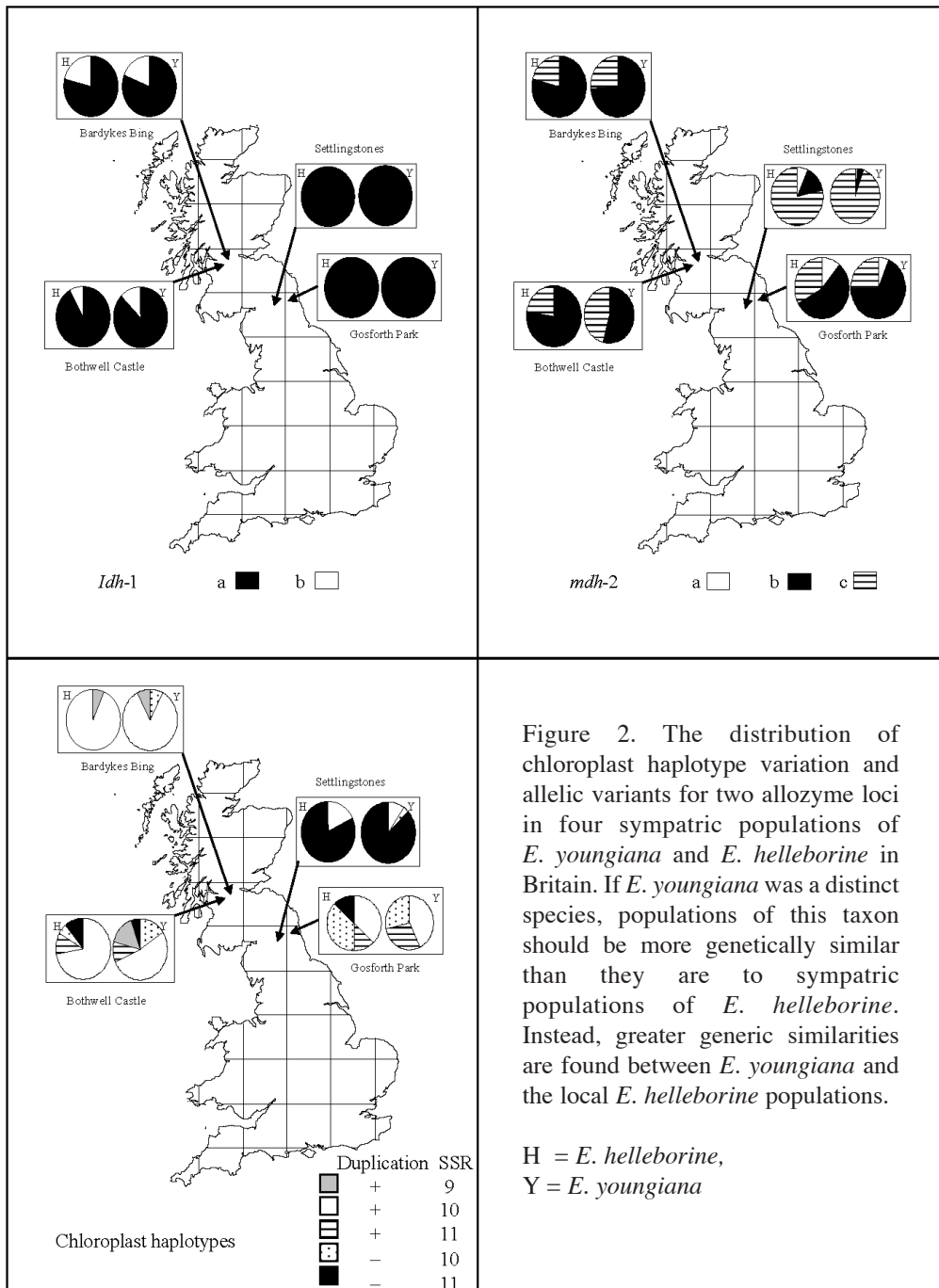
Figure 2. The distribution of chloroplast haplotype variation and allelic variants for two allozyme loci in four sympatric populations of *E. youngiana* and *E. helleborine* in Britain. If *E. youngiana* was a distinct species, populations of this taxon should be more genetically similar than they are to sympatric populations of *E. helleborine*. Instead, greater generic similarities are found between *E. youngiana* and the local *E. helleborine* populations.
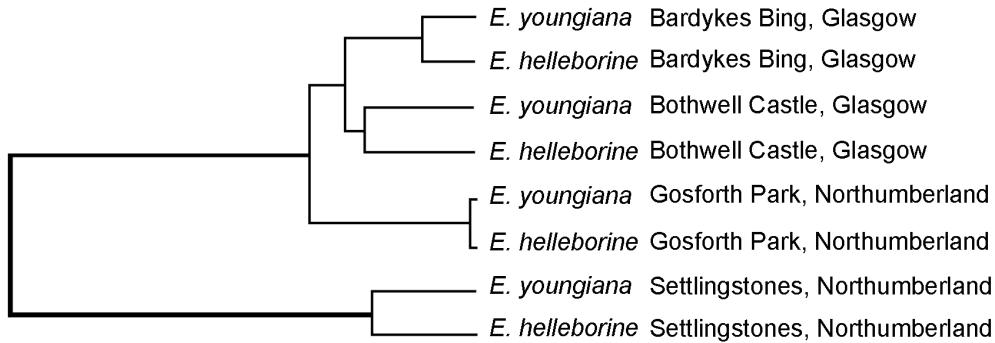
H = *E. helleborine*,
Y = *E. youngiana*

FIGURE 3. UPGMA clustering based on pairwise estimates of population differentiation (F_{ST}) for eight polymorphic allozyme loci from four sympatric populations of *E. youngiana* and *E. helleborine* in Britain. Populations of *E. youngiana* do not form a distinct cohesive genetic entity; instead, each population is more closely related to its local *E. helleborine* population.

arisen from outcrossing taxa on numerous occasions in *Epipactis*, perhaps it is not surprising to find populations in which there is a mixture of floral morphologies, even if there is as yet no clear divergence of taxa. It is possible that these polymorphic populations represent the type of population from which future autogamous lineages may originate.

The apparent absence of self-pollination in *E. youngiana*, despite the flowers having a selfing floral morphology, may reflect the presence of sympatric allogamous *E. helleborine* at these sites. Even if pollinia export is reduced in individuals with the *E. youngiana* morphology, these plants presumably can behave as functional females and receive pollinia import from neighbouring allogamous *E. helleborine* plants via visiting pollinators. To achieve reproductive isolation, plants with a selfing floral morphology may need to disperse to a site where no allogamous plants occur.

ARE THE *E. YOUNGIANA* POPULATIONS TYPICAL OF TAXONOMIC COMPLEXITY IN *EPIPACTIS*? (NO)

The populations of *E. youngiana* show a very different pattern of genetic variation from the consistent pattern seen in autogamous *Epipactis* species (all of which were homozygous and uniform for the allozyme loci considered here; Figure 4b). There is thus a clear difference between *E. youngiana* and the vast majority of other autogamous lineages recognised at the species level in *Epipactis* (Figs 4a, b; Table 2). The only close parallel we are aware of is *E. renzii* Robatsch, a taxon restricted to coastal dunes in Denmark. Based on allozyme electrophoresis, Pedersen & Ehlers (2000) concluded that it had arisen on multiple occasions from local populations of *E. helleborine* subsp. *neerlandica* (Verm.) Buttler. Like *E. young-iana*, *E. renzii* occurs sympatrically with populations of *E. helleborine* and at individual sites

the two putative taxa share the same alleles. Unlike *E. youngiana,* all three populations of *E. renzii* examined by Pedersen & Ehlers (2000) showed a strongly significant inbreeding coefficient ($F_{IS} = 0.486$, $F_{IS} = 0.832$, $F_{IS} = 1.0$, all $p < 0.001$). The authors concluded that the origin of self-pollination was recent and attributable to adaptation for reproductive assurance due to a short flowering season caused by water stress and early wilting of flowers. Based on the absence of extensive molecular and morphological divergence, Pedersen & Ehlers (2000) argued that *E. renzii* should be given varietal status rather than species status. This apparently intermediate phase represented by *E. renzii,* between allogamous populations of *E. helleborine* and homozygous uniform autogamous segregates is interesting, and a parallel taxonomic treatment for *E. youngiana* (varietal status) may be appropriate. This approach was adopted informally by Lang (2004) and Bateman (2006), and a formal transfer was performed by Kreutz (2004).

WHAT IS THE MECHANISM FOR THE RECURRENT EVOLUTION OF A SELFING FLORAL MORPHOLOGY WITHIN *EPIPACTIS*?

This assessment of patterns of genetic diversity in *Epipactis* has demonstrated the frequency of transitions in floral morphology from outcrossing to selfing types. Given the lability of this switch, it is worth evaluating the evolutionary processes hypothesised to underlie these transitions. Selection for reproductive assurance under conditions of poor pollinator availability and/or a short flowering season provide some explanations for the advantages of self-pollination, but not the mechanism underlying the transitions. Pedersen & Ehlers (2000) argued that recurrent mutations may be responsible and noted that a mutation resulting in paedomorphosis via an arrested development of the rostellum may be the key step required in the evolution of autogamy. Whilst we consider this hypothesis plausible, we also believe that an alternative hypothesis is worth considering: that hybridisation between autogamous and allogamous *Epipactis* species provides a mechanism for the transfer of genes encoding the selfing-floral morphology into novel heterozygous backgrounds from which new selfing lineages with new character combinations can arise. Under this scenario the evolution of autogamy could in some cases be considered as a cyclical process more akin to an 'evolutionary detour' than the 'evolutionary dead-end' proposed by Stebbins (1957). Selfing lineages evolve and differentiate, and at some future point occur in sympatry with allogamous taxa, hybridise and result in the production of further selfing lineages.

   As yet there is little evidence to support or refute this 'evolutionary detour' hypothesis. Evidence from mixed populations of autogamous taxa (e.g. mixed populations of *E. phyllanthes* and *E. dunensis* on the west coast of England) suggests that these taxa co-exist at high densities without undergoing any gene exchange (Fig. 5). However, where we have sampled populations of putative autogams occurring in sympatry with allogamous taxa in the complex *E. youngiana* sites, the pattern changes somewhat:

1 Plants with a morphology consistent with *E. dunensis* occur in the same complex sites that contain Scottish populations of *E. youngiana* and *E. helleborine*. At these sites, plants with the morphology of *E. dunensis* are genetically variable and heterozygous for the same alleles found in local populations of *E. helleborine* and *E. youngiana* (Fig. 4, Table 2), and plants of intermediate morphologies occur. This is a marked contrast with
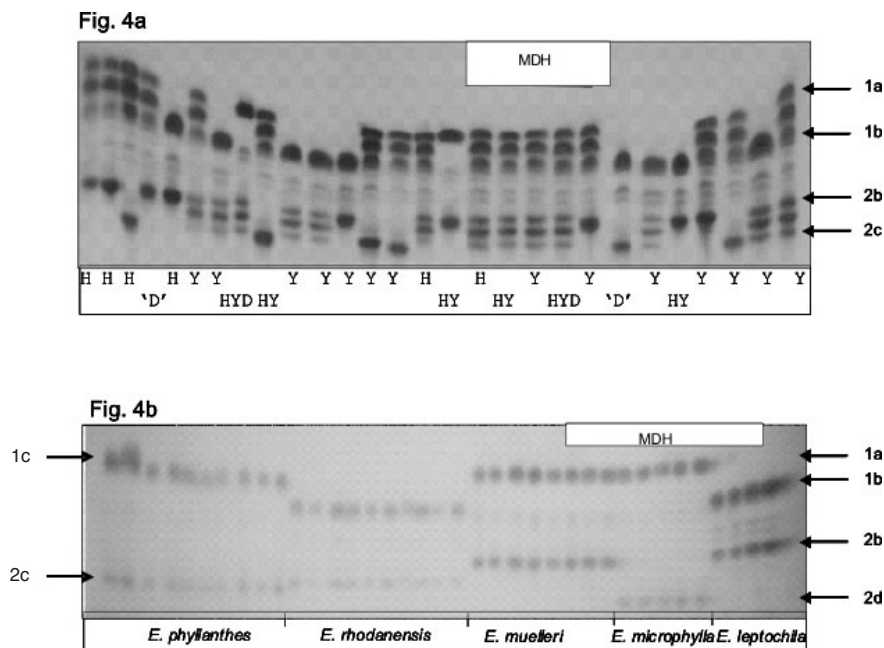
FIGURE 4. Patterns of allozyme diversity in a complex *E. youngiana* population compared with typical autogamous *Epipactis* taxa. (a) MDH variation at two loci from a mixed Scottish population (Bothwell Castle) of plants with the morphology of *E. youngiana*, *E. helleborine* and *E. dunensis* showing high levels of heterozygosity for the same set of alleles; H = *E. helleborine*, Y = *E. youngiana*, and 'D' = plants which resemble morphologically *E. dunensis* (no plants with the classic *E. dunensis* molecular genotype have yet been detected in Scotland). Combinations of these letters represent morphological intermediates. (b) MDH variation at two loci showing the classic autogamous genetic signature in *Epipactis*: fixed homozygous and uniform allozyme genotypes within species, but fixed differences for different allelic combinations between species.

   Arrows represent locus (number) and allele (letter) designations as used in Table 2. Unlabelled bands were not scored and are assumed to represent heterodimers and breakdown products.
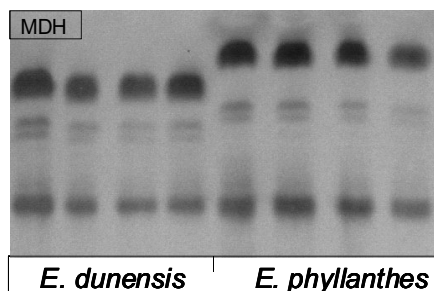
FIGURE 5. Representative MDH variation in plants of *E. dunensis* and *E. phyllanthes* from a mixed site on the north-west coast of England, demonstrating the maintenance of clear genetic differences between autogamous species growing in sympatry.

all other *E. dunensis* populations we have examined, in which the plants are all genetical-ly distinct, homozygous and uniform, and suggests that the plants of *E. dunensis*-type morphology in the Scottish sites are not 'pure'.

2 Likewise, at Settlingstones, the local plants of *E. phyllanthes* possess an MDH allele that is also found in the local *E. helleborine* and *E. youngiana* populations. This was absent from a survey of 408 plants from 26 other populations of *E. phyllanthes*, all of which showed a single uniform allozyme profile. This again may indicate some past hybridisa-tion, although *E. phyllanthes* at this site appears to be morphologically uniform and typical.

3 One additional curious feature of the Settlingstones site is that for both *E. helleborine* and *E. youngiana* there is a high frequency of an unusual chloroplast type (82% and 87% respectively: Fig. 2). Although present at similar frequencies in some populations of *E. helleborine* introduced to North American (Squirrell *et al.* 2001) this haplotype is typically absent or occurs at a low frequency in British populations (in a survey of ten populations it ranged from a frequency of zero to 9.5%, mean = 1%). This may just be chance, although the coincidence of this atypical marker in a taxonomically complex population also may suggest a genetic signature of past hybridisation. However, if this pattern is due to hybridity, it is not obvious which species was involved (this haplotype is not found in geographically proximal species such as *E. dunensis* or *E. phyllanthes*). The only other British species that possesses this chloroplast haplotype is *E. leptochila* s.s., a species with a much more southerly distribution in the UK, so a hybrid explanation for the unusually high frequency of this marker would require a rather convoluted scenario.

Thus the evidence for the evolutionary detour hypothesis is somewhat circumstantial and equivocal. Further research on mixed populations of allogamous and autogamous taxa is required to test the importance of hybridisation as a mechanism underlying the recurrent origins of self-pollination. However, it does at least seem plausible that a normally autoga-mous species such as *E. dunensis* may receive insect visits and pollinia import (cf. Richards 1986) if growing in sympatry with an outcrossing species, and thus potentially can serve as a conduit for the transfer of genes encoding selfing floral morphologies into novel hetero-zygous backgrounds.

IS A SPECIES-BASED APPROACH TO CONSERVATION APPROPRIATE FOR DEALING WITH DIVERSITY IN TAXONOMICALLY COMPLEX GROUPS?

Under current species-based conservation programmes, the conservation status of *E. young-iana* should be revised. The genetic data and the extreme difficulties of identifying morpho-logical discontinuities in the field all suggest that this does not represent a cohesive, distinct, reproductively isolated species that has stabilised by autogamy. Instead, it is best considered as a series of complex populations that have not currently achieved separate evolutionary trajectories from the sympatric populations of *E. helleborine.* Given the available evidence, it would in practice be exceedingly difficult to enforce the current legislative conservation protection of this 'species' under the Wildlife and Countryside Act.

However, it is equally important to note that casually dismissing the conservation value of complex populations like *E. youngiana* is a simplistic view, and that it typifies a wide-spread problem regarding the conservation of taxonomically complex groups in the post-glacial flora of Britain. Work on *Epipactis* has revealed a range of genetically variable allogamous taxa, a range of uniform homozygous lineages with a floral morphology consist-

ent with selfing, and some populations such as *E. youngiana* that fall someway between the two. This pattern of common variable species, local endemic entities, and morphologically complex populations containing individuals not readily assignable to any discrete taxon is paralleled in other actively diversifying taxonomically complex groups such as *Euphrasia* (French 2004, French *et al*. 2005) and *Sorbus* (Robertson *et al.* 2004). Taxonomic complexity, recent/ongoing diversification and endemism are all tightly associated in the British flora, and indeed in that of the broader North Atlantic region. If diversification is ongoing, one should not expect all diversity to fall conveniently into neat discrete packages. Therefore it seems appropriate to develop conservation strategies that encompass the broad range of diversity and evolutionary processes in these groups, rather than focusing attention and resources entirely on the taxonomic status of a fraction of this diversity. An alternative conservation goal for taxonomically complex groups would be to develop conservation strategies aimed at the diversification process itself, which recognise the value of all elements in the system such as progenitor species, endemics and taxonomically complex sites (Hollingsworth 2003, Ennos *et al.* 2005).

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