The relationship between flower size, inbreeding coefficient and inferred selfing rate in British *Euphrasia* species

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The genus *Euphrasia* in Britain comprises a taxonomically complex group of self-compatible, morphologically similar, hemi-parasitic, annual plant species of high conservation importance. The 19 diploid and tetraploid taxa currently recognised show striking variation in flower size. The objective of this paper is to determine whether a relationship exists between flower size and breeding system within *Euphrasia*. Following a survey of flower size variation among the 19 taxa, seven diploid populations, encompassing a broad range of flower sizes, were selected for detailed study. Four nuclear microsatellite loci were used to estimate the

inbreeding coefficient F_{is} within each population. F_{is} values varied from 0.17–0.77 and showed a significant, negative correlation with flower size. These results are best explained as the consequence of variation in selfing rate among the *Euphrasia* populations, with selfing rate increasing as flower size decreases. The potential factors influencing breeding system evolution in *Euphrasia* are discussed, together with the role of autogamy in generating taxonomic complexity and facilitating lineage differentiation within the genus.

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Introduction

Insect-pollinated self-compatible plants show a range of mating systems from complete outcrossing to complete selfing. Even within species, different populations may evolve contrasting rates of outcrossing (Charlesworth and Mayer, 1995; Vogler and Kalisz, 2001). The advantages of outcrossing include the production of variable progeny and avoidance of inbreeding depression. In addition, the higher effective population size of outcrossed populations reduces the rate at which rare alleles are lost by drift, and contributes to the maintenance of genetic variation (Jarne and Charlesworth, 1993). Factors favouring selfing include automatic selection for selfing genes and reproductive assurance in areas where there are constraints on allogamous pollination (Schoen *et al.*, 1996; Holsinger, 2000). Self-pollination may evolve by the reduction of herkogamy (anther-stigma separation) or dichogamy (timing of anther-stigma maturation) (Dole, 1992; Armbruster *et al.*, 2002), and secondary changes in flower structure may then take place with reduction in resource allocation to the reproductive organs (Barrett *et al.*, 1996). This may involve a reduction in flower size (Stebbins, 1970) or pollen:ovule ratio (Cruden, 1977).

A consequence of the evolution of selfing is the separation of a taxon into reproductively isolated units.

Transitions from cross- to self-pollination can hence have profound effects on evolutionary diversification within a plant group. Lack of gene exchange may result in the fixation of distinct multilocus genotypes possessing different combinations of character states. This can lead to difficulties in defining species limits. Selfing lines have been variously treated as full species through to unnamed intraspecific variants (Squirrell *et al.*, 2002; Hollingsworth, 2003). The choice of species concept can in turn have a marked impact on conservation strategies. Excessive splitting of a plant group into a high number of species can elevate their representation within a regional endemic flora and this may raise their conservation status (Hollingsworth, 2003). In contrast, lumping together a series of distinct taxa into a single species complex will reduce the conservation attention given to the group.

The genus *Euphrasia* (Scrophulariaceae) in Britain is a classic example of a taxonomically complex group (19 currently recognised species, of which at least seven are considered endemic, together with 64 accepted hybrid combinations; Silverside, 1991; Stace, 1997). Both diploid and tetraploid taxa occur (Yeo, 1978), and within each ploidy level, species with markedly different degrees of protogyny and flower size are recognised (Pugsley, 1930). Experimental evidence has shown that these annual plants are self-compatible (Darwin, 1876; Yeo, 1966) but not apomictic (Yeo, 1966). The *Euphrasia* complex could therefore be thought of as a collection of more or less selfing lines, with varying degrees of reproductive isolation among them. The extent of reproductive isolation within a ploidy level would be a function of the breeding system, with increased autogamy leading to increased isolation.

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At present, we have no quantitative information on the extent of self-pollination in *Euphrasia*, and there are conflicting views on its prevalence in the genus. On the basis of a paucity of observed pollinator visits (mainly hover-flies (Diptera: Syrphidae) and bumble-bees (*Bombus*, Hymenoptera: Apidae); Yeo, 1966), Pugsley (1930) and others have concluded that all British *Euphrasia* taxa are highly selfing, irrespective of their floral biology. An alternative view is that selfing rate varies with flower size (Vitek, 1998). Other authors have invoked outcrossing even within small-flowered *Euphrasia* to account for the parentage of certain hybrid taxa (Yeo, 1978).

Analysis of progeny arrays in the context of a mixed mating model is a common method for estimating outcrossing rates (Ritland, 1990). This requires genotyping of families from a range of maternal parents. The small seeds, poor germination and low survival of hemiparasitic *Euphrasia* seedlings on their host plants in a glasshouse situation made such an approach impractical (Yeo, 1961). An indirect method for estimating selfing rates is however available. This relies on the fact that, in a diploid population practising a mixture of selfing and random mating, the equilibrium inbreeding coefficient, F_{is} , is determined by the rate of self-pollination. By measuring F_{is} in such a population using selectively neutral, polymorphic, co-dominant markers, inferences about its selfing rate can be made (Brown, 1979).

The primary objective of this paper is therefore to investigate whether the observed variation in flower size among *Euphrasia* populations is associated with differences in their selfing rates. Given the biological constraints of the system, this question has been tackled by measuring the inbreeding coefficient, F_{is} , within a set of populations that span the full range of flower sizes. The relationship between F_{is} (used here as surrogate for selfing rate) and flower size has then been determined.

The second objective of the research stems from a need to understand how taxonomic complexity is generated in the *Euphrasia* group. Specifically, we wish to determine whether the rates of selfing inferred from estimation of F_{is} are sufficient to create significant reproductive barriers between taxa. Understanding the biological basis of taxonomic complexity is a priority for conservation of *Euphrasia* taxa that constitute almost 25% of the vascular plant species listed on the UK Biodiversity Action Plan 'Short List' (HMG, 1995).

Materials and methods

Flower size measurements

A comprehensive field survey of flower size variation was undertaken for the 19 recognised taxa of British *Euphrasia*. A total of 755 plants were sampled from 45 populations throughout the UK (Table 1). In the field single flowers were removed from the first flowering node of each individual and immediately placed on wet tissue, in a petri dish, to prevent drying out. At the same time, the total number of open flowers and total number of branches were recorded from each plant sampled.

Within 24 h of collection, the corollas were dissected into their upper and lower lobes. These were measured immediately or fixed onto card with a transparent tape to allow measurements to be taken later. Using this fixation method, no discernable shrinkage of corolla lobes was

observed. For each corolla, the depth and inner and outer width of the central lower lip were measured under a dissecting microscope, together with the length of the upper lip (Figure 1). By assuming that the lower lip approximated a trapezium in shape, it was possible to calculate its area, a character thought to be most indicative of the attractiveness of the flower to pollinators (Karlsson, 1982).

Genetic analyses

From the floral survey, seven populations were selected to represent the maximal variation in average corolla size among diploid taxa (Figure 1, Table 1). Tetraploid taxa were not analysed due to difficulties involved in the scoring of their genotypes, and subsequent estimation of F_{is} values. The diploid populations selected included three populations of *Euphrasia officinalis* subsp *rostrkoviana* (D3; D4; D5), two populations of *E. vigursii* (D15; D16), one population of *E. officinalis* subsp *anglica* (D1) and one population of *E. rivularis* (D10). The population of *E. officinalis* subsp *anglica* consisted of two subpopulations separated by approximately 50 m; these were analysed separately (D1A, D1B).

From each of the seven populations chosen, fresh plant tissue was collected from 15–30 individuals for DNA analysis. Samples consisting of a minimum of three leaves per plant were placed in a 1.5 ml eppendorf tube and frozen in the field with a Taylor Wharton cryopreserver (-197°C), before long-term storage at -80°C .

DNA extraction was performed directly in the eppendorf tubes, using the hexadecyltrimethylammonium bromide (CTAB) method, as described by Saghai-Marooof *et al* (1984), with the modifications of Doyle and Doyle (1987). 2-Mercaptoethanol (0.2%) and insoluble polyvinylpolypyrrolidone (PVPP; 0.1%) were added to the $2 \times$ CTAB buffer (1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% CTAB). Two chloroform/isoamyl alcohol extractions were performed, and after precipitation with freezer-cold isopropanol overnight, the resultant DNA pellet was resuspended in 50 μl of Tris-EDTA, pH 8 (TE). This DNA was then stored at -20°C .

Microsatellite markers were chosen for this study because they have high allelic diversity resulting from their high mutation rate (Jarne and Lagoda, 1996). They therefore displayed the allelic variation required for the estimation of F_{is} even in highly inbreeding species where gene diversities are typically lower than in outcrossing species (Hamrick and Godt, 1996).

Four microsatellite loci (*Ene1*, *Ene2*, *Ene3*, *Ene4*) were amplified using primers and PCR conditions as described by French *et al* (2003). The PCR products were run on an ABI 377 DNA sequencer, followed by gel image analysis and allele size scoring using ABI Genescan[®] software (version 3.1.2) (Applied Biosystems).

Data analysis

The microsatellite data were formatted for analysis using Microsatellite Toolkit (Park, 2001). For each population, the mean number of alleles per locus (A), the proportion of polymorphic loci (P), the mean observed heterozygosity (H_O) and the mean expected heterozygosity (H_E) at Hardy-Weinberg equilibrium were calculated using GDA: version 1.0 (Lewis and Zaykin, 2001). Wright's

Table 1 Location and average corolla measurements of 45 populations of British *Euphrasia* taxa

Pop.	Taxon	Region	Population	Grid reference	No.	Average area $\pm$ SD of lower lip (mm ²)	Average length $\pm$ SD of upper lip (mm)
<i>Diploid</i>							
D1	<i>E. officinalis</i> subsp <i>anglica</i>	South-west England	Kit Hill	SX/374.715	29	5.00 $\pm$ 1.17	6.71 $\pm$ 0.70
D3	<i>E. officinalis</i> subsp <i>rostkoviana</i>	Wales	Gower	SS/539.928	26	10.66 $\pm$ 1.61	9.53 $\pm$ 0.56
D4	<i>E. officinalis</i> subsp <i>rostkoviana</i>	Wales	Caeae Llety-cyli	SN/603.535	26	7.87 $\pm$ 1.13	8.28 $\pm$ 0.54
D5	<i>E. officinalis</i> subsp <i>rostkoviana</i>	Wales	Devil's Bridge	SN/751.735	25	7.89 $\pm$ 1.59	8.35 $\pm$ 0.71
D7	<i>E. rivularis</i>	Wales	Snowdon	SH/605.558	22	5.06 $\pm$ 1.05	6.13 $\pm$ 0.55
D8	<i>E. rivularis</i>	Wales	Cwm Glas	SH/616.565	19	5.69 $\pm$ 1.18	6.89 $\pm$ 0.68
D9	<i>E. rivularis</i>	North-west England	Stickle Tarn	NY/290.083	12	7.29 $\pm$ 1.17	6.94 $\pm$ 0.38
D10	<i>E. rivularis</i>	North-west England	Honiston Pass	NY/226.134	26	3.95 $\pm$ 0.85	5.85 $\pm$ 0.49
D12	<i>E. vigursii</i>	South-west England	Carvannel Downs	SW/641.446	27	4.65 $\pm$ 0.79	5.96 $\pm$ 0.36
D13	<i>E. vigursii</i>	South-west England	Porthtowan	SW/688.477	30	5.15 $\pm$ 1.15	6.26 $\pm$ 0.65
D15	<i>E. vigursii</i>	South-west England	Tregrethona Downs	SW/959.628	24	6.64 $\pm$ 1.19	7.10 $\pm$ 0.70
D16	<i>E. vigursii</i>	South-west England	Treslea Downs	SX/134.683	27	6.03 $\pm$ 1.63	6.30 $\pm$ 0.61
<i>Tetraploid</i>							
T3	<i>E. arctica</i> subsp <i>borealis</i>	North-west England	Rosthwaite	NY/255.159	28	6.37 $\pm$ 0.87	6.96 $\pm$ 0.42
T5	<i>E. arctica</i> subsp <i>borealis</i>	Mid Scotland	Tulloch Moor	NH/960.161	15	9.05 $\pm$ 2.02	7.90 $\pm$ 0.65
T7	<i>E. arctica</i> subsp <i>borealis</i>	North-west Scotland	Siabost	NB/271.473	19	11.27 $\pm$ 3.33	8.24 $\pm$ 0.77
T9	<i>E. arctica</i> subsp <i>borealis</i>	North Scotland	Durness	NC/405.669	11	10.63 $\pm$ 2.71	7.96 $\pm$ 1.16
T10	<i>E. arctica</i> subsp <i>borealis</i>	North Scotland	Reay	NC/958.653	20	13.07 $\pm$ 2.26	8.98 $\pm$ 0.72
T11	<i>E. cambrica</i>	Wales	Snowdon	SH/605.558	8	0.87 $\pm$ 0.30	3.64 $\pm$ 0.24
T13	<i>E. campbelliae</i>	North-west Scotland	Mealasta	NA/994.235	6	3.80 $\pm$ 0.83	6.59 $\pm$ 0.38
T16	<i>E. confusa</i>	South-west England	Kit Hill	SX/374.715	13	3.03 $\pm$ 0.92	5.85 $\pm$ 0.59
T17	<i>E. confusa</i>	North Scotland	Inchnadamp	NC/267.213	5	3.21 $\pm$ 0.32	5.82 $\pm$ 0.52
T18	' <i>E. fharaidensis</i> '	North Scotland	Inchnadamp	NC/272.210	12	2.51 $\pm$ 0.44	4.97 $\pm$ 0.21
T19	' <i>E. fharaidensis</i> '	North Scotland	Rhiconich	NC/253.523	11	2.25 $\pm$ 0.37	5.10 $\pm$ 0.43
T23	<i>E. foulaensis</i>	North-west Scotland	Mealasta	NA/993.240	6	2.08 $\pm$ 0.51	5.11 $\pm$ 0.22
T24	<i>E. foulaensis</i>	North-west Scotland	Mangurstadh	NB/005.315	10	2.33 $\pm$ 0.54	5.05 $\pm$ 0.28
T29	<i>E. frigida</i>	Mid Scotland	Creag an Lochain	NN/592.411	15	3.15 $\pm$ 0.61	6.46 $\pm$ 0.90
T36	<i>E. heslop-harrisonii</i>	North-west Scotland	Seilebost	NG/083.668	13	2.65 $\pm$ 0.98	5.62 $\pm$ 0.65
T37	<i>E. marshallii</i>	North Scotland	Betty Hill	NC/705.628	20	3.91 $\pm$ 0.31	6.09 $\pm$ 0.40
T38	<i>E. marshallii</i>	North Scotland	Melness	NC/577.653	12	6.69 $\pm$ 1.27	6.46 $\pm$ 0.39
T40	<i>E. micrantha</i>	South-west England	Wheal Busy	SW/742.423	20	2.46 $\pm$ 0.58	5.67 $\pm$ 0.37
T41	<i>E. micrantha</i>	South-west England	Chapel Porth	SW/697.494	9	1.44 $\pm$ 0.39	4.40 $\pm$ 0.68
T42	<i>E. micrantha</i>	Mid Scotland	Tulloch Moor	NH/962.167	17	3.25 $\pm$ 0.58	5.75 $\pm$ 0.56
T43	<i>E. nemorosa</i>	Wales	Cae Biaen Dyffryn	SN/606.447	14	2.34 $\pm$ 0.52	6.23 $\pm$ 0.65
T46	<i>E. nemorosa</i>	North-west Scotland	Dail Beag	NB/227.458	20	5.70 $\pm$ 1.08	6.83 $\pm$ 0.39
T48	<i>E. nemorosa</i>	North Scotland	Fharaid Head	NC/392.687	19	7.30 $\pm$ 1.46	7.27 $\pm$ 0.79
T49	<i>E. nemorosa</i>	North Scotland	Dunnett Bay	ND/223.700	20	10.76 $\pm$ 2.54	7.73 $\pm$ 0.73
T50	<i>E. ostenfeldii</i>	Wales	Snowdon	SH/602.556	10	2.18 $\pm$ 0.36	5.72 $\pm$ 0.51
T52	<i>E. ostenfeldii</i>	North Scotland	Inchnadamp	NC/272.210	14	1.54 $\pm$ 0.22	4.83 $\pm$ 0.27
T54	<i>E. pseudokernerii</i>	East England	Therfield Heath	TL/330.395	20	10.25 $\pm$ 1.87	9.43 $\pm$ 0.78
T55	<i>E. pseudokernerii</i>	East England	Fleam Dyke	TL/545.545	20	11.39 $\pm$ 2.12	9.28 $\pm$ 1.09
T56	<i>E. pseudokernerii</i>	East England	Devil's Ditch	TL/617.616	20	11.93 $\pm$ 1.77	9.17 $\pm$ 0.89
T57	<i>E. scottica</i>	North-west England	Honiston Pass	NY/230.136	5	1.74 $\pm$ 0.50	4.75 $\pm$ 0.43
T62	<i>E. scottica</i>	North-west Scotland	Carlabhagh	NB/190.413	14	2.62 $\pm$ 0.29	4.98 $\pm$ 0.32
T65	<i>E. tetraquetra</i>	South-west England	Wicca Pool	SW/465.403	9	2.08 $\pm$ 0.36	5.19 $\pm$ 0.39
T67	<i>E. tetraquetra</i>	Wales	Mewslade	SS/419.873	7	3.43 $\pm$ 1.16	5.81 $\pm$ 0.42

'*Euphrasia fharaidensis*' is an informal taxon name given to a recognisable entity from the NW of Scotland that has yet to be formally described. For all other taxa, nomenclature follows Yeo (1978) and Silverside (1991).

Figure 1 Average corolla size from the 45 *Euphrasia* populations listed in Table 1. Diploid populations used for the genetic analysis are represented by filled squares. Floral measurements indicated in figure, y = length of upper lip.

fixation index, F_{is} (Wright, 1965), was computed for each locus and averaged across all loci, for each population using FSTAT version 2.9.3.2 (Goudet, 2001). Standard errors and significance values were estimated based on randomisation tests. For each population, the mean and standard error were calculated for the area of the lower lip, number of open flowers per plant and number of branches. Correlations between the inbreeding coefficient of populations and population mean values for all morphological characters were calculated using the statistical package, MINITAB.

Results

Morphological variation

Across all taxa, the area of the lower lip ranged from 13.07 to 0.87 mm² (Table 1, Figure 1). The largest flowers were found in the widespread tetraploid species *E. arctica* subsp *borealis* and the smallest flowers in the endemic tetraploid *E. cambrica*. Within the seven diploid taxa selected for genetic analysis, the area of the lower lip ranged from 10.66 to 3.95 mm². *E. officinalis* subsp *rostkoviana* had the largest flowers, *E. officinalis* subsp *anglica* and *E. rivularis* the smallest and *E. vigursii* was intermediate (Table 1, Figure 1). In populations of these diploid taxa, the correlation between mean flower size and either mean total number of flowers per plant or mean number of branches was not significant ($r = 0.399$, $P = 0.328$; $r = 0.280$, $P = 0.502$, respectively). There was also no significant correlation between the mean number of branches and mean total number of flowers in a population ($r = 0.568$, $P = 0.142$).

Microsatellite variation

In the total data set all loci were polymorphic, with 38 alleles detected (12 from *Ene1*, 17 from *Ene2*, six from *Ene3* and three from *Ene4*). Within taxa, the proportion of loci that were polymorphic was 100% in *E. officinalis* subsp *rostkoviana*, *E. vigursii* and *E. rivularis*, but only three of the four loci were polymorphic in the population of *E. officinalis* subsp *anglica*. At the population level, the greatest allelic and gene diversity was found in population D5 of *E. officinalis* subsp *rostkoviana* ($A = 5.5$; $H_E = 0.606$; $H_O = 0.459$; Table 2). The lowest diversity was found in population D15 of *E. vigursii*, which was fixed for a single allele at each locus ($A = 1.0$; $H_E = 0.0$; $H_O = 0.0$), and also in one subpopulation of *E. officinalis* subsp *anglica* which was also invariant (Table 2). The lack of genetic variation in these populations precluded the calculation of their inbreeding coefficients.

In the remaining populations showing allelic variation, 21 of the 23 single-locus estimates of F_{is} were positive, showing a strong deficit of heterozygotes compared with Hardy-Weinberg expectations. Multilocus F_{is} values were all positive and ranged from $F_{is} = 0.166$ in population D4 to $F_{is} = 0.770$ in population D10 (Table 2). All but one population (D4) showed a significant deviation from panmixia ($P < 0.05$, Table 2).

Inbreeding coefficient and flower morphology

There was a significant negative correlation between flower size and F_{is} ($r = -0.893$, $P = 0.016$) (Figure 2) and this explained 80% of the variation. The populations with the smallest flowers (area of lower lip < 5 mm²) had the highest inbreeding coefficients ($F_{is} > 0.74$), whereas larger flowered populations (area of lower lip > 7.5 mm²) had smaller inbreeding coefficients ($F_{is} < 0.25$).

Table 2 Morphological measurements (mean $\pm$ SD), mean number of alleles per locus (A), percentage of polymorphic loci (P), expected and observed heterozygosities (H_E and H_O), single and multilocus inbreeding coefficients (F_{is}), for eight populations of diploid *Euphrasia*

Taxon	Pop.	No.	Lower lip area (mm ²)	Branch number per plant	Number of flowers per plant	A	P	F_{is} locus Ene1	F_{is} locus Ene2	F_{is} locus Ene3	F_{is} locus Ene4	H_E	H_O	F_{is} multilocus
<i>E. officinalis</i> subsp <i>Rostkoviana</i>	D3	28	10.66 $\pm$ 0.32	0.5 $\pm$ 0.13	3.33 $\pm$ 0.30	4.00	1.0	0.129 NS	0.151 NS	0.235 NS	0.439*	0.547	0.420	0.236*
<i>E. officinalis</i> subsp <i>Rostkoviana</i>	D5	30	7.89 $\pm$ 0.32	2.6 $\pm$ 0.36	4.17 $\pm$ 0.58	5.50	1.0	0.188*	0.560*	0.597*	-0.221 NS	0.606	0.459	0.246*
<i>E. officinalis</i> subsp <i>Rostkoviana</i>	D4	26	7.87 $\pm$ 0.22	1.37 $\pm$ 0.31	2.20 $\pm$ 0.28	2.75	1.0	0.136 NS	-0.020 NS	0.209 NS	0.212 NS	0.264	0.221	0.166 NS
<i>E. vigursii</i>	D15	28	6.64 $\pm$ 0.24	1.07 $\pm$ 0.27	1.83 $\pm$ 0.29	1.00	0.0	N/A	N/A	N/A	N/A	0.000	0.000	N/A
<i>E. vigursii</i>	D16	28	6.03 $\pm$ 0.31	1.17 $\pm$ 0.32	1.83 $\pm$ 0.34	4.25	1.0	0.526*	0.763*	0.332*	0.432*	0.582	0.264	0.551*
<i>E. officinalis</i> subsp <i>anglica</i>	D1A	15	5.56 $\pm$ 0.20	1.27 $\pm$ 0.23	4.73 $\pm$ 0.67	1.00	0.0	N/A	N/A	N/A	N/A	0.000	0.000	N/A
<i>E. officinalis</i> subsp <i>anglica</i>	D1B	15	4.4 $\pm$ 0.33	0.6 $\pm$ 0.19	1.93 $\pm$ 0.27	2.25	0.75	0.744*	1.000*	0.498*	N/A	0.377	0.100	0.741*
<i>E. ritularis</i>	D10	30	3.95 $\pm$ 0.17	0.19 $\pm$ 0.09	1.29 $\pm$ 0.16	3.75	1.0	0.736*	0.714*	0.914*	0.730*	0.430	0.100	0.770*

For population codes, see Table 1. NS = not significant, * $P < 0.05$.

Figure 2 Relationship between corolla size (the area of the central lower lobe) and inbreeding coefficient, F_{is} , for each of the six *Euphrasia* populations.

No significant correlations were found between F_{is} and either the mean number of flowers or mean number of branches per plant ($r = -0.701$, $P = 0.121$; $r = -0.583$, $P = 0.224$, respectively). There was also no significant correlation between flower size and levels of allelic diversity ($r = 0.324$, $P = 0.433$), gene diversity ($r = 0.260$, $P = 0.533$) or percentage of loci that were polymorphic ($r = 0.234$, $P = 0.577$).

Discussion

Interpretation of F_{is}

This study has revealed positive values of F_{is} in all populations of *Euphrasia* sampled. For five of the six populations F_{is} values are significantly greater than zero. In addition, a strong and statistically significant negative correlation has been demonstrated between the inbreeding coefficient F_{is} and the mean population flower size.

Positive estimates of F_{is} usually indicate an inbreeding mating system. However, positive F_{is} values may also be recorded in outcrossing populations under certain circumstances. The first is where high frequencies of null alleles occur at the marker loci used to estimate F_{is} , and the second is where there is strong population substructure.

Two observations suggest that null alleles do not account for the positive F_{is} values reported here. The first is that within populations F_{is} estimates are relatively consistent over loci, and do not appear to be caused by abnormal behaviour of particular loci (Table 2). The second is that nonamplifying individuals (homozygous nulls) were not detected at any of the microsatellite loci in either the populations scored here or in the samples used initially to screen the markers.

Evidence to demonstrate that population substructure may generate positive F_{is} estimates comes from reanalysis of data from the *E. officinalis* subsp *anglica* population (Table 2). This comprises two subpopulations, D1A and D1B, separated by approximately 50 m. Subpopulation D1B is polymorphic at three microsatellite loci and is genetically differentiated from population

D1A, which is fixed at all microsatellite loci. When data from the genetically differentiated populations are combined, the estimate of F_{is} increased from 0.741 (subpopulation D1B only) to 0.875 (combined subpopulations). Thus, population subdivision could potentially account for a fraction of the observed inbreeding values recorded. However, it should be noted that strong population subdivision is not a feature of outcrossing species (Ennos, 2001) and it is unlikely that simple population substructure within predominantly outcrossing taxa could account for either the levels or patterns of variability of F_{is} reported in this study. Thus, the most likely explanation for the results is that the variation in estimated F_{is} in *Euphrasia* reflects differences in rates of selfing among populations. High selfing rates are associated with small flower size.

Where populations are at inbreeding equilibrium, the value of F_{is} can be used to calculate an estimate of the rate of selfing s using the relationship $s = (2F_{is}) / (1 + F_{is})$ (Allard et al, 1969). If inbreeding equilibrium were assumed in our populations, then the smallest inferred selfing rates would be $s = 0.4$, found in *E. officinalis* subsp. *rozkoviana*, whose populations have large showy flowers. In contrast, the maximum inferred rate of selfing would be $s = 0.85$, found in the small-flowered taxa, *E. officinalis* subsp. *anglica* and *E. rivularis*. This extremely high selfing rate is in keeping with other features of these taxa that include poorly developed nectaries, and a minimal degree of herkogamy and dichogamy (Schulz, as reported by Knuth, 1909).

While the correlation between flower size and inbreeding coefficient suggests that within flower pollination is the most likely route for self-pollination, geitonogamous pollination could also occur in *Euphrasia*. If this were the case, a positive relationship would be expected between F_{is} estimates and flower number and branch number per plant. However, no significant correlations were detected. This suggests that geitonogamy is unlikely to be important in determining inbreeding coefficients within *Euphrasia* populations.

Potential factors influencing the breeding system in British *Euphrasia*

This study indicates that substantial self-pollination is likely to be occurring within *Euphrasia*, but has not examined the ecological and genetic factors driving the evolution of autogamy. Selection for high levels of self-pollination is likely under conditions of poor pollinator availability, and such a breeding behaviour has been proposed for the small-flowered *E. willkommii*, which occurs in dense populations, on high mountains, in Spain. There, despite insect visitation as low as 3%, a 2-year study found that approximately 80% of the ovules were pollinated (Gómez, 2002).

In addition to the observed poor pollinator service (Yeo, 1966), it is possible that the genetic load is below the level at which selfing genes are at a selective advantage (Jarne and Charlesworth, 1993). We may speculate that high biparental inbreeding from recurrent fluctuations in population size and limited gene flow in these annual plants (Molau, 1993) may be important in the purging of deleterious alleles and so reducing the genetic load. Once genetic load is low, selfing may easily evolve.

Stability of intermediate selfing rates

It is not clear whether populations with intermediate flower sizes represent (a) transient flower sizes from which an increase or a decrease in size will eventually be selected, or (b) an equilibrium situation in which facultative autogamy is promoted due to pollinator unpredictability, resulting in selection for the capacity to either self or outcross (Vogler and Kalisz, 2001). In large-flowered taxa such as *E. officinalis* subsp. *rozkoviana*, some selfing occurs (Figure 2, Table 2). The potential reasons for this include geitonogamy, facilitated selfing and delayed selfing. Indeed, marked corolla tube elongation occurs following anther dehiscence, which reduces initial anther–stigma separation (Pugsley, 1930). This elongation may represent such a delayed selfing mechanism and serve as a useful adaptive mechanism of reproductive assurance for these annual plants.

Reproductive isolation and the potential for taxonomic diversification

The correlation between flower size and inbreeding coefficient is informative with regard to diversification mechanisms in the genus. High levels of self-pollination are one potential mechanism behind taxon diversification and differentiation. As homozygosity increases by 50% for each generation of complete selfing, a large genetic distance can quickly develop between selfing lines and outcrossing progenitor taxa, and selfing taxa can acquire novel multilocus character combinations (Baker, 1959; Squirrell et al, 2002). Thus, as with other plants (Jain, 1976), isolation caused by breeding system transitions may contribute towards the high diversity of often sympatric *Euphrasia* species in Britain. If one extrapolates from the inferred selfing rates measured in this study to all British *Euphrasia* species, the predominance of self-pollination is evident (Figure 3). Out of the 19 taxa, 11 have flower sizes that fall within a zone of inferred selfing of >80%. A further four taxa have mean flower sizes which are of a similar or smaller size than the population of *E. vigursii* (mean area of central lower lip 6.03 mm²) that had a mean inferred selfing rate of $s = 0.711$ ($F_{is} = 0.551$). Thus, the recurrent evolution of autogamous lineages provides one explanation for the

Figure 3 The number of British *Euphrasia* species in relation to their mean flower sizes, based on the data in Table 1. The area to the left of the line is the area in which inferred selfing rates exceed 80% (eg an F_{is} estimate >0.66), based on extrapolation from the genetic analysis of the populations examined in the current study.

presence of high levels of *Euphrasia* diversity in recently glaciated landscape such as the British Isles. A similar process has been described within British *Epipactis*. Using isozyme and chloroplast RFLP analyses, Squirrell et al (2002) showed that lineage diversification within the *Epipactis leptochila* complex has probably been the result of the recurrent origin of selfing lines from the outcrossing progenitor, *Epipactis helleborine*.

Confirmation that *Euphrasia* has a mixed mating system with differing rates of inbreeding associated with variation in floral morphology is an important first step in the understanding of the processes driving the morphological diversification within this taxonomically complex plant group. Further examination of the ecological conditions promoting recurrent allogamous-autogamous shifts in *Euphrasia* may help towards a greater understanding of processes involved in evolutionary diversification of *Euphrasia* and other taxonomically complex plant groups. This will help in establishing and setting conservation priorities, and offers the potential to design conservation programmes in which the evolutionary process, and not just its products, is an integral conservation goal (Hollingsworth, 2003).

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