Conservation genetics of the critical plant genus *Euphrasia* L. in Britain

A thesis submitted to the University of Edinburgh for the degree of Doctor of Philosophy

Graham Christopher French

PhD Thesis

The University of Edinburgh

2003



Abstract

Recent national and international conservation legislation has led to an increased focus on 'prioritised species lists' for the allocation of conservation resources. Where the delimitation of a species is routine, this approach can be appropriate, and used to specify clear conservation targets. However, prioritised species lists become problematic when there are difficulties in species delimitation. This issue is particularly pertinent for the UK Biodiversity Action Plans where nearly half of the species on the high priority short list for conservation are taxonomically controversial. One particularly complex group accounting for the majority of these problems is *Euphrasia*. Interactions among several factors have been hypothesised as underlying the taxonomic complexity in the genus including breeding system shifts, hemi-parasitism, hybridisation, ploidy variation and local ecotypic adaptation. Six endemic species are currently recognised as having conservation importance in the UK but the taxonomic complexity of the group makes implementing conservation measures difficult.

The goal of this thesis is to use molecular markers to evaluate taxon limits and evolutionary processes in British *Euphrasia* to clarify the most appropriate approach for conservation. The results show that the major reproductive barrier in the group corresponds to ploidy level, with AFLP and chloroplast data both significantly differentiated between diploid and tetraploid species, although occasional gene flow via inter-ploidal hybridisation appears to contribute towards diversification of the diploid group. The genetic data support the current species level taxonomy for the diploid but not the tetraploid species, where a considerable overlap between taxa was detected. The chloroplast data detected four discrete lineages, the distribution of these among species suggest at least three allopolyploid events in the formation of the tetraploid taxa, within which distinct ecological groups occur. Variation in the breeding system was detected at the intra- and inter-specific levels and estimates of the inbreeding coefficient showed a strong correlation with flower size and support the importance of multiple shifts in breeding system as contributing towards the overall diversification within the group.

Given the lack of clear species limits in the tetraploid group and the dynamic nature of *Euphrasia* evolution, a change from a species- to process-based conservation approach is recommended. This change includes recognising the importance of progenitor taxa, and ecological and morphological diversity, and a decrease in the importance presently given to individual named endemic taxa.

Declaration

I hereby declare that this thesis is composed of work carried out by myself unless otherwise acknowledged and that this thesis is of my own composition. The research was carried out in the period of October 1999 to October 2002. This thesis has not in whole or in part been previously presented for any other degree.

Graham Christopher French

September 2003

Acknowledgements

During my PhD I have had the good fortune to have been supported by three supervisors, whose combined strengths, within their own particular fields of expertise, have made this thesis possible. I therefore wish to thank Pete Hollingsworth and Richard Ennos, for imparting some of their depth of understanding in plant population genetics and Alan Silverside, for his assistance in the field. Without Alan's impressive field identification skills, interpretation of the molecular data would not have had the ecological perspective so important in the understanding of the diversification processes in a complex group, like *Euphrasia.*

This PhD was funded by NERC, with CASE partners Scottish Natural Heritage, Countryside Council for Wales and English Nature. I wish to acknowledge their financial support as well as the insights they gave me in merging academic research into the development of practical conservation management strategies. I wish to particularly thank Chris Sydes, Barbara Jones and Ian Taylor for their willing assistance and patient wait for tangible results. I hope any recommendations that come out of this work will not only be of benefit to conserving the wealth of *Euphrasia* diversity I have enjoyed seeing but also to the protection of some of the few remaining fragments of botanically rich habitats left.

During my research I have benefited from the experience of working at both the University and Royal Botanic Garden in Edinburgh. Moving freely between these two laboratories has not only allowed me to pool their individual strengths but also has greatly enhanced the "PhD" experience. I wish to thank all the people I have met during this time in both institutions, but particularly would like to acknowledge the help and friendship I have received from John Morman, Michelle Hollingsworth, Alex Ponge, Sarah Rendell, Ashley Robertson, Jeremy Milne, Mark Hughes, Cécile Bâcles, Sarah Bekessey, Alan Forrest, Simoneta Negrete-Yankelevich and Fabiola Lopez-Barrera. I would like to thank Colin Legg and Alan Gray for their help with the analysis of the ecological data and wish to acknowledge all the people that work to keep these two institutions going. In particular, the breadth of the journals I have

consulted has only been possible thanks to the library facilities at Royal Botanic Garden Edinburgh.

An important part of this research has been the collection of plant material from the field. The insights I gained, seeing these populations in their natural environment, contributed an important part to my overall understanding of how *Euphrasia* diversify. I would therefore like to acknowledge everybody who has assisted in my fieldwork, in particular the overwhelming help I received from various vice-county recorders, reserve managers, landowners and crofters. I would especially like to thank Andy Jones and Helen Hughes from Countryside Council for Wales, Christina Oliver for her help in Cornwall, Quentin Kay for his help in South Wales and Peter Yeo for his assistance in Cambridgeshire. The conclusions that I will draw from this molecular study have relied heavily on the work done from numerous past and present amateur and professional botanists, some of the more influential ones repeatedly cropping up during this thesis. I would like to acknowledge these botanists not only for their contribution to the wealth of knowledge of *Euphrasia*, but also for their obvious enthusiasm for botany in general.

Three names have been so far been missing from this list of acknowledgements. My parents have always been there to support me and their help during this PhD has been no exception. I hope they have enjoyed their caravan holidays during the visits to various field sites around Britain and am sure they would be the first to remind me that, despite my meticulous gathering of site locations, one fortuitous population in particular would not have been found without their help. Finally I would like to thank Jane, molecular adviser, field assistant and proof reader all rolled into one, whose love and support I have greatly treasured during this PhD research. Our regular Sundays off exploring and photographing natural history in and around Edinburgh have made this "marathon" achievable.

They were black and iridescent green and without number; and in front of Simon, the Lord of the Flies hung on his stick and grinned.

William Golding: Lord of the Flies (1954)

Table of Contents

Abstract i
Declaration iii
Acknowledgements iv
Table of Contents vii
List of Figures xii
List of Tables xv
List of Abbreviations xvii

CHAPTER 1: Using molecular markers to aid the conservation of taxonomically complex plant groups 1

1.1. Introduction 1

1.2. Using molecular markers in taxonomically complex groups 2

1.3. The taxonomically complex plant group, *Euphrasia* 7

1.4. Aims of the molecular study in British *Euphrasia* 9

1.5. Materials and Methods 10

1.5.1. Overview of sampling strategy 10

1.5.2. Molecular markers used in this study 11

1.5.2.1. Co-dominant nuclear markers 11

1.5.2.2. Dominant markers 13

1.5.2.3. Organelle markers 14

1.5.3. Microsoft® Excel™ Macros 15

1.6. Layout of the thesis 16

1.7. *Euphrasia* nomenclature 17

CHAPTER 2: Isolation of polymorphic microsatellite markers for British *Euphrasia* 19

2.1. Introduction 19

2.2. Isolation of microsatellite markers 19

2.3. Screening of microsatellite markers 20

CHAPTER 3: The relationship between flower size and mating behaviour in British *Euphrasia* species 24

3.1. Introduction 24

3.2. Materials and Methods 27

3.2.1. Flower size measurements 27

3.2.2. Genetic analyses 31
3.2.3. Data analysis 31
3.3. Results 32
3.3.1. Flower size measurements 32
3.3.2. Microsatellite variation 32
3.3.3. Inbreeding coefficient and flower morphology 33
3.4. Discussion 35
3.4.1. Estimates of breeding behaviour 35
3.4.2. Potential factors influencing the breeding system in British *Euphrasia* 37
3.4.3. Stability of intermediate selfing rates 38
3.4.4. Reproductive isolation and the potential for taxonomic diversification 38

CHAPTER 4: Partitioning of genetic variation within British *Euphrasia*. Is there an association with ploidy level? 40
4.1. Introduction 40
4.2. Methods 44
4.2.1. Materials 44
4.2.2. DNA extraction 44
4.2.3. Molecular marker analyses 49
4.2.3.1. Chloroplast PCR-RFLP analysis 49
4.2.3.1.1. PCR amplification 49
4.2.3.1.2. Restriction digestion 50
4.2.3.1.3. Electrophoresis of restriction digestion products 50
4.2.3.2. AFLP analysis 50
4.2.3.2.1. Digestion and ligation 50
4.2.3.2.2. Pre-amplification 51
4.2.3.2.3. Selective amplification 51
4.2.3.2.4. Electrophoresis of selective AFLP PCR products 52
4.2.4. Scoring and data analysis 52
4.2.4.1. Chloroplast PCR-RFLP analysis 52
4.2.4.2. AFLP analysis 52
4.2.4.3. Unique molecular marker distribution 53
4.2.4.4. Geographical and ploidy structuring 53
4.3. Results 54

4.3.1. Chloroplast PCR-RFLP haplotype and AFLP diversity 54
4.3.2. Chloroplast PCR-RFLP haplotype and AFLP band distribution 54
4.4. Discussion 61
4.4.1. Differentiation of ploidy groups 61
4.4.2. Is the barrier between ploidy levels absolute? 61
4.4.3. Is there evidence that the tetraploids are allopolyploid? 62
4.4.4. Evolutionary significance of polyploids in *Euphrasia* 63
4.4.5. Broad scale population structure 64
4.4.6. Summary 65

CHAPTER 5: Partitioning of genetic variation within the diploid group of British *Euphrasia*. Is there an association with current taxonomic species delimitation? 66
5.1. Introduction 66
5.2. Materials and Methods 72
5.2.1. Materials 72
5.2.2. DNA extraction and molecular marker analyses 72
5.2.3. Data analysis 74
5.2.3.1. Population Genetic analyses 75
5.2.3.2. Phenetic analyses 75
5.3. Results 76
5.3.1. Chloroplast PCR-RFLP haplotype and AFLP band distribution 76
5.4. Discussion 81

CHAPTER 6: Partitioning of genetic variation within the tetraploid group of British *Euphrasia*. Is there an association with current taxonomic delimitation? 87
6.1. Introduction 87
6.1.1. Taxonomy of tetraploid *Euphrasia* 88
6.1.2. Series *Boreales* 88
6.1.3. Series *Nemorosae* 89
6.1.4. Series *Parviflorae* 918
6.1.5. Other tetraploid taxa 99
6.1.6. Morphological variation within the tetraploid group 99
6.2. Materials and Methods 100

6.2.1. Widespread sampling 101
6.2.2. Paired sampling of two species 106
6.2.3. DNA extraction and molecular marker analyses 106
6.2.4. Data analysis 106
6.2.5. Widespread taxonomic analyses 110
6.2.6. Paired population analyses 112
6.3. Results 113
6.3.1. Widespread sampling of tetraploid species 113
6.3.2. Paired population sampling of two tetraploid species 125
6.4. Discussion 131
6.4.1. Taxonomic grouping at the species level 131
6.4.2. Taxonomic grouping at the series level 132
6.4.3. Grouping at the regional level 134
6.4.4. Summary 134

CHAPTER 7: A pilot study into ecological specialisation within tetraploid British *Euphrasia* 136
7.1. Introduction 136
7.2. Materials and Methods 139
7.3. Data analysis 139
7.4. Results 143
7.5. Discussion 145

CHAPTER 8: Conservation genetics of British *Euphrasia* 146
8.1. Introduction 146
8.2. Genetic congruence with the current taxonomic treatment 147
8.3. Species orientated conservation 151
8.4. Process orientated conservation 153
8.5. Diversifying processes in British *Euphrasia* 155
8.5.1. Diversification Process I: Multiple allopolyploid events 155
8.5.2. Diversification Process II: Post glacial migration of progenitor groups into Britain 160
8.5.3. Diversification Process III: Formation of restricted British *Euphrasia* taxa 163
8.6. Summary 169

CHAPTER 9: Conservation strategy for British *Euphrasia* **170**
9.1. Introduction **170**
9.2. Process action plans **173**
9.3. Process action plan for British *Euphrasia* **176**
9.3.1. Proposed processes involved in generating diversity 176
9.3.2. Progenitor populations 177
9.3.3. "Hotspot" areas of diversification 179
9.3.4. Connecting progenitor populations with "hotspot" areas 179
9.3.5. Ecological habitat diversity 179
9.3.6. Current end products of diversification 180
9.4. Summary **180**

References **182**

Appendix I **200**
Appendix II **203**
Appendix III **232**

List of Figures

Figure 3:1. Measurements of corolla size. 30

Figure 3:2. Average corolla size from the 45 different *Euphrasia* populations..... 30

Figure 3:3. The relationship between corolla size and a) inbreeding coefficient, F_{is}; b) inferred population selfing rate, *s* for each of six *Euphrasia* populations..... 35

Figure 3:4. The number of British *Euphrasia* species in relation to their mean flower sizes..... 39

Figure 4:1. Summary of fertility results taken from artificial crossing experiments between British diploid and tetraploid taxa. 43

Figure 4:2. Distribution of diploid and tetraploid sites within Britain, grouped according to region..... 45

Figure 4:3. Polymorphism detected in the chloroplast genome of British *Euphrasia*, revealed by RFLP analysis of four chloroplast regions. 55

Figure 4:4. Relationships between chloroplast haplotypes, represented as a minimum spanning network. 56

Figure 4:5. Distribution of molecular markers across individuals and ploidal groups. i) number of AFLP bands per individual; ii) chloroplast haplotypes. 57

Figure 4:6. Number of AFLP bands per individual, grouped according to ploidy level. i) south-west England: ii) Wales. 58

Figure 4:7. Number of total, polymorphic and ploidy limited AFLP bands, grouped according to ploidy group. 59

Figure 5:1. British *Euphrasia* species, collected within the diploid series *Euphrasia*, section *Euphrasia*..... 69

Figure 5:2. Distribution of diploid sites within Britain, grouped according to region. 74

Figure 5:3. Relationships between chloroplast haplotypes, represented as a minimum spanning network. 77

Figure 5:4. Percentage of chloroplast haplotypes within each taxa. 79

Figure 5:5. Number of total, polymorphic and taxon limited AFLP bands, grouped according to taxa. 79

Figure 5:6. Principal coordinate analysis of genetic distance between individuals from four diploid taxa. 80

Figure 5:7. Schematic diagram of hypothesed formation of diploid endemic species via inter-ploidal hybridisation. 85

Figure 6:1. British *Euphrasia* species, collected within the tetraploid series *Boreales*, *Nemorosae* and *Parviflorae*, section *Euphrasia*. 90

Figure 6:2. Number of populations of each tetraploid species sampled from seven regions within Britain 105

Figure 6:3. Paired populations of *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*, from across north-west and north Scotland regions. 107

Figure 6:4. Haplotype groups defined according to relationships between chloroplast haplotypes, represented as a minimum spanning network 111

Figure 6:5. Percentage of chloroplast haplotypes within each of Yeo's series 114

Figure 6:6. Percentage of chloroplast haplotypes within each region. 115

Figure 6:7. Percentage of chloroplast haplotypes within each species. 116

Figure 6:8. Number of total, polymorphic and species limited AFLP bands, grouped according to species. 117

Figure 6:9. Number of total, polymorphic and series limited AFLP bands, grouped according series. 118

Figure 6:10. Number of total, polymorphic and region limited AFLP bands, grouped according to region 119

Figure 6:11. Principal coordinate analysis of genetic distance between indiviudals from 12 tetraploid species 120

Figure 6:12. Principal coordinate analysis of genetic distance between individuals from three series 121

Figure 6:13. Principal coordinate analysis of genetic distance between individuals from six regions. 122

Figure 6:14. Percentage of chloroplast haplotypes within *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*. 126

Figure 6:15. Distribution of chloroplast haplotypes, grouped according to species within geographical area, across north-west and north Scotland regions. 126

Figure 6:16. Number of total, polymorphic and species limited AFLP bands for *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa* sampled from four different geographic areas. 127

Figure 6:17. Number of total, polymorphic and area limited AFLP bands, grouped according area. 127

Figure 6:18. Principal coordinate analysis of genetic distance between individuals from two tetraploid species across four areas, within north-west and north Scotland regions. 128

Figure 6:19. Principal coordinate analysis of genetic distance between individuals from two tetraploid species across four areas, within north-west and north Scotland regions. 129

Figure 6:20. Percentage of chloroplast haplotype groups within each series. 133

Figure 7:1. Haplotype-taxonomic groups defined according to relationships between chloroplast haplotypes, represented as a minimum spanning network. 137

Figure 7:2. Percentage of three chloroplast haplotypes groups within each species. 140

Figure 7:3. Mean Ellenberg values for four environmental variables, grouped within two tetraploid haplotype-taxonomic groups in British *Euphrasia*. 144

Figure 8:1. Modern taxonomic divisions within the genus *Euphrasia* in Britain.... 148

Figure 8:2. Partitioning of chloroplast haplotype and AFLP band variation within the subsection *Euphrasia* in Britain. 150

Figure 8:3. Hypothetical scheme of some possible genomic relationships in British *Euphrasia*. 158

Figure 8:4. Haplotype groups defined according to relationships between chloroplast haplotypes, represented as a minimum spanning network 159

List of Tables

Table 1:1. British taxonomically complex plant groups containing taxa recognised as either being of national or international conservation importance. 3

Table 2:1. Characterization of five *Euphrasia* microsatellite loci. 21

Table 2:2. Sampling of diploid *Euphrasia* taxa for microsatellite loci analysis. 22

Table 2:3. p-value for genotypic disequilibrium between all loci across six diploid populations. 22

Table 2:4. Cross-species amplification of five microsatellite loci within four diploid *Euphrasia* taxa. 23

Table 3:1. Location of 45 populations of *Euphrasia*, taken from all British taxa and their mean corolla measurements. 28

Table 3:2. Morphological measurements, mean number of alleles per locus, proportion of polymorphic loci, expected and observed heterozygosities, single and multilocus inbreeding coefficients, and inferred selfing rate, for eight populations of diploid *Euphrasia*. 34

Table 4:1. Hierarchical sampling of *Euphrasia* throughout Britain. 46

Table 4:2. PCR amplification conditions and estimated product size for the amplification of four regions used to assess the variation in the chloroplast genome of British *Euphrasia*. 49

Table 4:3. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia* 55

Table 4:4. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. 59

Table 4:5. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes for two regions (south-west England and Wales). 60

Table 4:6. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. 60

Table 5:1. Sampling of diploid *Euphrasia* taxa from four British regions for chloroplast PCR-RFLP and AFLP analysis. 73

Table 5:2. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*. 77

Table 5:3. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. 82

Table 5:4. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. 82

Table 6:1. Sampling of tetraploid *Euphrasia* within seven British regions, for chloroplast PCR-RFLP and AFLP analysis. 102

Table 6:2. Paired sampling of two tetraploid species within north-west and north Scotland regions, for chloroplast PCR-RFLP and AFLP analysis 109

Table 6:3. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*. 111

Table 6:4. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to 15 species. 123

Table 6:5. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to series. 124

Table 6:6. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to regions. 124

Table 6:7. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to the two species; *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*. 130

Table 6:8. Analysis of molecular variance for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to four areas in north-west and north Scotland. 130

Table 7:1. Chloroplast haplotypes detected by RFLP analysis of chloroplast regions in British *Euphrasia*. 137

Table 7:2. Associated species recorded within 37 populations of 10 British tetraploid taxa. 141

Table 7:3. Nested analysis of variance of mean Ellenberg values for four environmental variables. 143

Table 8:1. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*. 159

List of Abbreviations

A	mean number of alleles per locus
AMOVA	analysis of molecular variance
BAP	Biodiversity Action Plan
CTAB	hexadecyltrimethylammonium bromide
$D_{(ij)}$	genetic distance between individuals i and j
dCTP	deoxycytosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
F_{is}	Wright's inbreeding coefficient
GR	grid reference
G_{ST}	level of differentiation among populations
H_E	mean expected heterozygosity
H_O	mean observed heterozygosity
h_S	average intra-population gene diversity
h_T	total gene diversity
HTG	haplotype-taxonomic group
I.C.B.N.	*International Code of Botanical Nomenclature*
kb	kilobase pairs
P	proportion of polymorphic loci
PCR	polymerase chain reaction
PVPP	polyvinylpolypyrrolidine
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
s	inferred selfing rate
$S_{(ij)}$	genetic similarity between individuals i and j
UK	United Kingdom

CHAPTER 1:Using molecular markers to aid the conservation of taxonomically complex plant groups

Abstract: Taxonomic uncertainty pervades the lists of plant species of conservation importance in Britain. To aid with their conservation prioritisation a range of molecular markers may be used to assess species delimitation or improve understanding of processes involved in generating diversity within these taxonomically complex groups. However, for the most cost effective and applicable inferences from a particular molecular study, the appropriate use and analyses of these molecular markers is important. British *Euphrasia* represents one such complex plant group of high conservation importance. After reviewing which molecular techniques have been commonly used in the study of other taxonomically complex groups, highlighting their advantages and disadvantages within conservation projects, I discuss the techniques I have used in this molecular study of British *Euphrasia*. I also introduce the objectives of the study, in terms of examining the partitioning of genetic variation with *Euphrasia,* its relationship to existing *Euphrasia* taxonomy, the understanding of evolutionary process involved in generating diversity and the formulation of a more appropriate strategy for conservation prioritisation of *Euphrasia* taxa in Britain. Finally I highlight the advantages of using Visual Basic for Application Macros to analyse molecular data sets.

1.1. Introduction

Conservation practitioners are often faced with decisions regarding the allocation of limited resources to seemingly overwhelming numbers of species in need of protection (Milligan *et al.*, 1994). Useful aids for making these decisions include a range of molecular markers that can gather genetic information on these rare species (Burke *et al.*, 1992). In an ideal world the use of molecular markers would be relatively straightforward. Species would conform to the taxonomic "ideal", being obligately outcrossing and non-hybridising discrete units, with matching morphological and reproductive boundaries (Stace, 1978). Estimates of gene and genotype frequencies and patterns of diversity can then help us to infer the presence of any detrimental effects of species rarity, for example, isolation of populations by habitat fragmentation, and inbreeding depression due to population decline (Ellstrand and Elam, 1993; Amos and Blamford, 2001).

Plants do not, however, generally fit this "ideal" species framework. Combinations of varying levels of outcrossing, chromosome number differences and hybridisation all contribute to the mismatching of morphological and reproductive boundaries (Stace, 1975; Stace, 1978). While this taxonomic complexity leads to problems in a relatively small proportion of the flora as a whole (e.g. only 1-3% of the UK flora present major taxonomic problems caused by hybridisation (Stace, 1975; McDade, 1995), where this complexity does occur, it causes problems for conservation prioritisation. An examination of the high conservation priority lists in the British flora shows that taxonomic uncertainty is pervasive. The UK Biodiversity Action Plan (BAP) "short" list of 27 vascular plant species, for example, contains 11 species where taxonomic uncertainty is particularly acute (Anon, 1995b). Setting conservation strategies for these taxonomically complex species are difficult. Extensive hybridisation leads to a mismatch between entities defined by morphological or reproductive criteria, blurring species boundaries and producing taxonomic confusion. In addition, processes other than random sexual mating predominate (Squirrell, 2002), generating potentially endless series of restricted endemic taxa. In the agamospermous group, *Hieracium* L., for example, 80 species are included in the Red Data Book (RDB) for vascular plants (Wigginton, 1999). With many of the 250+ named *Hieracium* taxa in Britain being equally local rare endemics (Stace, 1997), this is undoubtedly a compromise measure.

By taking one taxonomically complex group and examining it in detail, this thesis explores the role that molecular markers can play in the understanding of taxonomically complex groups and the setting of their conservation priorities.

1.2. Using molecular markers in taxonomically complex groups

British plant groups of conservation importance, containing species with high degrees of taxonomic uncertainty, are listed in Table 1:1. Combinations of hybridisation, varying levels of outcrossing and differences in ploidy levels all contribute to taxonomic difficulties within these groups. Following Stace (1978), complex plant groups may be classified according to whether morphological or

Table 1:1. British taxonomically complex plant groups containing taxa recognised as either being of national or international conservation importance.

Species/Groups	No. of Taxa	*Highest BAP status	#Highest RDB status	†Schedule 8 WCS list	‡European status	Causes of taxonomic difficulty	Reference
Alchemilla L. spp.	6		Endangered			Polyploid agamospermous complex with hybridisation	Walters (1949)
Calamagrostis Adans. spp.	1	Middle	Vulnerable			Morphological similarity between C.*scotica* (Druce) Druce and *C.stricta* (Timm) Koeler	Stewart (1997)
Carex flava L. agg.	1		Vulnerable			Morphologically similar species complex	Jermy *et al.* (1982)
Cochlearia L. spp.	3	Short	Lower risk			Polyploid species complex with low morphological differentiation	Rich (1991) Rich & Dalby (1996)
Cotoneaster Medikus spp.	2	Short	Endangered	Yes		Morphological similarities between *C.integerrimus* Medikus & *C.cambricus* J.Fryer & B. Hylmö	Stace (1997) Fryer & Hylmö (1994)
Dactylorhiza Necker ex Nevski spp.	3	Long	Cr.endangered	Yes	Yes	Polyploid species complex, hybridisation and continuous morphological variation	Bateman & Denholm (1983; 1985; 1988)
Epipactis Zinn spp.	2	Short	Endangered	Yes		Allo- and autogamous taxa	Richards & Porter (1982) Richards (1997)
Euphrasia L. spp.	6	Short	Endangered			Polyploid species complex, hybridisation and continuous morphological variation	Yeo (1978a)
Gentianella Moench spp.	3	Short	Cr.endangered	Yes	Yes	Hybridisation with high intra-specific morphological variation	Pritchard (1959; 1960a; 1960b)
Hieracium L. spp.	80	Middle	Not given	Yes		Polyploid agamospermous complex	Stace (1997)
Limonium Mill. spp.	10	Middle	Vulnerable			Polyploid agamospermous complex, low morphological differentiation	Ingrouille & Stace (1986)
Orobanche L. spp.	2	Long	Endangered	Yes		Low morphological differentiation with poor herbarium character preservation	Rumsey & Jury (1991)

Table 1:1. *Continued.*

Species/Groups	No. of Taxa	*Highest BAP status	#Highest RDB status	+Schedule 8 WCS list	‡European status	Taxonomic difficulty	Reference
Rubus fructicosus L. agg.	28		Not given			Polyploid agamospermous complex and phenotypic variation	Eddees & Newton (1988)
Sorbus L. spp.	13	Long	Cr.endangered			Polyploid agamospermous complex	Warburg (1962) Hampton & Kay (1995)
Taraxacum Weber spp.	10		Not given			Polyploid sexual and agamospermous complex	Dudman & Richards (1997)
Ulmus minor Mill agg.	1	Long				Clonal reproduction within *Ulmus minor* Mill species complex.	Richens (1968) Melville (1975) Armstrong & Sell (1996) Stace (1997)
Total	161						

*UK Biodiversity Action plan with increasing conservation status recognised in long, middle and short lists. Middle and short lists contain taxa that are globally threatened or rapidly declining in the UK (over 50% decline in last 25 years) (Anon, 1995a; Anon, 1995b).

#British Red Data Book with highest threat categories as defined by The World Conservation Union. Cr. Endangered = Critically Endangered (Wigginton, 1999).

+ Wildlife and Countryside Act, 1981: Schedule 8.

‡ Included in one or more of EC Habitats and Species Directive Annex IIb or IVb; Bern Convention Appendix I or CITES Appendix II.

reproductive criteria are stronger predictors of discrete taxonomic unit recognition within each particular group.

1. Reproductive criteria stronger predictor of discrete taxonomic units
   a. Morphologically similar taxa with allogamous breeding systems (e.g. *Carex flava* agg., *Orobanche* spp.).
   b. Morphologically similar taxa within a polyploid complex (e.g. *Cochlearia* spp., *Dactylorhiza* spp., *Euphrasia* spp.).
   c. Morphologically similar taxa with autogamous breeding systems (e.g. *Epipactis* spp., *Euphrasia* spp.).
   d. Morphologically similar taxa with agamospermous breeding systems (e.g. *Alchemilla* spp., *Cotoneaster* spp., *Hieracium* spp., *Limonium* spp., *Rubus* spp., *Sorbus* spp., *Taraxacum* spp.).
   e. Morphologically similar taxa with extensive clonal spread (e.g. *Ulmus minor* agg.).
2. Morphological criteria stronger predictor of discrete taxonomic units
   a. Extensive hybridisation between two morphologically distinct taxa (e.g. *Calamagrostis* spp., *Dactylorhiza* spp., *Euphrasia* spp., *Gentianella* spp.).

In reality, taxonomically complex groups are not as easily polarised, with varying degrees and combinations of reproductive and morphological criteria used to distinguish discrete taxonomic units. Discrepancies between the use of morphological or reproductive criteria to define taxonomic units, however, leads to disagreements over the placing of taxonomic boundaries within the Linnean hierarchical classification system. These disagreements centre mainly on whether taxonomic units warrant recognition at the species or other taxonomic levels (section, subspecies, variants, microspecies etc.). The assignment of taxonomic rank has important consequences under current conservation legislation. Taxa defined at the species level may be included on lists of conservation importance whereas taxa defined at other taxonomic levels often are not (Hollingsworth, 2003).

One current use of molecular markers in conservation is to support or dispute current species delimitation, giving additional weight to either the morphological or

reproductive criteria used to define the taxonomic boundaries. For example, partitioning of neutral genetic markers among morphologically distinct potentially hybridising taxa may be used as evidence of the presence or absence of reproductive barriers between these taxa. In other cases, partitioning of neutral markers may support the recognition of two taxa at the species level despite poorly defined character separation (cryptic species). Such approaches have been used in a number of taxonomically complex groups. These include groups with predominant sexual reproduction; *Carex flava* agg. (Hedrén, 2002), *Orobanche* (Paran *et al.*, 1997; Román *et al.*, 2003) and *Dactylorhiza* (Hedrén *et al.*, 2001), groups with autogamous taxa; *Epipactis* (Harris and Abbott, 1997; Hollingsworth, 2001) and *Euphrasia* (Oliver, 1999), groups with agamospermous reproduction; *Alchemilla* (Sepp *et al.*, 2000), *Hieracium* (Stace *et al.*, 1997), *Limonium* (Palacios *et al.*, 2000), *Rubus* (Waugh *et al.*, 1990), *Sorbus* (Hampton and Kay, 1995) and *Taraxacum* (Van Oostrum *et al.*, 1985) and groups with predominant vegetative spread; *Ulmus minor* complex (Coleman *et al.*, 2000; Hollingsworth *et al.*, 2000).

In the recently colonised flora of Britain, many of the taxonomically complex groups have undergone active diversification during the relatively short time period since the retreat of the last ice sheet (Brochmann *et al.*, 2003). Protection of future adaptive potential could therefore be considered an especially important conservation goal in these groups. By increasing the understanding of processes that have been involved in the diversification within these taxonomically complex groups, conservation strategies may be more effectively directed towards protecting these processes. This may enable better protection of the evolutionary potential within a group, allowing it to continually adapt and diversify to future changing environmental conditions. Molecular markers may have an important role in the understanding of these processes. This approach has already been used in a number of taxonomically complex groups, including highlighting the importance of hybridisation and polyploidisation in both *Cochlearia* (Koch *et al.*, 1996) and *Dactylorhiza* (Hedrén, 1996), shifts in breeding system in the *Epipactis leptochila* (Godf.) Godf. complex (Squirrell *et al.*, 2002) and the formation of endemic agamospermous *Sorbus* taxa on the Scottish island of Arran (Robertson *et al.*, submitted).

1.3. The taxonomically complex plant group, *Euphrasia*

With its high level of diversification, and numerous restricted endemic taxa of conservation importance, the taxonomically complex group I shall be examining in detail is British *Euphrasia*. Although six *Euphrasia* taxa are on the Biodiversity Action Plan (BAP) "short" list (Table 1:1), this genus is one of the least studied in terms of using molecular markers in taxonomically complex groups. There are currently no published conservation genetic papers using molecular markers within *Euphrasia*, and the only presently completed work of which I am aware is the examination of genetic variation in four Scottish taxa, using allozyme and RAPD markers (Oliver, 1999).

In contrast, the genus has attracted a great deal of taxonomic interest (Wettstein, 1896; Townsend, 1897; Pugsley, 1930; Bobear, 1969; Yeo, 1978a), and has been the subject of field study (Karlsson, 1982; 1984), chromosomal counts (Yeo, 1954), cultivation and cross-fertilisation experiments (Yeo, 1956; 1959; 1962; 1964; 1966; 1976). The extensive literature on British *Euphrasia* hence allows an overall comparison between current conservation policy, as determined from present taxonomy, and that proposed following the use of molecular markers.

The taxonomy of British *Euphrasia* has remained relatively stable since the first extensive treatment of the group in Britain (Townsend, 1897), following the first world monograph (Wettstein, 1896). Most subsequent changes have been of nomenclature, description of new species (Pugsley 1930; Pugsley, 1940) or demotion of species to lower taxonomic levels (Yeo, 1971; Silverside, 1991d). Generally characters used to delimit species and other taxonomic levels have not changed. The main exceptions to this involve the character of elongating corolla tube, which is no longer used for series delimitation (Jørgensen, 1919), and the single character difference, presence of hairs, which is no longer considered sufficient for species delimitation (Yeo, 1971; Zopfi, 1997). The most recent European *Euphrasia* monograph, formulated in preparation for the account of the genus in *Flora Europaea*, currently defines 48 species (Yeo, 1978a). Out of these 19 occur in the British Isles (20 if one species restricted to Guernsey is included), 11 of which are

endemic or predominantly restricted to this country. Six of these species are given high conservation priority status, being included on the BAP "short list" for vascular plants (Anon, 1995b).

Species definition within *Euphrasia* does not conform to the "taxonomic ideal". The annual hemi-parasitic taxa, though not agamospermous (Yeo, 1959; Yeo, 1966), are self-compatible (Darwin, 1876; Yeo 1966), with markedly different degrees of protogyny and flower size that suggest the presence of high rates of inbreeding (Pugsley, 1930). With 64 presently accepted hybrid combinations (Stace, 1997), hybridisation is presumed to be common. Fertile hybrid offspring are produced both between homoploid taxa (Yeo, 1959; Yeo, 1966) and across ploidal levels. However, the diploid (2n=22) and tetraploid (2n=44) taxa are thought to be separated by a partial, or perhaps, complete reproductive barrier (Yeo, 1956). Hence, in this group, there is a marked mismatch between species boundaries as defined by morphological criteria, and those defined using reproductive criteria. In addition, taxonomic complexities produced from high intra-populational morphological variation, plus a lack of discrete discriminating morphological characters, have led to the definition of many more species than would be expected under a strict biological species concept (Sell and Yeo, 1970).

The present species concept in *Euphrasia* (Yeo, 1978a), reflects a balance between the need to describe the high morphological variation present, without resorting to too much cumbersome polynomial nomenclature. Other workers have taken a broader species approach to *Euphrasia* in Europe. Hultén (1975) stated that the genus in the Northern Hemisphere behaved as a single complex species. Karlsson (1976), whilst feeling that this view was too extreme, grouped 18 Swedish taxa into five species complexes. These complexes were considered relatively natural units because hybridisation within complexes is more common than between complexes. Vitek (1998) has taken a less extreme view still, recognising 25-30 natural specific units within *Euphrasia* in Europe. This is still less than the 48 species as defined by Yeo (1978a). Because the conservation of British *Euphrasia* is presently species-based, these differences of opinion on how best to define taxonomic units have

important consequences for the effectiveness of conservation prioritisation in the group. In this thesis, I shall examine whether the partitioning of genetic variation is concordant with the current taxonomy of the group. I will also ask whether taxonomic description of this variation provides the best framework within which to develop conservation programmes for the unique variation found within this genus in Britain.

1.4. Aims of the molecular study in British *Euphrasia*

This thesis will examine the use of molecular markers in the genus *Euphrasia*, aiming to compare the effectiveness of the current species-based conservation approach with one produced in the light of new molecular data. The use of molecular markers will be undertaken using two differing approaches;

1. Examination and comparison of the partitioning of genetic variation within the group;
   a. Among the two ploidal groups (diploid and tetraploid).
   b. Among the species within the diploid group.
   c. Among the series and species within the tetraploid group.

   Within these taxonomic divisions the degree of geographical partitioning of genetic variation will also be examined.
2. Examination of the processes that have generated diversity within British *Euphrasia*;
   a. Degree of inbreeding within taxa and the relationship to flower size.
   b. Degree of ecological specialisation among tetraploid taxa and association with chloroplast molecular marker distributions.
   c. Origin and maintenance of diversity during the putative post-glacial migration of the tetraploid group into and throughout this country.
   d. Generation of the current British taxonomic diversity, within both diploid and tetraploid groups.

These two approaches will then be compared and a new conservation action plan formulated to optimally protect the unique diversity found in British *Euphrasia*.

1.5. Materials and Methods

1.5.1. Overview of sampling strategy

Many British *Euphrasia* species are thought to have a high selfing rate (Pugsley, 1930). Therefore, within this group, partitioning of genetic variation is likely to be predominantly among, rather than within, populations. To achieve the greatest assessment of genetic variation of British *Euphrasia*, the sampling strategy was therefore designed to maximise the number of populations sampled, rather than include large numbers of individuals from few populations. By applying this widespread strategy, it was accepted that overall genetic variation was not fully assessed within British *Euphrasia*. However, this approach allowed an assessment of the relationship between the partitioning of genetic and morphological variation among taxa and examination of the processes generating diversity within the group. A more focused sampling strategy was also conducted to more adequately assess variation within populations of two tetraploid species.

In the widespread sampling strategy, 18 out of the 20 British species were sampled. Two species and two subspecies were excluded, either because too few individuals were found (e.g. only four individuals of *Euphrasia rotundifolia* and one individual *Euphrasia officinalis* subsp. *monticola*), or insufficient time was available to visit field sites (*Euphrasia stricta* in Guernsey and *Euphrasia arctica* subsp. *arctica* in Orkney and Shetland). Within each of the sampled taxa, with three exceptions, individuals from at least three populations were collected. Only two populations of *Euphrasia officinalis* subsp. *anglica* and *Euphrasia frigida* were visited in the allocated fieldwork period and only one population of *Euphrasia cambrica* was found. The taxonomic identities of all populations were confirmed by the present BSBI recorder for British *Euphrasia*, Dr Alan Silverside, with most identifications performed in the field or from herbarium specimens. Herbarium specimens have been deposited at **E** (Royal Botanic Garden Edinburgh).

By concentrating on material from populations determined to represent one of the currently recognised *Euphrasia* species, the sampling strategy excluded variation

present in populations with character combinations from differing species or from taxa not currently given taxonomic recognition. Therefore, a significant proportion of the total genetic variation in British *Euphrasia* was not sampled. Given the taxonomic complexity, and the fact that this is an initial molecular study into the group, such an approach was deemed the most appropriate. Results obtained from this study may be used as a platform for future studies in the genus, where these additional populations may be incorporated.

1.5.2. Molecular markers used in this study

A range of molecular markers may be used to study both taxonomic relationships and processes of diversification within plant groups (Bachmann, 1994). Important factors to be taken into account when determining which markers are most appropriate to the study of taxonomically complex groups include the requirements to screen large numbers of individuals, relatively cheaply, at the appropriate taxonomic level. The available marker types fall into three main groups; co-dominant nuclear, dominant, and organelle markers.

1.5.2.1. Co-dominant nuclear markers

Analysis of allozyme variation, using electrophoresis of enzymes from single protein-coding loci, offers a relatively cheap, quick method of determining variation both within and between populations. The advantages of co-dominance and a sound understanding of the genetic behaviour of the markers (May, 1998), have resulted in this technique being commonly used for examining genetic variation within a number of taxonomically complex groups. Examples include *Carex* (Hedrén, 2002), *Dactylorhiza* (Hedrén, 1996), *Epipactis* (Harris and Abbott, 1997; Squirrell *et al.*, 2002), *Hieracium* (Shi *et al.*, 1996; Stace *et al.*, 1997), *Sorbus* (Proctor *et al.*, 1989; Hampton and Kay, 1995; Robertson *et al.*, submitted) and *Ulmus* (Machon *et al.*, 1995). In addition, this has also been one of the most commonly used marker systems for the estimation of plant mating systems (Brown, 1989).

Allozymes have been used to examine genetic variation within British *Euphrasia* (Oliver, 1999). However, from four populations of the tetraploid small flowered species, *Euphrasia heslop-harrisonii*, examined, only two out of seven enzyme

systems produced polymorphic, scorable markers. These markers showed fixed heterozygosity within individuals of this species, with no intra-populational allelic polymorphism.

Assessment of the mating system of *Euphrasia* species is likely to be important in understanding the partitioning of genetic variation and the process of diversification within this plant group. Part of this study, therefore, aimed to assess the mating system within British *Euphrasia*, and its relationship with flower size. Estimation of outcrossing is commonly performed by the analysis of progeny genotype arrays, (Ritland, 1990). However, the small seed size, poor germination and low survival of the hemi-parasitic seedlings on host plants made this approach impractical. An alternative, indirect method of measuring the inbreeding coefficient, $F_{is,}$ in populations with differing flower sizes was therefore adopted (Brown, 1979). This method requires intra-population variation within the marker system used. With the previous finding of no intra-populational variation (Oliver, 1999), allozymes are unsuitable in this context. A more polymorphic co-dominant marker was therefore required. With higher allelic diversity produced from a higher rate of mutation (Jarne and Lagoda, 1996), the use of microsatellite markers seemed more appropriate, especially where estimation of inbreeding coefficients were required in the potentially selfing, small flowered populations. These DNA based markers, consisting of variable lengths of between 1 to 10 base-pair tandem repeat motifs, are highly reproducible, have high resolution and are relatively easy to score (Jarne and Lagoda, 1996).

Microsatellite markers have not, however, been the first marker of choice for many conservation projects. A time consuming and expensive process of isolation of the loci is often required. Combined with poor cross-specific amplification and low frequency in plant genomes, this has deterred researchers from using them (Zane *et al.*, 2002). To date there are no published studies using these markers in taxonomically complex plant groups of conservation importance in Britain. However, recent improvements of isolation techniques, and the development of automated DNA sequencing, have resulted in a decrease in the time and cost required

for this development phase, so that enough microsatellite markers for population genetic studies may now be relatively quickly isolated (Zane *et al.*, 2002; Squirrell *et al.*, 2003). Microsatellites have recently, for example, been isolated for two rare endemic species of *Limonium* in Spain (Palop *et al.*, 2000; Palop and González-Candelas, 2002). Given the advantages of microsatellite markers, they were chosen to estimate inbreeding coefficients in diploid *Euphrasia* populations. No microsatellite loci had, however, previously been isolated within the genus *Euphrasia*, and so part of this molecular study involved microsatellite isolation, prior to their application.

1.5.2.2. Dominant markers

Partitioning of genetic variation among taxa has commonly been estimated by a number of genetic fingerprinting techniques and analysed using a phenetic approach to estimate genetic similarities between individual genotypes. In this molecular approach, PCR-based techniques have allowed the use of small amounts of DNA compared to membrane hybridisation methods, which additionally require the use of radioactive probes (Weising *et al.*, 2000). One of the mostly commonly used PCR-based techniques, in taxonomically complex groups, has been randomly amplified polymorphic DNA (RAPD). Since its initial development (Williams *et al.*, 1990), it has been used in a wide range of these plant groups, including *Alchemilla* (Sepp *et al.*, 2000), *Cochlearia* (Koch *et al.*, 1996), *Hieracium* (Shi *et al.* 1996; Stace *et al.*, 1997), *Limonium* (Palacios and González-Candelas, 1997a; 1997b; Rodriguez *et al.*, 2003), *Orobanche* (Paran *et al.*, 1997; Román *et al.*, 2003) and the *Ulmus minor* agg. (Coleman *et al.*, 2000; Hollingsworth *et al.*, 2000). RAPDs were also used to examine genetic variation among four Scottish *Euphrasia* species (Oliver, 1999).

Despite uncertainty over independence, homology and co-migration of individual amplified bands, as well as their dominant expression, RAPDs have the advantages of ease and speed of use, and cost effectiveness of large scale screening (Hadrys *et* al., 1992; Karp *et al.*, 1996). They are, however, commonly perceived to suffer from further problems of unclear band scoring and low reproducibility (Bachmann, 1994). More recently, a different PCR-based DNA fingerprinting technique has been developed, termed AFLP (Vos *et al.*, 1995). Though still having the same dis-

advantages of a dominant marker system, and being more technically demanding, this seems to be a more reproducible technique, producing a higher number of scorable bands than RAPDs (Karp *et al.*, 1996; Muller and Wolfenbarger, 1999). This AFLP technique has only recently been applied to the molecular study of taxonomically complex groups. Examples include *Dactylorhiza* (Hedrén *et al.*, 2001) and *Limonium* (Palacios and González-Candelas, 1999; Palacios *et al.*, 1999). Given the advantages over RAPDs, I used the AFLP technique to generate markers across the range of *Euphrasia* species. The distributions of these bands were used to examine both the partitioning of genetic variation and processes of diversification within British *Euphrasia*.

1.5.2.3. Organelle markers

Insights into processes of diversification may be more clearly gained from data on genetic variation from both the nuclear and organelle genomes. Compared with the biparentally inherited nuclear genome, the distribution of markers from the uniparental haploid organelle genomes may give a clearer view of both the evolutionary and postglacial colonisation history of a taxonomic group (Ennos *et al.*, 1999). Chloroplast markers have, for example, been used to infer evolutionary history within the taxonomically complex groups of *Cochlearia* (Koch *et al.*, 1996), *Epipactis* (Squirrell *et al.*, 2002) and *Sorbus* (Robertson *et al.*, submitted).

In contrast to animals, plants have two organelle genomes, one in the mitochondria and the other in the chloroplast. Both of these genomes have been used in plant population studies, but use of chloroplast genome is more common, mainly because mitochondrial DNA undergoes frequent intragenomic recombination. The chloroplast genome, on the other hand, behaves as a haploid non-recombining unit (Ennos *et al.*, 1999), making it an easier molecule with which to work (Weising *et al.*, 1995). However, "selective sweeps" produced by advantageous mutations going to fixation, may lead to low levels of genetic variation (Ennos *et al.*, 1999). Variation may be further reduced by the smaller effective population sizes and lower mutation rates of this genome, as compared to the nuclear genome. This has often resulted in variation being too low for the use of chloroplast genome in intra-specific studies (Ennos *et al.*, 1999). Levels of chloroplast variation may, however, vary among species. Within

the holo-parasitic taxa of the genus *Orobanche*, high levels of variation have been described. High variation has been proposed to have result from relaxed selection pressures, with subsequent lower rates of "selective sweeps" experienced within this chloroplast genome (Wolfe and dePamphilis, 1997). By a similar process, variation within the chloroplast genome of hemi-parasitic *Euphrasia* taxa may similarly be high.

As the chloroplast genome is relatively conserved (Ennos *et al.*, 1999), it has been possible to develop a number of universal chloroplast primers that amplify specific regions across broad taxonomic groups (see Taberlet *et al.*, 1991; Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997; Chiang *et al.*, 1998; Hamilton, 1998 for examples). Screening genomes, using these primer pairs, across a range of species provides a relatively quick method for detecting sequence variation by restriction fragment length polymorphisms (RFLP). In addition to the use of microsatellite and AFLP molecular markers, I therefore used chloroplast PCR-RFLPs to assess the partitioning of genetic variation and processes of diversification within British *Euphrasia*.

1.5.3. Microsoft® Excel™ Macros

During this molecular study a large amount of population genetic data were generated from 76 *Euphrasia* populations. This dataset included a range of data, namely microsatellite fragment lengths, presence or absence of AFLP bands, and chloroplast restriction fragment profiles. As an aid to the analysis of these molecular data sets, a number of "Visual Basic for Applications" macros were written in Excel. This use of the Microsoft object orientated programming language (Green *et al.*, 2002), allowed the automation of many repetitive, time consuming organisational tasks, performed both during the formatting of the data sets and the production of differing population genetic software input files. The use of these macros also had the advantage of eliminating human errors, and increasing the range and depth of analyses that were possible.

A macro for microsatellite analysis (Park, 2001), was used for the analysis of the microsatellite data. For the AFLP and chloroplast data, a number of macros were

written. The visual basic codes for these are given in Appendix II. These macros, incorporated as "Add in" programs into the Excel application, aided the formation of Arlequin, R and Haplodiv software input files, for both chloroplast and AFLP data sets, as well as identifying different chloroplast haplotypes from a number of restriction fragment profiles, the presence and frequencies of chloroplast haplotypes and AFLP bands for each population, and the production of principal coordinate analysis plots from eigenvector values.

1.6. Layout of the thesis

The isolation of five microsatellite loci, by a membrane enrichment procedure, is first described in Chapter 2. These loci were used to examine the relationship between flower size and mating system behaviour within the diploid group of British *Euphrasia*. This is the topic of Chapter 3. Both of these two chapters have been submitted as papers and so some repetition of content was unavoidable. The next three chapters describe the results obtained from microsatellite, AFLP and chloroplast PCR-RFLP techniques, as applied to examination of the partitioning of genetic variation within British *Euphrasia*. In Chapter 4, this partitioning is related to the inter-ploidal barrier between the diploid and tetraploid groups. Partitioning of variation, and its congruence with current taxonomy within each of these groups are then examined in more detail in Chapters 5 (diploid) and 6 (tetraploid). In both of these chapters the relevant taxonomy and geographical distribution of taxa are discussed in more detail.

The next two chapters discuss the results obtained from both AFLP and chloroplast PCR-RFLP techniques, as applied to understanding the processes involved in the diversification of *Euphrasia* in Britain. Chapter 7 is a preliminary investigation into the importance that ecological specialisation may have had in the generation of diversity within the tetraploid group. Chapter 8 synthesises the previous knowledge provided by cultivation, fertilisation, ecological and taxonomic work, with the ecological and genetic understanding gained in this study, to develop a hypothesis to explain the overall process of diversification within British *Euphrasia*.

The final chapter, Chapter 9, examines the findings of this study with the objective of developing a strategy for conservation prioritisation within British *Euphrasia*. This strategy is summarised by the formulation of a combined Biodiversity Action Plan for British *Euphrasia*, given in Appendix III. By developing this strategy for conservation prioritisation, this chapter also explores whether molecular markers are more usefully applied to the analysis of species delimitation, or to the understanding of the evolutionary processes involved in the overall diversification within *Euphrasia* in Britain. The determination of whether a species- or process-based conservation approach is more appropriate for British *Euphrasia* may help with better targeting of molecular marker use in the other taxonomically complex groups that are of high conservation importance in Britain.

1.7. *Euphrasia* nomenclature

The nomenclature of *Euphrasia* taxa mentioned in this thesis follows Yeo (1978a), with modifications required within the current rules of the *International Code of Botanical Nomenclature* (*I.C.B.N.*), described by Silverside (1991d). Under the *I.C.B.N.* the use of the same taxonomic name at both species and aggregate level was rejected if it was used in different senses and had become a long-persistent source of error (Stafleu *et al.*, 1972, as reported by Silverside, 1991d). As the *Euphrasia* aggregate has commonly been referred to as *Euphrasia officinalis* agg., Yeo (1978a), rejected the use of the name *Euphrasia officinalis* at the species level as a *nomen ambiguum*.

However, after 1975, the rule governing the multiple use of the same taxonomic name was changed. Now under Article 69.1 of the current *I.C.B.N.* rules, the multiple use of the same name is valid if the species is the type species of the aggregate (Stafleu *et al.*, 1978, as reported by Silverside, 1991d). This is presently the case for *Euphrasia* (Silverside, 1991d). Since this alteration of Article 69.1, there have been further proposals to amend this rule so that the use of identical names at both species and aggregate level is rejected in *Euphrasia* (Brummitt, 1992; Weber, 1998). As these proposals have been rejected (Greuter *et al.*, 1994; Greuter *et al.*, 2000), in this thesis, I shall use the name *Euphrasia officinalis* at both species and aggregate level.

In addition, under *I.C.B.N.*, Article 22.1, any subdivision of the genus containing the type species must take the same name as the genus. As reported by Silverside (1991d), by accepting *Euphrasia officinalis* as the type species, both the series and subsection containing this species should be called *Euphrasia*. I shall adopt this nomenclature in this thesis.

For ease of reading, authorities for all *Euphrasia* taxa are not included within the main text of the thesis. Instead they are listed within Appendix I. This list also contains changes in nomenclature and identification revisions that have taken place within British *Euphrasia*, since 1891.

CHAPTER 2: Isolation of polymorphic microsatellite markers for British *Euphrasia*

Abstract: *Euphrasia* species in Britain attract a large amount of conservation attention due to the recognition of numerous endemic taxa in what is essentially a species-poor flora. To develop a set of research tools to investigate the evolutionary processes underlying this diversification, a membrane enrichment procedure has been used to isolate five polymorphic microsatellite loci from *Euphrasia nemorosa* (Pers.) Wallr. These loci amplify polymorphic products in several other British *Euphrasia* species.

2.1. Introduction

In Britain, the genus *Euphrasia* is taxonomically complex, with extreme difficulty concerning the placement of species boundaries. There are 19 recognised species (Yeo, 1978a), along with 64 recorded hybrid combinations (Stace, 1997; Yeo, 1978a). This high level of taxonomic complexity, and the associated recognition of numerous local endemic taxa, has resulted in *Euphrasia* species being disproportionately represented in the UK Biodiversity Action Plan conservation priority 'short-list' (Anon, 1995b). One of the potential biological mechanisms that may have contributed towards the evolution of such large numbers of localised taxa is self-pollination leading to the presence of a series of closely related inbreeding lines. Flower size varies in *Euphrasia* species, and this variation has been hypothesised to impact on outcrossing rates (Vitek, 1998).

2.2. Isolation of microsatellite markers

In order to investigate the relationship between the breeding system and taxonomic complexity in British *Euphrasia* I isolated a set of nuclear microsatellite loci. Genomic DNA, from the tetraploid species *Euphrasia nemorosa* (Wheal Busy, south-west England; GR SW/742.423; Population T68) was enriched for microsatellite sequences following a modified method based on Edwards *et al.* (1995) and Squirrell and Wolff (2001). Modifications were the use of *Tsp509*I restriction enzyme (AATT; New England Biolabs Inc.) and *Tsp509*I PCR adaptors (White and Powell, 1997), as described by Hughes *et al.* (2002). The digested DNA

was hybridized to 8 x 8mm Hybond® N+ membranes (Amersham Pharmacia Biotech), previously prepared by the fixation of 10 μg each of $(GA)_{15}$, $(CA)_{15}$ and $(AAG)_8$.

The enriched DNA was cloned using a PCR-Script™ Amp Cloning Kit (Stratagene) and the insert sequence of the recombinant clones was determined using a Thermosequenase II dye terminator cycle sequencing kit (Amersham) and an ABI 377 sequencer (Applied Biosystems). Of the 70 clones successfully sequenced, 75% contained microsatellites, ranging from 5-23 repeat units. The most common motif was $(GT)_n$, accounting for 60% of the microsatellite loci. 8% of the microsatellite loci were compound. One trinucleotide microsatellite $(CTT)_{15}$ was found. Suitable flanking sequence for primer design was present in 17 of the microsatellites. Primer design was carried out using PRIMER-3 (Rozen and Skaletsky, 2000), starting initially with the modified parameters as described by Beasley *et al.* (1999).

2.3. Screening of microsatellite markers

Primers were tested using 25 μl polymerase chain reactions (PCR) containing 5 ng genomic DNA, 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20), 2 mM $MgCl_2$, 100 μM dNTPs, 200 nM of forward and reverse primer (MWG Biotech) and 1 unit of *Taq* DNA polymerase (Bioline), under the following conditions: 95°C for 12 minutes, 30 cycles of 95°C for 15 seconds, annealing temperature (T_{an})°C for 15 seconds, 72°C for 15 seconds; followed by a final extension phase at 72°C for 10 minutes, using a GeneAmp 9600 thermocycler (Perkin Elmer). The PCR products were visualised by electrophoresis on a 2% agarose gel. Of the 17 primer pairs tested, 11 gave a product of similar size to that of the clone. Using 4 μM TAMRA labelled dCTP (Applied Biosystems) analysis of the PCR products on an ABI 377 sequencer resulted in five polymorphic loci which worked in a sample of three populations of the diploid species, *Euphrasia officinalis* subsp. *rostkoviana* (Table 2:1). The forward primer from each of these primer pairs was labelled with a fluorescent dye (JOE or FAM; MWG Biotech) and used in screening further populations. Cross species amplification occurred in all related British diploid species that were analysed: *Euphrasia officinalis* subsp. *anglica*,

Table 2:1. Characterization of five *Euphrasia* microsatellite loci. Allele numbers, size range and observed and expected heterozygosity were determined from three populations of *Euphrasia officinalis* subspecies *rostkoviana* (total number of individuals = 84).

Locus	Repeat motif	Primer sequence (5' - 3')	T_{an} (°C)	Size (bp)	*A*	H_E	H_O
Ene1	$(GT)_7$	‡AATTCCATCACTGCCAGAAAGAA TGCACAAACACTCCCTAAGTTTG	53	152-177	8	0.73	*0.48
Ene2	$(CA)_{15}N_{25}(CA)_9(TA)_9$	+TCAGGTCTTCATCCCTAGAAAGA ATATAGGTGAGGACCAGATGATG	54	119-197	13	0.82	*0.37
Ene3	$(CA)_{10}$	‡ATGAAGCAGCTCAGTCGTCTTT AGCAAGCAACATCATAGAGAACA	53	109-119	6	0.57	*0.19
Ene4	$(CA)_9$	+TTGATACTTGAATGGCTGTCAAA AAGTTGACCAGAGGAGCTCTGTA	53	134-138	3	0.67	*0.45
Ene5	$(CA)_7$	‡CCGTCACATACTCACATTACACA CCATTGACTTCGATTTGAAGATT	52	115-130	4	0.41	*0.20

‡ labelled with JOE fluorescent dye;+ labelled with FAM fluorescent dye, T_{an} = annealing temperature; *A* = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; * significant deviation from Hardy-Weinberg equilibrium ($p < 0.05$).

Euphrasia rivularis and *Euphrasia vigursii* (Table 2:2). All loci were in linkage equilibrium in all populations (Table 2:3) but there was a significant deficit of heterozygotes compared with Hardy-Weinberg expectations in six out of the seven populations (Table 2:4). Although null alleles are a possible source of this deficit, it is likely to be primarily attributable to varying levels of self-pollination. Estimates of the multi-locus inbreeding coefficient ranged from $F_{is} = 0.166$ to $F_{is} = 0.770$ and showed a strong and significant negative correlation with the mean flower size of each population (Chapter 3). These markers thus represent useful tools to investigate breeding system evolution and population genetic structure in British *Euphrasia*. This is the subject of the next chapter.

Table 2:2. Sampling of diploid *Euphrasia* taxa for microsatellite loci analysis. N = number of individuals sampled in each population.

Number	Region	Population	Grid reference	N
Euphrasia officinalis* subsp. *rostkoviana				
D3	Wales	Gower	SS/539.928	28
D4	Wales	Caeae Llety-cyli	SN/603.535	26
D5	Wales	Devil's Bridge	SN/751.735	30
Euphrasia officinalis* subsp. *anglica				
D1	south-west England	Kit Hill	SX/374.715	30
Euphrasia vigursii				
D15	south-west England	Tregrethona Downs	SW/959.628	29
D16	south-west England	Treslea Downs	SX/134.683	28
Euphrasia rivularis				
D10	north-west England	Honiston Pass	NY/226.134	30
Total				201

Table 2:3. p-value for genotypic disequilibrium between all loci across six diploid populations (8000 permutations), calculated using the software package FSTAT (Goudet, 2001). * significant disequilibrium ($p < 0.05$) after sequential Bonferroni correction (Rice, 1989). pop. = population; NA = unable to test significance.

	pop. D3	pop. D4	pop. D5	pop. D1	pop. D16	pop. D10
Ene1 x *Ene2*	0.220	1.000	*0.000	0.307	0.061	0.242
Ene1 x *Ene3*	0.189	0.422	0.256	0.216	0.039	0.946
Ene1 x *Ene4*	0.665	0.199	0.065	NA	0.088	0.157
Ene1 x *Ene5*	0.191	0.729	0.515	NA	0.944	0.305
Ene2 x *Ene3*	0.106	0.166	0.250	0.082	*0.001	0.650
Ene2 x *Ene4*	0.679	0.071	*0.008	NA	0.014	0.343
Ene2 x *Ene5*	0.187	1.000	0.786	NA	0.699	0.936
Ene3 x *Ene4*	0.587	0.531	0.254	NA	0.087	1.000
Ene3 x *Ene5*	0.445	0.715	0.230	NA	0.107	0.827
Ene4 x *Ene5*	0.430	0.782	0.965	NA	0.413	0.985

Population D15 and subpopulation of D1 monomorphic across all 5 loci.

Table 2:4. Cross-species amplification of five microsatellite loci within four diploid *Euphrasia* taxa.

	Ene1			*Ene2*			*Ene3*			*Ene4*			*Ene5*	
	N	*A* (sizes)	H_o (H_E)	N	*A* (sizes)	H_o (H_E)	N	*A* (sizes)	H_o (H_E)	N	*A* (sizes)	H_o (H_E)	N	*A* (sizes)	H_o (H_E)
E. officinalis* ssp. *rostkoviana															
D3	28	3 (169-175)	0.39 (0.45)	28	6 (181-197)	0.68 (0.80)	28	4 (109-117)	0.29 (0.37)	28	3 (134-138)	0.32 (0.57)	28	2 (119-130)	0.14 (0.20)
D4	26	4 (152-173)	0.35 (0.40)	26	2 (156-168)	0.08 (0.08)	26	3 (109-113)	0.19 (0.24)	26	2 (134-138)	0.27 (0.34)	14	2 (117-119)	0.21 (0.39)
D5	28	8 (152-177)	0.68 (0.83)	30	7 (119-179)	*0.33 (0.75)	30	4 (109-119)	*0.10 (0.25)	29	3 (134-138)	*0.72 (0.60)	12	3 (115-119)	0.33 (0.65)
E. officinalis ssp. anglica															
D1	30	3 (152-175)	*0.07 (0.52)	28	2 (168-176)	*0.00 (0.44)	30	3 (109-113)	*0.13 (0.62)	30	1 (138)	m	20	1 (115)	m
E. vigursii															
D15	28	1 (177)	m	29	1 (168)	m	28	1 (109)	m	29	2 (134-138)	0.00 (0.07)	12	1 (115)	m
D16	28	5 (152-179)	*0.36 (0.75)	27	6 (162-176)	*0.19 (0.77)	28	3 (109-113)	0.32 (0.48)	26	3 (134-138)	0.19 (0.34)	10	3 (115-119)	*0.00 (0.61)
E. rivularis															
D10	30	8 (165-185)	*0.20 (0.75)	30	2 (156-160)	*0.10 (0.35)	30	2 (109-111)	*0.03 (0.38)	30	3 (134-138)	*0.07 (0.24)	16	2 (115-117)	0.06 (0.18)
Total		12			17			6			3			4	

N = number of individuals; *A* = number of alleles; H_E =expected heterozygosity; H_O = observed heterozygosity; m = monomorphic * significant deviation from Hardy-Weinberg equilibrium ($p < 0.05$).

CHAPTER 3: The relationship between flower size and mating behaviour in British *Euphrasia* species

Abstract: The genus *Euphrasia* in Britain consists of a taxonomically complex group of morphologically similar hemi-parasitic annual plant species that are of high conservation importance. The complex consists of 19 currently recognised diploid and tetraploid species which show intra- and inter-specific variation in flower size. The primary objective of this chapter has been to investigate the relationship between flower size and mating behaviour. A survey of flower size variation was undertaken from 45 populations of 19 taxa (755 individual plants in total). From these populations, seven were selected that encompass the broadest range of flower sizes in diploid species. These populations were used for genetic assessments of mating behaviour using four nuclear microsatellite loci. Selfing rates (inferred from F_{is} estimates) were significantly and strongly correlated with flower size; the smaller the flower, the higher the rate of selfing. The highest selfing rate was estimated at 87% in a population of *Euphrasia rivularis* whose flower size (mean area of lower lip) was 3.95 mm^2. In contrast, selfing rates of < 40% were observed from populations of *Euphrasia officinalis* subsp. *rostkoviana* whose flower sizes were > 7.8 mm^2. Populations of *Euphrasia vigursii* with intermediate flower sizes, had intermediate inferred selfing rates. The potential factors influencing self-pollination in *Euphrasia* are discussed, as is the role of autogamy in generating taxonomic complexity and lineage differentiation within the genus.

3.1. Introduction

Insect pollinated self-compatible plants have a range of mating systems, from complete outcrossing to complete selfing, and even within species, different populations may evolve different rates of outcrossing (Charlesworth and Mayer, 1995; Vogler and Kalisz, 2001). The factors favouring outcrossing are advantages associated with the production of variable progeny, different from the parents, and avoidance of inbreeding depression. In addition, higher effective population sizes, reducing the loss of rare alleles by drift, contribute towards the maintenance of genetic variation (Jarne and Charlesworth, 1993). Selfing is favoured by the natural advantage of selfing genes, and by reproductive assurance in areas where there are constraints on allogamous pollination (Schoen *et al.*, 1996). Self-pollination may evolve by the reduction of herkogamy (anther-stigma separation) or dichogamy (timing of anther-stigma maturation) (Armbruster *et al.*, 2002; Dole, 1992) 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 result in 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 separate reproductive units. Transitions from cross- to self-pollination can thus have profound effects on evolutionary diversification within a plant group, with limited gene exchange between the selfing lines. This may result in the evolution of distinct multilocus genotypes possessing different combinations of character states. This can lead to difficulties in defining species limits, and selfing lines have been varyingly treated from full species to un-named intra-specific variants (Squirrell *et al.*, 2002; Hollingsworth, 2003). The subsequent choice of a species concept for a given taxonomic group 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 have important consequences for conservation prioritisation (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* in Britain is a classic example of a taxonomically complex group (19 currently recognised species of which at least seven are considered endemic, and 64 accepted hybrid combinations; Stace, 1997; Silverside, 1991). Both diploid and tetraploid taxa occur (Yeo, 1978a), 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 may therefore be thought to consist of a number of more or less selfing lines, with varying degree of isolation from one another. The extent of isolation will be a function of the breeding system, with increased autogamy leading to increased isolation. However, there are conflicting views as to the extent of self-pollination in the genus. Based on a paucity of observed pollinator visits, some researchers have concluded that all British *Euphrasia* taxa are highly selfing, irrespective of their floral biology (Pugsley, 1930). Other authors have suggested that

flower size has an impact on the level of selfing (Vitek, 1998), and the fact that even small flowered *Euphrasia* species have been invoked in the parentage of hybrid taxa (Pugsley, 1930; Yeo, 1978), indicates some outcrossing.

The object of this paper is to investigate the extent and variability of selfing among *Euphrasia* taxa, and the relationship of selfing with flower size and other plant characteristics. As *Euphrasia* species constitute almost 25% of the vascular plant species listed on the UK Biodiversity Action Plan priority 'Short List' for conservation (Anon, 1995b), understanding the biology underlying the taxonomic complexity in the group is a high priority. Specifically we wished to determine:

1. At what rate does selfing occur within the diploid taxa?
2. Does sufficient selfing occur to represent a significant reproductive barrier between taxa?
3. Is there variation amongst taxa with respect to selfing rates?
4. Is the degree of selfing within a population related to flower size?

Analysis of progeny genotype arrays in the context of the mixed mating model is a common method for estimating individual and mean population outcrossing rates (Ritland, 1990). This requires successful genotyping of families from a range of maternal parents. The small seeds, poor germination and low survival of hemi-parasitic *Euphrasia* seedlings on their host plants in a glasshouse situation makes such an approach impractical (Yeo, 1961). An alternative indirect method for estimating selfing rates was therefore adopted. 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 directly by the rate of self-pollination. By estimating F_{is} in a population using polymorphic co-dominant markers, the selfing rate of these populations can therefore be estimated (Brown, 1979).

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 display 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). The study was confined to four diploid taxa chosen to represent the full spectrum of flower size found in British *Euphrasia* species. Tetraploid taxa were not examined, due to difficulties of assessing the genotypes. Estimates of selfing rate inferred from F_{is} estimates were compared with measures of flower size and other plant characteristics.

3.2. Materials and Methods

3.2.1. Flower size measurements

A survey of flower size in British *Euphrasia* species was undertaken by measuring the corolla sizes of a total of 755 plants from 45 populations of 19 taxa (Table 3:1). Single flowers were removed from the first flowering node of each individual plant and placed in an eppendorf tube in the field. Within 24 hours of collection, the corollas were dissected into their upper and lower lobes. These were measured immediately or fixed onto card with sellotape to allow measurements to be taken later. For each corolla, the depth, and inner and outer width of the middle lower lobe were measured under a dissecting microscope. By assuming that the lower middle lobe shape approximated to a trapezium, it was possible to calculate the area of the middle lower lobe, a character thought to be most indicative of the attractiveness of the flower to pollinators (Karlsson, 1982; Figure 3:1). The length of the upper lip of the corolla was also measured. The total number of open flowers and total number of branches were recorded from each plant sampled.

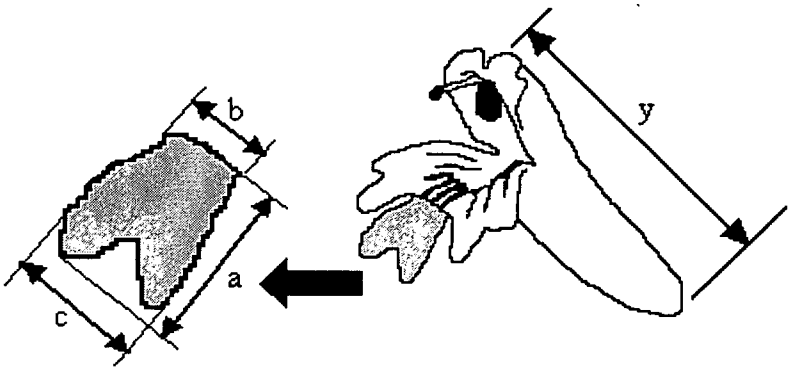
From this survey, seven populations were selected to represent the maximal variation in average corolla size among diploid taxa (Figure 3:2). These included three populations of *Euphrasia officinalis* subsp. *rostkoviana* (D3; D4; D5), two populations of *Euphrasia vigursii* (D15; D16), one population of *Euphrasia officinalis* subsp. *anglica* (D1) and one population of *Euphrasia rivularis* (D10). The population of *Euphrasia officinalis* subsp. *anglica* consisted of two subpopulations separated by approximately 50m; these were analysed separately (D1a, D1b).

Table 3:1. Location of 45 populations of *Euphrasia*, taken from all British taxa and their mean corolla measurements: area of middle lower lobe (mm^2) and length of upper lip (mm). "*Euphrasia fharaidensis*" is an informal taxon name given to a recognisable entity from the NW of Scotland that has yet to be formerly described.

Pop.	Taxon	Region	Population	Grid reference	No.	Mean area ± St.Dev. of lower lip (mm^2)	Mean length ± St.Dev. of upper lip (mm)
	Diploid						
D1	*Euphrasia officinalis* subsp. *anglica*	south-west England	Kit Hill	SX/374.715	29	5.00 ± 1.17	6.71 ± 0.70
D3	*Euphrasia officinalis* subsp. *rostkoviana*	Wales	Gower	SS/539.928	26	10.66 ± 1.61	9.53 ± 0.56
D4	*Euphrasia officinalis* subsp. *rostkoviana*	Wales	Caeae Llety-cyli	SN/603.535	26	7.87 ± 1.13	8.28 ± 0.54
D5	*Euphrasia officinalis* subsp. *rostkoviana*	Wales	Devil's Bridge	SN/751.735	25	7.89 ± 1.59	8.35 ± 0.71
D7	*Euphrasia rivularis*	Wales	Snowdon	SH/605.558	22	5.06 ± 1.05	6.13 ± 0.55
D8	*Euphrasia rivularis*	Wales	Cwm Glas	SH/616.565	19	5.69 ± 1.18	6.89 ± 0.68
D9	*Euphrasia rivularis*	north-west England	Stickle Tarn	NY/290.083	12	7.29 ± 1.17	6.94 ± 0.38
D10	*Euphrasia rivularis*	north-west England	Honiston Pass	NY/226.134	26	3.95 ± 0.85	5.85 ± 0.49
D12	*Euphrasia vigursii*	south-west England	Carvannel Downs	SW/641.446	27	4.65 ± 0.79	5.96 ± 0.36
D13	*Euphrasia vigursii*	south-west England	Porthtowan	SW/688.477	30	5.15 ± 1.15	6.26 ± 0.65
D15	*Euphrasia vigursii*	south-west England	Tregrethona Downs	SW/959.628	24	6.64 ± 1.19	7.10 ± 0.70
D16	*Euphrasia vigursii*	south-west England	Treslea Downs	SX/134.683	27	6.03 ± 1.63	6.30 ± 0.61
	Tetraploid						
T3	*Euphrasia arctica* subsp. *borealis*	north-west England	Rosthwaite	NY/255.159	28	6.37 ± 0.87	6.96 ± 0.42
T5	*Euphrasia arctica* subsp. *borealis*	mid Scotland	Tulloch Moor	NH/960.161	15	9.05 ± 2.02	7.90 ± 0.65
T7	*Euphrasia arctica* subsp. *borealis*	north-west Scotland	Siabost	NB/271.473	19	11.27 ± 3.33	8.24 ± 0.77
T9	*Euphrasia arctica* subsp. *borealis*	north Scotland	Durness	NC/405.669	11	10.63 ± 2.71	7.96 ± 1.16
T10	*Euphrasia arctica* subsp. *borealis*	north Scotland	Reay	NC/958.653	20	13.07 ± 2.26	8.98 ± 0.72
T11	*Euphrasia cambrica*	Wales	Snowdon	SH/605.558	8	0.87 ± 0.30	3.64 ± 0.24
T13	*Euphrasia campbelliae*	north-west Scotland	Mealasta	NA/994.235	6	3.80 ± 0.83	6.59 ± 0.38
T16	*Euphrasia confusa*	south-west England	Kit Hill	SX/374.715	13	3.03 ± 0.92	5.85 ± 0.59
T17	*Euphrasia confusa*	north Scotland	Inchnadamp	NC/267.213	5	3.21 ± 0.32	5.82 ± 0.52
T18	"*Euphrasia fharaidensis*"	north Scotland	Inchnadamp	NC/272.210	12	2.51 ± 0.44	4.97 ± 0.21

Table 3:1. *Continued*

Pop.	Taxon	Region	Population	Grid reference	No.	Mean area ± St.Dev. of lower lip (mm^2)	Mean length ± St.Dev. of upper lip (mm)
T19	*"Euphrasia fharaidensis"*	north Scotland	Rhiconich	NC/253.523	11	2.25 ± 0.37	5.10 ± 0.43
T23	*Euphrasia foulaensis*	north-west Scotland	Mealasta	NA/993.240	6	2.08 ± 0.51	5.11 ± 0.22
T24	*Euphrasia foulaensis*	north-west Scotland	Mangurstadh	NB/005.315	10	2.33 ± 0.54	5.05 ± 0.28
T29	*Euphrasia frigida*	mid Scotland	Creag an Lochain	NN/592.411	15	3.15 ± 0.61	6.46 ± 0.90
T36	*Euphrasia heslop-harrisonii*	north-west Scotland	Seilebost	NG/083.668	13	2.65 ± 0.98	5.62 ± 0.65
T37	*Euphrasia marshallii*	north Scotland	Betty Hill	NC/705.628	20	3.91 ± 0.31	6.09 ± 0.40
T38	*Euphrasia marshallii*	north Scotland	Melness	NC/577.653	12	6.69 ± 1.27	6.46 ± 0.39
T40	*Euphrasia micrantha*	south-west England	Wheal Busy	SW/742.423	20	2.46 ± 0.58	5.67 ± 0.37
T41	*Euphrasia micrantha*	south-west England	Chapel Porth	SW/697.494	9	1.44 ± 0.39	4.40 ± 0.68
T42	*Euphrasia micrantha*	mid Scotland	Tulloch Moor	NH/962.167	17	3.25 ± 0.58	5.75 ± 0.56
T43	*Euphrasia nemorosa*	Wales	Cae Biaen Dyffryn	SN/606.447	14	2.34 ± 0.52	6.23 ± 0.65
T46	*Euphrasia nemorosa*	north-west Scotland	Dail Beag	NB/227.458	20	5.70 ± 1.08	6.83 ± 0.39
T48	*Euphrasia nemorosa*	north Scotland	Fharaid Head	NC/392.687	19	7.30 ± 1.46	7.27 ± 0.79
T49	*Euphrasia nemorosa*	north Scotland	Dunnett Bay	ND/223.700	20	10.76 ± 2.54	7.73 ± 0.73
T50	*Euphrasia ostenfeldii*	Wales	Snowdon	SH/602.556	10	2.18 ± 0.36	5.72 ± 0.51
T52	*Euphrasia ostenfeldii*	north Scotland	Inchnadamp	NC/272.210	14	1.54 ± 0.22	4.83 ± 0.27
T54	*Euphrasia pseudokerneri*	east England	Therfield Heath	TL/330.395	20	10.25 ± 1.87	9.43 ± 0.78
T55	*Euphrasia pseudokerneri*	east England	Fleam Dyke	TL/545.545	20	11.39 ± 2.12	9.28 ± 1.09
T56	*Euphrasia pseudokerneri*	east England	Devil's Ditch	TL/617.616	20	11.93 ± 1.77	9.17 ± 0.89
T57	*Euphrasia scottica*	north-west England	Honiston Pass	NY/230.136	5	1.74 ± 0.50	4.75 ± 0.43
T62	*Euphrasia scottica*	north-west Scotland	Carlabhagh	NB/190.413	14	2.62 ± 0.29	4.98 ± 0.32
T65	*Euphrasia tetraquetra*	south-west England	Wicca Pool	SW/465.403	9	2.08 ± 0.36	5.19 ± 0.39
T67	*Euphrasia tetraquetra*	Wales	Mewslade	SS/419.873	7	3.43 ± 1.16	5.81 ± 0.42



$$\text{area of trapezium} = \frac{a(b+c)}{2}$$

Figure 3:1. Measurements of corolla size. y = length of upper lip taken from base of corolla tube to base of the lobes of the upper lip. Area of middle lower lobe, as indicated by grey shading, calculated by approximation to the area of a trapezium.

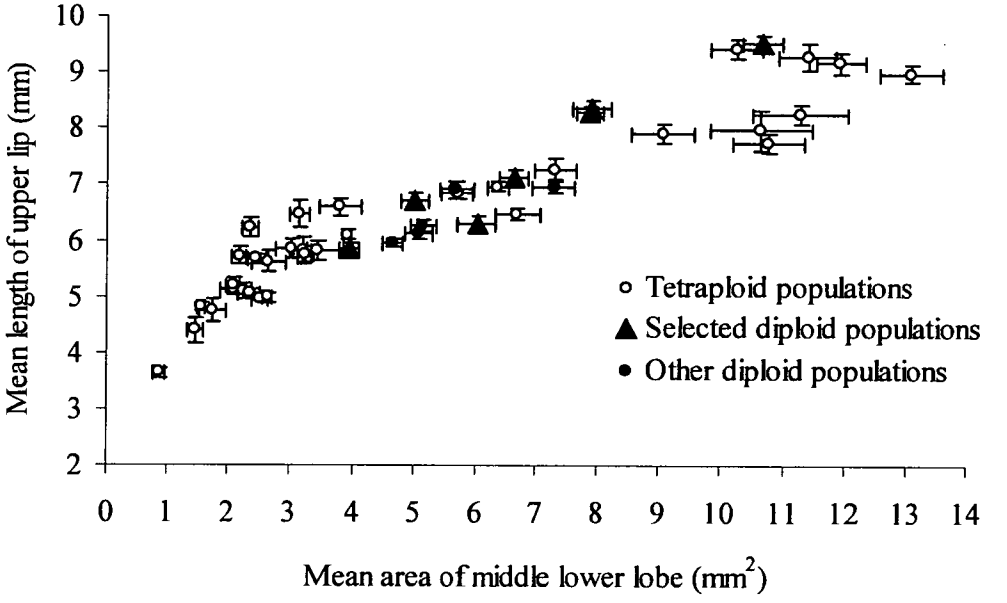


Figure 3:2. Average corolla size from the 45 different *Euphrasia* populations listed in Table 3:1. The triangles represent the diploid populations used for the genetic analysis.

3.2.2. Genetic analyses

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 1.5 ml eppendorf tubes, using the hexadecyltrimethylammonium bromide (CTAB) method, as described by Saghri-Maroof *et al.* (1984), with modifications, as described by Doyle and Doyle (1987). 0.2% 2-mercaptoethanol and 0.1% insoluble polyvinylpolypyrrolidine (PVPP) were added to the 2x 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.

Four microsatellite loci (*Ene1*, *Ene2*, *Ene3*, *Ene4*) were amplified using primers and PCR conditions as described in Chapter 2. 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).

3.2.3. Data analysis

The microsatellite data were formatted for analysis using the Microsatellite Toolkit (Parks, 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 fixation index, F_{is} (Wright, 1965), was computed for each locus and across all loci, for each population using FSTAT: version 2.9.3.2 (Goudet, 2001). Significance values were estimated based on randomisation tests. The selfing rate was estimated from the multilocus estimate of F_{is} using the equation, $s = (2F_{is})/(1+F_{is})$ (Allard *et al.*, 1969). For each population, the mean and standard error were calculated for the area of middle lower

lobe, number of branches and number of open flowers per plant. Correlations between all the pairwise population mean statistics were explored using the statistical package, MINITAB®.

3.3. Results

3.3.1. Flower size measurements

Across all taxa, the area of the middle lower lip ranged from 13.07 mm^2 to 0.87 mm^2 (Table 3:1; Figure 3:2). The largest flowers were found in the tetraploid species *Euphrasia arctica* subsp. *borealis*, but most of the taxonomic diversity in Britain (15 out of 19 taxa) was associated with flowers in the smaller half of this size range (e.g. on average < 6.5 mm^2; Table 3:1). The smallest flowers of all were found in the diminutive tetraploid endemic *Euphrasia cambrica* (mean area of the middle lower lip = 0.87 mm^2). Within the seven diploid taxa selected for genetic analysis, the area of the middle lower lip ranged from 10.66 mm^2 to 3.95 mm^2. *Euphrasia officinalis* subsp. *rostkoviana* had the largest flowers, *Euphrasia officinalis* subsp. *anglica* and *Euphrasia rivularis* the smallest and *Euphrasia vigursii* was intermediate (Table 3:1, Figure 3:2). In populations of these taxa, the correlation between the mean flower size and either mean number of branches or mean total number of flowers per plant, was positive but not significant ($r = 0.280$, $p = 0.502$; $r = 0.399$, $p = 0.328$, respectively). The mean number of branches was also positively correlated with the mean total number of flowers in a population but this was also not significant ($r = 0.568$, $p = 0.142$).

3.3.2. Microsatellite variation

In the total dataset, all loci were polymorphic, with at total of 38 alleles detected (12 from *Ene1*, 17 from *Ene2*, 6 from *Ene3* and 3 from *Ene4*). Within taxa, the proportion of loci that were polymorphic was 100% in *Euphrasia officinalis* subsp. *rostkoviana, Euphrasia vigursii* and *Euphrasia rivularis*, but only three of the four loci were polymorphic in the population of *Euphrasia officinalis* subsp. *anglica*. The mean number of alleles per locus and gene diversities at the taxon level were: *Euphrasia officinalis* subsp. *rostkoviana* $A = 7.5$, $H_E = 0.696$; *Euphrasia officinalis* subsp. *anglica* $A = 2.25$, $H_E = 0.395$; *Euphrasia vigursii* $A = 4.25$, $H_E = 0.499$; and

Euphrasia rivularis $A = 3.75$, $H_E = 0.430$. At the population level, the greatest allelic and gene diversity was found in population D5 of *Euphrasia officinalis* subsp. *rostkoviana* ($A = 5.5$ and $H_E = 0.606$; Table 3:2). The lowest diversity was found in population D15 of *Euphrasia vigursii* which was fixed for a single allele at each locus ($A = 1.0$ and $H_E = 0.0$), and also in one sub-population of *Euphrasia officinalis* subsp. *anglica* which was also invariant (Table 3:2). The lack of genetic variation in these populations precluded the calculation of the inbreeding coefficient from these plants.

From the remaining populations which showed allelic variation, of the 23 single locus estimates of F_{is}, 21 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 3:2). All but one (population D4) showed a significant deviation from panmixia (Table 3:2).

3.3.3. Inbreeding coefficient and flower morphology

There was a significant negative correlation between flower size, and F_{is} or the inferred selfing rate, explaining well over 80% of the variation in each case ($r = -0.893$, $p = 0.016$; $r = -0.877$, $p = 0.022$, respectively) (Figure 3:3). The smallest flowers (area of middle lower lobe < $5mm^2$) had the highest inferred selfing rates (> 80%) whereas larger flowered taxa (area of middle lower lobe > $7.5mm^2$) showed evidence of increased outcrossing ($s < 50\%$).

Negative correlations were found between F_{is} and both the mean number of flowers and branches per plant, but in neither case was the correlation significant ($r = -0.701$, $p = 0.121$; and $r = -0.583$, $p = 0.224$, respectively). There was 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 proportion of loci that were polymorphic ($r = 0.234$; $p = 0.577$).

Table 3:2. Morphological measurements, mean number of alleles per locus (A), proportion of polymorphic loci (P), expected and observed heterozygosities (H_E and H_O), single and multilocus inbreeding coefficients (F_{is}), and inferred selfing rate (s), for eight populations of diploid *Euphrasia*. For population codes see Table 3:1. NS = not significant, * = $p < 0.05$.

Taxon	Pop.	No.	Mean lower lip area (mm^2)	Mean branch number per plant	Mean 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	s multilocus
E.officinalis subsp. *rostkoviana*	D3	28	10.66 ± 0.32	0.50 ± 0.13	3.33 ± 0.30	4.00	1.00	0.129 NS	0.151 NS	0.235 NS	0.439 *	0.547	0.420	0.236 *	0.382
E.officinalis subsp. *rostkoviana*	D5	30	7.89 ± 0.32	2.60 ± 0.36	4.17 ± 0.58	5.50	1.00	0.188 *	0.560 *	0.597 *	-0.221 NS	0.606	0.459	0.246 *	0.395
E.officinalis subsp. *rostkoviana*	D4	26	7.87 ± 0.22	1.37 ± 0.31	2.20 ± 0.28	2.75	1.00	0.136 NS	-0.020 NS	0.209 NS	0.212 NS	0.264	0.221	0.166 NS	0.285
E.vigursii	D15	28	6.64 ± 0.24	1.07 ± 0.27	1.83 ± 0.29	1.00	0.00	N/A	N/A	N/A	N/A	0.000	0.000	N/A	N/A
E.vigursii	D16	28	6.03 ± 0.31	1.17 ± 0.32	1.83 ± 0.34	4.25	1.00	0.526 *	0.763 *	0.332 *	0.432 *	0.582	0.264	0.551 *	0.711
E.officinalis subsp. *anglica*	D1A	15	5.56 ± 0.20	1.27 ± 0.23	4.73 ± 0.67	1.00	0.00	N/A	N/A	N/A	N/A	0.000	0.000	N/A	N/A
E.officinalis subsp. *anglica*	D1B	15	4.40 ± 0.33	0.60 ± 0.19	1.93 ± 0.27	2.25	0.75	0.744 *	1.000 *	0.498 *	N/A	0.377	0.100	0.741 *	0.851
E.rivularis	D10	30	3.95 ± 0.17	0.19 ± 0.09	1.29 ± 0.16	3.75	1.00	0.736 *	0.714 *	0.914 *	0.730 *	0.430	0.100	0.770 *	0.870

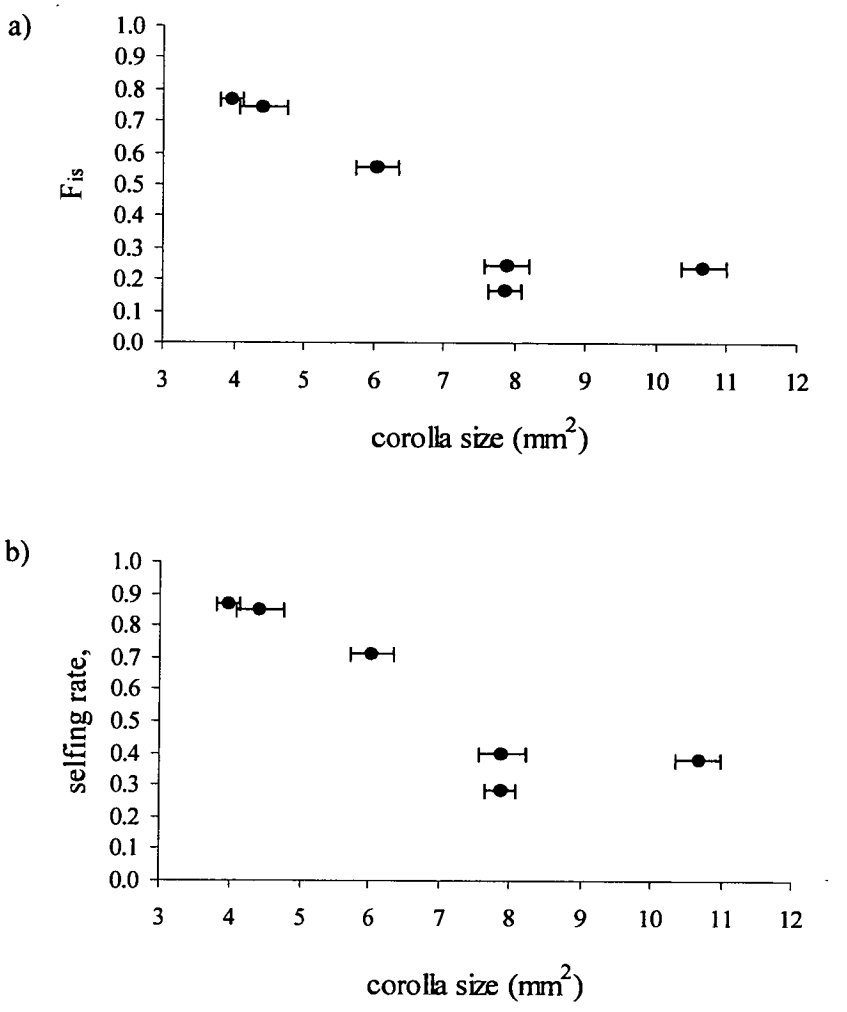


Figure 3:3. The relationship between corolla size (the area of the middle lower lobe) and a) inbreeding coefficient, F_{is}; b) inferred population selfing rate, s for each of six *Euphrasia* populations.

3.4. Discussion

3.4.1. Estimates of breeding behaviour

Selfing occurs in all four taxa, varying among populations; there was a strong correlation between the inferred selfing rate and the mean flower size of a population. *Euphrasia officinalis* subsp. *rostkoviana*, whose populations have larger more showy flowers, was found to be predominantly outcrossing with the least degree of inferred selfing (populations D3, D4, and D5; Figure 3:3, Table 3:2).

Estimates of selfing rates from this taxon were the lowest in this study, and in one of the three populations, the F_{is} estimate was not significantly different from zero. The smaller flowered taxa, *Euphrasia officinalis* subsp. *anglica* and *Euphrasia rivularis*, on the other hand, had the highest degree of inferred selfing (s > 80%; Table 3:2). This is correlated with their smaller, less showy corollas, poor nectaries, and a minimal degree of herkogamy and dichogamy (Schulz, as reported by Knutt, 1909). The medium flowered *Euphrasia vigursii* population had an intermediate degree of inferred selfing.

Thus, despite observations of pollinator behaviour leading to suggestions that all taxa are predominantly selfing (Pugsley, 1930), our study has shown varying levels of self-pollination with a clear correlation with flower size. In this respect it is worth stressing that the diploid taxa studied here represent a nested subset of the range of flower sizes in British *Euphrasia* species (Figure 3:2). It may be that the range of selfing rates we have observed, in turn, reflect a nested subset of the selfing rates for the group as a whole. While we did not investigate any of the tetraploid species, many of these have smaller flowers than the diploid taxa examined here. It seems a reasonable extrapolation from our data that these very small flowered taxa will show very high levels of self-pollination and some may be completely autogamous.

While the correlation between flower size and inferred selfing rates suggest that within flower pollination is the most obvious cause of selfing, the extent to which geitonogamous pollination occurs in *Euphrasia* is unknown. In contrast to the significant correlation between flower size and inferred selfing rate, our study showed a less marked, non-significant correlation between inferred selfing rate and the number of flowers or branches. The greater the number of flowers on a plant, the greater the potential for geitonogamous pollination, but the issue is complicated by the fact that greater floral displays may serve to increase pollinator visits both to individual plants and populations as a whole. Further work is required to establish the extent to which any of the inferred selfing in *Euphrasia* is attributable to geitonogamy. Likewise, it is not possible to draw any conclusions regarding the maintenance of genetic variation with respect to outcrossing rates from this study. No

correlation was detected between levels of selfing and various metrics of genetic diversity, but the high mutation rates of microsatellite loci, coupled with population size effects (which were not controlled for in the study) prevent conclusions being drawn from these data.

3.4.2. Potential factors influencing the breeding system in British *Euphrasia*

This study has shown the presence of mixed mating system, related to variation in corolla size, but did not examine the ecological and genetic factors driving the evolution of autogamy in this group. Selection for high levels of self-pollination is likely under conditions of poor pollinator availability, and such breeding behaviour has been proposed for the occurrence of high population densities of the small flowered European species, *Euphrasia willkommii* on high mountains in Spain. Here, despite insect visitation as low as 3%, a two year study found that approximately 80% of the ovules were pollinated (Gómez, 2002).

In Britain, the variation in corolla size and its correlation with outcrossing rates raises the question whether there are distinct geographical/ecological distributions of flower sizes that suggest local adaptations with respect to local pollinator availability. There are, however, no obvious trends and small and large flowered taxa often occur in sympatry. The greatest density of small flowered taxa in Britain is on the north-west coast of Scotland (Preston *et al.*, 2002) where pollinator density is comparatively low but this area is also the home to the largest flowered British *Euphrasia* species, *Euphrasia arctica*.

In addition to the observed poor pollinator service (Yeo, 1966), it is possible that genetic load within outcrossing taxa may frequently occur below the level at which selfing genes are at a selective advantage and so are able spread within the population (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 this genetic load.

3.4.3. 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 for each flower to potentially either self or outcross (Vogler and Kalisz, 2001). The fact that some named taxa, such as *Euphrasia vigursii*, have consistently intermediate flower sizes suggests that there is some stability in these intermediate flower sizes, and potentially intermediate selfing rates. Even in large flowered taxa, such as *Euphrasia officinalis* subsp. *rostkoviana*, some selfing occurs (Figure 3:3, Table 3:2). This may be attributable to a delayed selfing mechanism. Marked corolla tube elongation occurs following anther dehiscence, and reduces initial anther-stigma separation (Pugsley, 1930). The possibility of some level of self-pollination, even in the predominantly outcrossing taxa, may serve as a useful mechanism of reproductive assurance for these annual plants.

3.4.4. Reproductive isolation and the potential for taxonomic diversification

The correlation between flower size and inferred selfing rates, and particularly the high selfing rates found in the smaller flowered populations, 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% under each generation of complete selfing, genetic differences can quickly be fixed between selfing lines and their outcrossing progenitor, and selfing taxa can acquire characteristic multi-locus character combinations (Squirrell *et al.*, 2002). Thus, in common with other plant genera (Jain, 1976), isolation caused by breeding system transitions may contribute towards the high diversity of often sympatric *Euphrasia* species in Britain. Certainly if one extrapolates from the inferred selfing rates to the total range of flower sizes in British *Euphrasia* species, the importance of self-pollination is evident (Figure 3:4). 11 out of the 19 taxa have flower sizes which fall within the zone of inferred selfing rates of > 80%. A further four taxa have mean flower sizes which are similar or smaller than

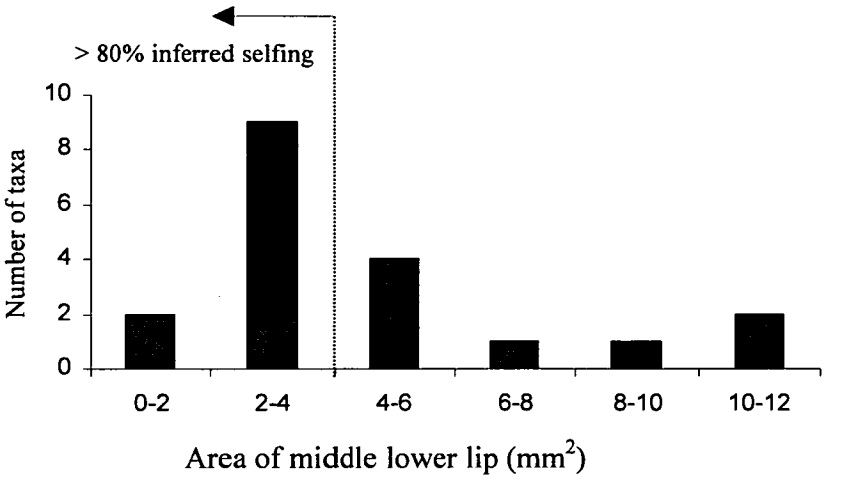


Figure 3:4. The number of British *Euphrasia* species in relation to their mean flower sizes, based on the data in Table 3:1. The area to the left of the dotted line is the area in which inferred selfing rates exceed 80% based on genetic analysis of the populations examined in the current study (see Figure 3:3).

the population of *Euphrasia vigursii* (mean area of middle lower lip 6.03 mm^2) that had a mean inferred selfing rate of s = 0.711. Thus the recurrent evolution of autogamous lineages provides one explanation for presence of high levels of *Euphrasia* diversity in a recently glaciated landscape such as the British Isles. A similar process has been described within a British orchid complex. 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* Crantz.

Confirmation that *Euphrasia* has a mixed mating system, with differing rates of selfing 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.

CHAPTER 4: Partitioning of genetic variation within British *Euphrasia*. Is there an association with ploidy level?

Abstract: British *Euphrasia* consists of a polyploid complex containing both diploid and tetraploid taxa. Evidence from field observations and past cross-fertilisation experiments suggest that gene flow between these two ploidal groups is rare but that the barrier is not absolute. This is supported by the molecular study of British *Euphrasia* described in this chapter. Here significant structuring of both chloroplast haplotypes and AFLP bands are found across ploidal groups. Evidence for hybridisation between two diploid populations and the tetraploid group is presented. In addition high numbers of AFLP bands unique to the tetraploid group support the hypothesis that this group arose from allopolyploidy, with the distribution of chloroplast haplotypes further suggesting that this polyploid event occurred on at least three occasions.

4.1. Introduction

Polyploidy has been estimated to have occurred in the ancestry of 50 - 80% of angiosperms (Masterson, 1994). This suggests that polyploidy has been an important process in the generation and maintenance of variation amongst flowering plants (Thompson and Lumaret, 1992). Chromosomal doubling, either directly within an individual plant (autopolyploidy), or from hybridisation between two genetically dissimilar individuals (segmental allopolyploidy or allopolyploidy), can lead to the immediate generation of an isolated gene pool. In the extreme case, this can lead to the formation of a new species. In Britain, for example, *Senecio cambrensis* Rosser (Ashton and Abbott, 1992) and *Spartina anglica* C.E.Hubbard (Raybould *et al.*, 1991) are thought to have originated from recent allopolyploidisation, following the introduction of one of their parental taxa into the British flora.

Within polyploid groups, molecular evidence has increasingly been used to show multiple origins for polyploid taxa (Soltis and Soltis, 1993; Soltis and Soltis, 1999). Phylogenetic studies of chloroplast RFLP and sequences, for example, led Segraves *et al.* (1999) to suggest that the tetraploid *Heuchera grossulariifolia* Rydb. has potentially arisen up to seven times along the rivers of Idaho and western Montana, USA. Even this high rate of polyploid origin may be an underestimate in some polyploid groups. Using RAPD markers, Cook *et al.* (1998) suggested that each

population of the tetraploid taxa, *Tragopogon miscellus* Ownbey and *Tragopogon mirus* Ownbey may represent independent origins of polyploidy.

High rates of polyploidy may make important contributions to the overall taxonomic complexity within a polyploid complex (Leitch and Bennet, 1997). On investigating polyploids within the *Draba* L. polyploid complex in the Nordic area, Brochmann *et al.* (1992) suggested that multiple origins may have been so extensive as to cause blurring of original discontinuities in morphology and make the establishment of the evolutionary history of populations difficult, if not impossible. Reticulation between populations may be further increased if the two ploidy gene pools are incompletely isolated. Meiotic irregularities can act as a barrier to gene flow between ploidy levels, with the production of infertile hybrids. However, a number of studies have shown that fertile hybrids, though rare, may be produced. Lord and Richards (1977) showed that morphological variation in the lower labellum of an orchid population near Durham, England, was the result of introgressive hybridisation between diploid *Dactylorhiza fuchsii* (Druce) Soó and tetraploid *Dactylorhiza purpurella* (T. & T. A. Steph.) Soó. The production of such hybrid swarms may act as a bridge between ploidy levels and transfer unique molecular markers from one parental gene pool to another. For example, introduced tetraploid populations of *Dactylis glomerata* L. have atypical alleles in low frequencies, thought to be attributable to gene flow from the native sympatric diploid populations in Galicia, Spain (Lumaret and Barrientos, 1990).

Within *Euphrasia,* the annual European species belong to a polyploid complex containing both tetraploid (2n=44) and diploid (2n=22) taxa (von Witsch, as reported by Yeo, 1954). Chromosome counts of British taxa have confirmed that the same two chromosomal groups are present (Yeo, 1954; Löve, 1956, as reported by Yeo, 1966). Representatives of the two ploidy groups occur sympatrically within Britain (Yeo, 1956). Despite this they remain morphologically distinct with qualitative differences within the indumentum, with diploid species possessing higher numbers of glandular hairs on the upper stem and leaves. These glands differ from the tetraploids in being ellipsoidal, not globular in shape and, occur on longer, more variable stalks, that

contain three or more cells (Silverside, 1990b). This has led to taxonomic separation of diploid and tetraploid taxa in Britain, with the diploid taxa all ascribed to a single series, *Euphrasia* within the subsection *Euphrasia* (Pugsley, 1930; Yeo, 1978a).

Cross-fertilisation experiments have shown poor seed set and low fertility of hybrids between the two ploidy levels (Yeo, 1959; Yeo, 1966; Karlsson, 1982; summarised in Figure 4:1). This significant reproductive barrier supports the taxonomic split of these two sympatric groups. However, the production of viable seed in approximately 0.1% of interploidal crosses and the detection of a triploid individual from a natural population (Yeo, 1954), suggests that this reproductive barrier is not absolute. Partially fertile F_1 hybrids may be produced between sympatric populations of differing ploidy level, with the formation of subsequent hybrid generations (Yeo, 1956).

The two ploidy groups have extensive ranges throughout Britain. The northern limit of the diploid group extends as far as mid Scotland and the tetraploid group extends further to the northern limits of Britain (Preston *et al.*, 2002). The overlapping distribution of two ploidy levels within British *Euphrasia*, and hence the potential for gene flow between diploid and tetraploid groups, may be an important factor to the generation of the diversity and taxonomic complexity of this group. The object of this chapter is to determine the extent of the reproductive barrier between the diploid and tetraploid British taxa of *Euphrasia*. Under the null hypothesis that there is no reproductive barrier, allowing inter-ploidal gene flow, mixing and sharing of molecular markers between groups implies few unique markers within either group. Marker variation among groups will be no greater than within groups. By estimating the amount and distribution of any unique molecular markers within and among the two groups I test this null hypothesis. Rejection of the null hypothesis implies the presence of a reproductive barrier between diploid and tetraploid taxa.

Given limited dispersal within and between populations (Barker, 1982; Molau, 1993), any significant structuring of markers between groups could be confounded

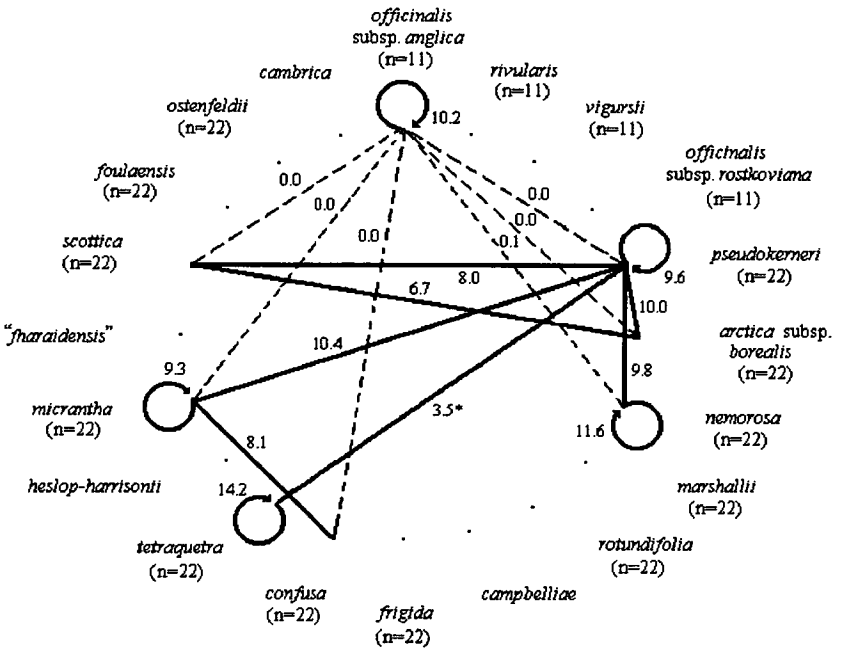


Figure 4:1. Summary of fertility results taken from artificial crossing experiments between British diploid and tetraploid taxa (Yeo, 1966; Karlsson, 1982). Heavy line represents fertile cross, dotted line represents infertile cross. n = haploid chromosome number; figures on lines = average number of seeds produced per capsule; * = some seeds lost during count.

by geographical structuring of markers, independent of any reproductive isolation. Given limited dispersal within and between populations (Barker, 1982; Molau, 1993), any significant structuring of markers between groups could be confounded by geographical structuring of markers, independent of any reproductive isolation. For example, if samples of diploid and tetraploid taxa are taken from different geographical localities then limited dispersal rather than ploidy level barriers could lead to differentiation. By employing a hierarchical sampling strategy across the range of taxa throughout Britain, I specifically wish to determine:

1. The distribution of molecular markers within and between ploidy groups.
2. The presence of unique molecular markers within each group.
3. The importance of geographical structuring and/or an inter-ploidal reproductive barrier in determining the distribution of these markers.

In addition previous cytological studies have not detected multivalents in tetraploid or triploid individuals (Yeo, 1954; Yeo, 1959). This led Yeo (1976) to suggest that

tetraploid taxa originated from past hybridisation event(s). By examining two classes of molecular markers across both diploid and tetraploid taxa, I also wish to determine whether there is any further evidence to support the hypothesis of an allopolyploid, as opposed to autopolyploid, origin of the tetraploids.

4.2. Methods

4.2.1. Materials

72 populations of *Euphrasia* were sampled from seven regions throughout Britain. These regions were south-west England, east England, north-west England, Wales, mid Scotland, north-west Scotland and north Scotland (Figure 4:2; Table 4:1). Representative taxa were sampled within each region, so that overall sampling reflected the range of British taxa. Plants from these sampled taxa were allocated to ploidal groups based on previously published chromosomal counts for individual species (Yeo, 1954), rather than de novo chromosome counts for each plant or population. 16 populations were sampled from four diploid taxa and 56 populations from 15 tetraploid taxa (Table 4:1). For each population, fresh plant tissue was collected from between 3 to 13 individuals (Table 4:1). Samples, consisting of a minimum of three leaves per plant, were either placed in i) a 1.5 ml eppendorf tube and frozen in the field, with a Taylor Wharton cryopreserver (temp -197°C), before long term storage at -80°C or ii) silica gel.

4.2.2. DNA extraction

DNA extraction was performed using the CTAB method, as described by Saghai-Maroof *et al.* (1984), with modifications as described by Doyle and Doyle (1987) and the inclusion of 0.2% 2-mercaptoethanol and 0.1% insoluble polyvinylpoly-pyrrolidine (PVPP) to the 2x CTAB buffer (1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% hexadecyltrimethylammonium bromide). Two chloroform/ isoamyl alcohol extractions were performed and after overnight precipitation with freezer-cold isopropanol, the resultant DNA pellet was resuspended in 50 µl of Tris-EDTA, pH 8 (TE). Using this procedure, approximately 0.5 to 1 µg of DNA was extracted from each individual plant. The quality and quantity of extracted DNA was assessed on 1.0% agarose (Bioline) gels.

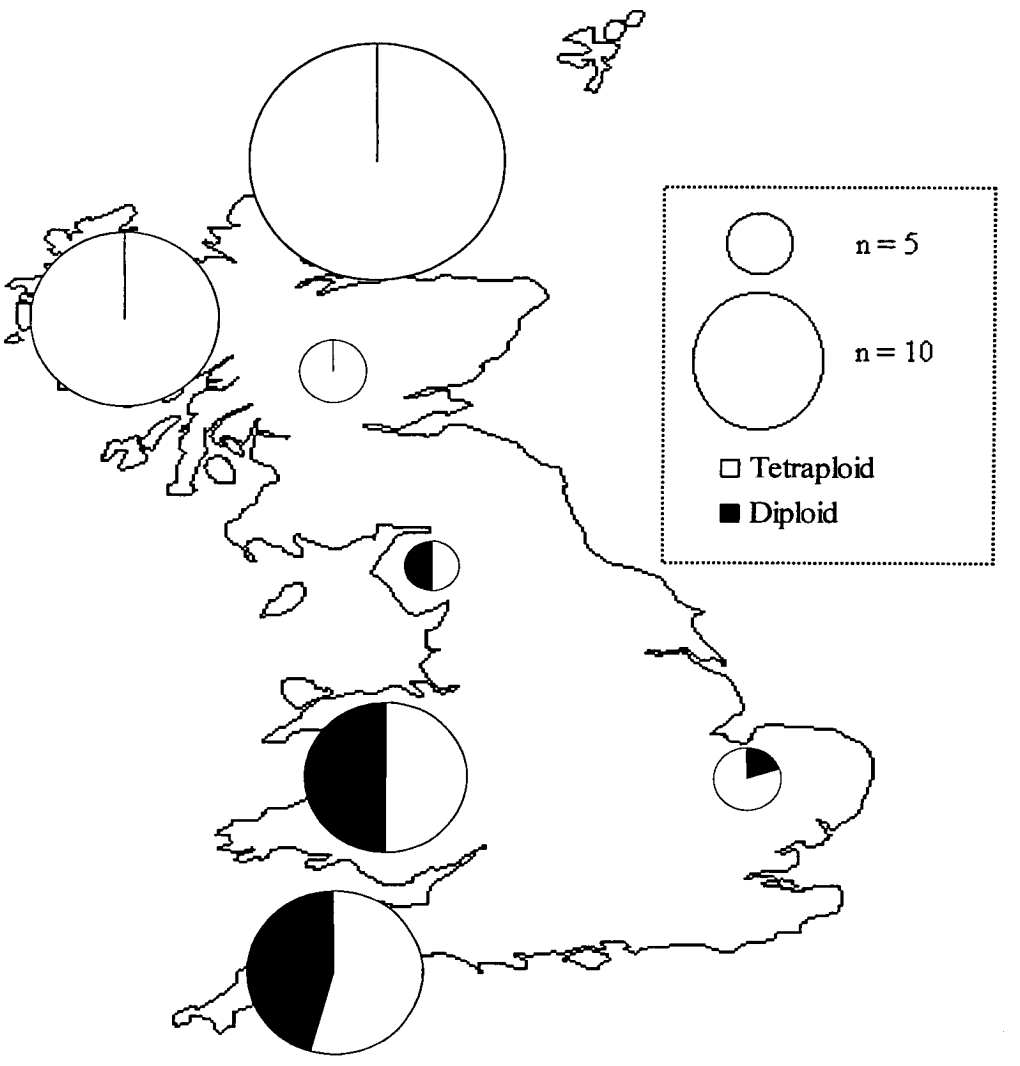


Figure 4:2. Distribution of diploid (black) and tetraploid (white) sites within Britain, grouped according to region. The areas of the circles represents number of populations within each region. n = number of populations.

Table 4:1. Hierarchical sampling of *Euphrasia* throughout Britain. Within seven regions, both diploid and tetraploid populations were sampled, across a range of taxa, for chloroplast PCR-RFLP and AFLP analysis. N (cp) = number of individuals sampled in each population for chloroplast PCR-RFLP analyses; N (AFLP) = number of individuals sampled in each population for AFLP analyses.

Number	Ploidy	Taxon	Population	Grid reference	N (cp)	N (AFLP)
South-west England						
D1	Diploid	*Euphrasia officinalis* subsp. *anglica*	Kit Hill	SX/374.715	5	3
D11	Diploid	*Euphrasia vigursii*	Wicca Pool	SW/465.403	5	3
D12	Diploid	*Euphrasia vigursii*	Carvannel Downs	SW/641.446	9	-
D13	Diploid	*Euphrasia vigursii*	Porthtowan	SW/688.477	10	3
D14	Diploid	*Euphrasia vigursii*	Ventogimps Moor	SW/781.511	10	-
D15	Diploid	*Euphrasia vigursii*	Tregrethona Downs	SW/959.628	10	3
D16	Diploid	*Euphrasia vigursii*	Treslea Downs	SX/134.683	8	3
T15	Tetraploid	*Euphrasia confusa*	Bunny Hill	SX/119.678	4	3
T16	Tetraploid	*Euphrasia confusa*	Kit Hill	SX/374.715	4	3
T40	Tetraploid	*Euphrasia micrantha*	Wheal Busy	SW/742.423	5	3
T41	Tetraploid	*Euphrasia micrantha*	Chapel Porth	SW/697.494	5	2
T65	Tetraploid	*Euphrasia tetraquetra*	Wicca Pool	SW/465.403	4	3
T66	Tetraploid	*Euphrasia tetraquetra*	Porthtowan	SW/689.479	4	2
Total					83	31
Wales						
D3	Diploid	*Euphrasia officinalis* subsp. *rostkoviana*	Gower	SS/539.928	5	2
D4	Diploid	*Euphrasia officinalis* subsp. *rostkoviana*	Caeae Llety-cyli	SN/603.535	5	2
D5	Diploid	*Euphrasia officinalis* subsp. *rostkoviana*	Devil's Bridge	SN/751.735	5	2
D6	Diploid	*Euphrasia rivularis*	Moel Siabod	SH/711.549	9	2
D7	Diploid	*Euphrasia rivularis*	Snowdon	SH/605.558	10	1
D8	Diploid	*Euphrasia rivularis*	Cwm Glas	SH/616.565	9	-
T1	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Rhos Common	SN/791.078	5	-
T2	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Craig Branddu	SN/715.458	5	-
T11	Tetraploid	*Euphrasia cambrica*	Snowdon	SH/605.558	3	1
T43	Tetraploid	*Euphrasia nemorosa*	Cae Biaen Dyffryn	SN/606.447	5	-
T50	Tetraploid	*Euphrasia ostenfeldii*	Snowdon	SH/602.556	5	2

Table 4:1. *Continued.*

Number	Ploidy	Taxon	Population	Grid reference	N (cp)	N (AFLP)
T67	Tetraploid	*Euphrasia tetraquetra*	Mewslade	SS/419.873	5	3
Total					71	15
East England						
D2	Diploid	*Euphrasia officinalis* subsp. *anglica*	Box Hill	TQ/170.510	5	2
T44	Tetraploid	*Euphrasia nemorosa*	Haslingfield	TL/408.517	5	-
T54	Tetraploid	*Euphrasia pseudokerneri*	Therfield Heath	TL/330.395	5	-
T55	Tetraploid	*Euphrasia pseudokerneri*	Fleam Dyke	TL/545.545	5	-
T56	Tetraploid	*Euphrasia pseudokerneri*	Devil's Ditch	TL/617.616	5	-
Total					25	2
North-west England						
D9	Diploid	*Euphrasia rivularis*	Stickle Tarn	NY/290.083	5	2
D10	Diploid	*Euphrasia rivularis*	Honiston Pass	NY/226.134	10	3
T3	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Rosthwaite	NY/255.159	4	-
T57	Tetraploid	*Euphrasia scottica*	Honiston Pass	NY/230.136	5	1
Total					24	6
Mid Scotland						
T4	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Straloch	NO/035.636	3	-
T5	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Tulloch Moor	NH/960.161	4	-
T29	Tetraploid	*Euphrasia frigida*	Creag an Lochain	NN/592.411	5	-
T42	Tetraploid	*Euphrasia micrantha*	Tulloch Moor	NH/962.167	5	3
T59	Tetraploid	*Euphrasia scottica*	Dalwhinnie	NN/642.850	5	-
Total					22	3
North-west Scotland						
T7	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Siabost	NB/271.473	5	2
T13	Tetraploid	*Euphrasia campbelliae*	Mealasta	NA/994.235	5	2
T14	Tetraploid	*Euphrasia campbelliae*	Islibhig	NA/993.280	5	2
T23	Tetraploid	*Euphrasia foulaensis*	Mealasta	NA/993.240	4	3
T24	Tetraploid	*Euphrasia foulaensis*	Mangurstadh	NB/005.315	5	3
T30	Tetraploid	*Euphrasia frigida*	Bealach na ba	NG/780.417	5	-
T33	Tetraploid	*Euphrasia heslop-harrisonii*	Ardtoe	NM/627.712	5	-

Table 4:1. *Continued.*

Number	Ploidy	Taxon	Population	Grid reference	N (cp)	N (AFLP)
T35	Tetraploid	*Euphrasia heslop-harrisonii*	Ardnish	NG/674.238	5	-
T36	Tetraploid	*Euphrasia heslop-harrisonii*	Seilebost	NG/083.668	5	2
T46	Tetraploid	*Euphrasia nemorosa*	Dail Beag	NB/227.458	5	-
T51	Tetraploid	*Euphrasia ostenfeldii*	Storr, Skye	NG/510.540	5	3
T62	Tetraploid	*Euphrasia scottica*	Carlabhagh	NB/190.413	5	3
T63	Tetraploid	*Euphrasia scottica*	Dail Beag	NB/226.462	5	-
Total					69	22
North Scotland						
T8	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Betty Hill	NC/700.621	3	2
T9	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Durness	NC/405.669	5	2
T10	Tetraploid	*Euphrasia arctica* subsp. *borealis*	Reay	NC/658.653	5	2
T17	Tetraploid	*Euphrasia confusa*	Inchnadamp	NC/267.213.	5	3
T18	Tetraploid	"*Euphrasia fharaidensis*"	Inchnadamp	NC/272.210	5	3
T19	Tetraploid	"*Euphrasia fharaidensis*"	Rhiconich	NC/253.523	4	2
T20	Tetraploid	"*Euphrasia fharaidensis*"	Fharaid Head	NC/387.713	5	3
T25	Tetraploid	*Euphrasia foulaensis*	Scourie Bay	NC/144.449	5	-
T27	Tetraploid	*Euphrasia foulaensis*	Coldbackie	NC/611.598	4	-
T37	Tetraploid	*Euphrasia marshallii*	Betty Hill	NC/605.627	5	2
T38	Tetraploid	*Euphrasia marshallii*	Melness	NC/577.653	5	2
T39	Tetraploid	*Euphrasia marshallii*	Strathy Point	NC/827.694	5	1
T47	Tetraploid	*Euphrasia nemorosa*	Betty Hill	NC/702.608	5	2
T48	Tetraploid	*Euphrasia nemorosa*	Fharaid Head	NC/392.687	5	2
T49	Tetraploid	*Euphrasia nemorosa*	Dunnett Bay	ND/223.700	3	1
T52	Tetraploid	*Euphrasia ostenfeldii*	Inchnadamp	NC/272.210	5	1
T53	Tetraploid	*Euphrasia ostenfeldii*	An-t-sron	NC/443.580	5	1
T60	Tetraploid	*Euphrasia scottica*	Newton	NC/153.308	5	-
T64	Tetraploid	*Euphrasia scottica*	Lochan Hackel	NC/572.533	5	-
Total					89	29

4.2.3. Molecular marker analyses

Molecular markers were generated by two different techniques. Chloroplast PCR-RFLPs were used to score the diversity of different chloroplast haplotypes and AFLPs were used to score the presence or absence of dominant bands in the genome.

4.2.3.1. Chloroplast PCR-RFLP analysis

4.2.3.1.1. PCR amplification

DNA for chloroplast PCR-RFLP analysis was diluted 1:20 with autoclaved deionised water. Four universal chloroplast primer pairs were used to amplify the chloroplast regions, shown in Table 4:2 (Demesure *et al.*, 1995). Amplification reactions were performed in 25 µl, with 1x *Taq* buffer (Bioline: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20), 2 mM $MgCl_2$ (Bioline), 100 µM dNTPs (Sigma), 200 nM of forward and reverse primer (MWG-Biotech), 2 µg bovine serum albumin (Promega) and 1 unit of *Taq* DNA polymerase (Bioline), under the following conditions: 94°C for 4 minutes, 30 - 35 cycles of 92°C for 45 seconds, 47.5 - 62 °C for 45 seconds (according to primer annealing temperature, Table 4:2), 72°C for 2 - 4 minutes (according to length of fragment to be amplified, Table 4:2), then 72°C for 10 minutes using GeneAmp® PCR system 9700 (Applied Biosystems). PCR products were electrophoresed on 1% agarose (Hybaid), followed by ultra-violet visualisation after ethidium bromide staining. Approximate sizes of PCR product were assessed by comparison with migration distance of standard markers (1kb ladder, Gibco-BRL Life Technologies).

Table 4:2. PCR amplification conditions and estimated product size for the amplification of four regions, used to assess the variation in the chloroplast genome of British *Euphrasia*.

Chloroplast region	Annealing temp. (°C)	Extension time (min)	Estimated size (bp)
*psa*A - *trn*S	58.0	3	3820
*trn*C - *trn*D	58.0	3	2160
*trn*H - *trn*K	62.0	2	1610
$trnK_1$ - $trnK_2$	56.0	3	2580

4.2.3.1.2. Restriction digestion

PCR products were digested with two restriction enzymes: *Hinf*I (New England Biolabs) and *Hae*III (New England Biolabs). Digestions were carried out in 20 µl reactions, at 37°C for 4 hours, containing autoclaved deionised water, 1x buffer (50 mM NaCl, 10 nM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9)), 5 U restriction enzyme and 5 µl PCR product. Enzyme activity was stopped by adding 2 µl 10x stop buffer (0.25 M disodium-EDTA (pH 8.0), 50% glycerol, 0.1% sodium dodecyl sulphate, 0.25% bromophenol blue, 0.25% xylene cyanol), which also acted as a loading dye.

4.2.3.1.3. Electrophoresis of restriction digestion products

5 µl of the digested PCR products were separated by electrophoresis through 8% polyacrylamide gels (Sambrook *et al.*, 1989). A 1 Kb ladder (Gibco-BRL Life Technologies) was included as a size marker on each gel. Gels were run in 1x TBE at a constant voltage of 20V/cm for 2.5 hours in a SE600 vertical gel apparatus (Hoefer). Fragments were visualised under ultra-violet light after ethidium bromide staining.

4.2.3.2. AFLP analysis

DNA for AFLP analysis was extracted immediately prior to amplification to minimise possible artifacts resulting from degradation of the stored DNA. The AFLP procedure was carried out using the restriction enzymes, *Mse*I and *Eco*RI, followed by a two step amplification strategy, as described by Vos *et al.* (1995), with some modifications.

4.2.3.2.1. Digestion and ligation

Digestion and ligation of adaptors were performed under the following conditions: approximately 100 ng of total genomic DNA were incubated for 2 hours at 37°C with 1 U *Mse*I (New England Biolabs), 5 U *Eco*RI (New England Biolabs), 4.55 µM *Mse*I - adapters (Forward: 5'-GACGATGAGTCCTGAG-3'; Reverse: 5'-TACTCAG-GACTCAT-3'), 0.455 µM *Eco*R1 - adapters (Forward: 5'-CTCGTAGACTGCGT-ACC-3'; Reverse: AATTGGTACGCAGTC-3') (MWG Biotech) and 1 U DNA T_4 ligase (New England Biolabs) in 11 µl volume with 1x T_4 ligase buffer, 0.5 M NaCl,

0.55 ng BSA (New England Biolabs). Complete digestion was checked by electrophoresis of 5 µl of digested and ligated DNA on 1.5% agarose (Bioline), followed by ultra-violet visualisation, after ethidium bromide staining. The remaining ligated DNA was diluted with 103 µl $TE_{(0.1)}$ (20 mM Tris-HCl, 0.1 mM EDTA, pH 8).

4.2.3.2.2. Pre-amplification

Pre-amplification was performed using *Mse*I and *Eco*RI primers which contained the addition of one selective nucleotide on 3' end (*Mse*I primer: 5'-GACGATGA-GTCCTGAGTAAC-3'; *Eco*RI primer: 5'-AGACTGCGTACCAATTCA-3' (MWG Biotech)). Amplification reactions were performed on 12 µl of diluted ligated DNA, in 20 µl volumes, with 1x *Taq* buffer (Bioline: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20), 1.5 mM $MgCl_2$ (Bioline), 200 µM dNTPs (Sigma), 1.5 mM *Mse*I primer and 0.25 mM *Eco*RI primer and 0.08 units of *Taq* DNA polymerase (Bioline), under the following conditions: 72°C for 2 minutes, 20 cycles of 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 2 minutes, then 60°C for 30 minutes using PTC-200 Peltier Thermal Cycler (MJ Research). Pre-amplification was checked by electrophoresis of 10 µl of PCR product on 1.5% agarose (Bioline), followed by ultra-violet visualisation after ethidium bromide staining. The remaining PCR product was diluted 1:20 with $TE_{(0.1)}$.

4.2.3.2.3. Selective amplification

Selective PCR amplification was performed using two combinations of *Mse*I and *Eco*RI primers with addition of three selective nucleotides on the 3' end. The *Eco*RI primer was additionally labelled at the 5' end with a fluorescent dye (JOE or FAM; MWG Biotech). The selective primer combinations were i) *Mse*I primer + CAT: *Eco*RI primer + ACC; ii) *Mse*I primer + CAC: *Eco*RI primer + AGC. Amplification reactions were performed on 2 µl of pre-amplified DNA, in 10 µl volumes, with 1x *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM *Mse*I primer and 0.05 µM *Eco*RI primer and 0.05 units of *Taq* DNA polymerase. After initial denaturation at 94°C for 2 minutes, the PCR conditions, using a PTC-200 Peltier Thermal Cycler consisted of further denaturation at 94°C for 20 seconds, followed by initial primer annealing at 66°C for 30 seconds and primer extension at 72°C for 2 minutes. With each successive cycle, the primer annealing temperature was then reduced by 1°C

until reaching 56°C. At this point, 20 further cycles at an annealing temperature of 56°C were performed. A final extension at 60°C for 30 minutes was then performed.

4.2.3.2.4. Electrophoresis of selective AFLP PCR products

The selective AFLP PCR products were analysed on an ABI 377 DNA sequencer (Applied Biosystems), followed by gel image analysis and band size scoring using ABI Genescan® software (version 3.1.2) (Applied Biosystems) and ABI Genotyper® software (version 2.5) (Applied Biosystems).

4.2.4. Scoring and data analysis

4.2.4.1. Chloroplast PCR-RFLP analysis

Polymorphisms were scored as unordered binary (point mutations) or multistate (insertion/deletions) characters. Chloroplast Excel macros (Chapter 2) were used to define and format chloroplast haplotypes from the chloroplast restriction fragment data. Total diversity (h_T), average intrapopulation diversity (h_S), and the level of differentiation among populations (G_{ST}) were calculated following the methods described by Pons and Petit (1995), using the computer software program HAPLODIV. Relatedness between haplotypes were represented by a minimum spanning network, calculated using the computer software program ARLEQUIN v. 2.000 (Schneider *et al.*, 2000).

4.2.4.2. AFLP analysis

Gels were scored for presence and absence of bands across individuals. Band size ranged from 50 to 450 base pairs. Unresolved bands were scored as missing data. To retain consistency of scoring between gels, bands were compared across gels and a standard individual was amplified and run on all gels. Loci and individuals that were unclear were excluded from further analysis. Both monomorphic and polymorphic bands were included in the analysis. AFLP Excel macros (Chapter 2) were used to determine presence of unique bands within regions and ploidy groups, and to format the data set for relevant population software packages.

4.2.4.3. Unique molecular marker distribution

With low numbers of individuals sampled per population, and significant and variable levels of inbreeding within and among populations (Chapter 3), a phenetic approach was used to investigate AFLP band distribution. For dominant AFLP bands, the total number of bands for each individual was calculated. Chloroplast haplotypes and number of individual AFLP bands were grouped according to ploidy level. This was performed across all regions and within the two regions: south-west England and Wales. These regions had equal numbers of individuals from each ploidy group. The null hypothesis of a random distribution of both sets of molecular markers were tested with either χ^2 or non-parametric Mann Whitney U test, using the statistical package, MINITAB®. For each ploidy group, the number of unique molecular markers (chloroplast haplotypes or dominant AFLP bands) was determined.

4.2.4.4. Geographical and ploidy structuring

To test geographical structuring and differentiation by ploidy level, analysis of molecular variance (AMOVA) was used to partition the genetic variation. Whilst AMOVA was initially developed specifically for restriction fragment data (Excoffier *et al.*, 1992), it has also been shown to be applicable to the analysis of dominant data (Huff *et al.*, 1993). Pairwise Euclidean distance matrices were therefore calculated for both chloroplast haplotypes and presence/absence AFLP data, using the equations of Excoffier *et al.* (1992). These matrices were used to perform AMOVA, using the software program ARLEQUIN (Schneider *et al.*, 2000). The significance of AMOVA variance was tested using a non-parametric permutation procedure (1000 randomisations) (Excoffier *et al.*, 1992). To examine variation among ploidy levels over all regions, the genetic variation was partitioned between three hierarchial levels: among regions, among ploidy level within regions and among individuals within each ploidy group. This was repeated at a smaller geographical scale, between two regions containing equal numbers of individuals from each ploidy level (Figure 4:2). To examine the degree of variation partitioned between ploidy groups across all individuals sampled, similar nested AMOVAs were performed for both sets of

molecular marker, the hierarchial levels were among ploidy groups, among populations within ploidy groups and among individuals within populations.

4.3. Results

4.3.1. Chloroplast PCR-RFLP haplotype and AFLP diversity

Restriction enzyme digestion of the four chloroplast regions produced a total of 37 restriction fragments. This resulted in the detection of 10 mutations, including seven indels and three point mutations. Combining information from these mutations allowed the identification of 12 haplotypes (Table 4:3; Figure 4:3) separated by one or two mutations (Figure 4:4). Overall each population was predominantly fixed for one chloroplast haplotype with 83.9% of the variation partitioned between populations, G_{ST} = 0.839 (h_T = 0.887, h_S = 0.143). The two AFLP primer pairs produced 139 scorable loci. Of these loci, 136 were polymorphic and three were monomorphic.

4.3.2. Chloroplast PCR-RFLP haplotype and AFLP band distribution

The distribution of chloroplast haplotypes was significantly structured across ploidy groups (χ^2 = 195.51, d.f. = 3, $p < 0.000$; Figure 4:5). Seven out of 12 chloroplast haplotypes were only found in the tetraploid group, while a further two haplotypes were unique to the diploid group (Figure 4:5). The remaining three chloroplast haplotypes were present in both groups (Figure 4:5).

The numbers of AFLP bands per individual were significantly different between the ploidy groups (U = 668.0, $p < 0.000$; Figure 4:5). The mean number of AFLP bands per diploid individual was lower than per tetraploid individual (30.26 ± 6.79 and 44.09 ± 4.73, respectively). The significant structuring of AFLP bands was repeated in both south-west England and Wales regions (U = 126.0, $p < 0.000$; U = 53.0, p = 0.029, respectively; Figure 4:6). The tetraploid group contained a higher number of unique AFLP bands than the diploid group (55 bands compared to 10 bands, respectively; Figure 4:7).

Table 4:3. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*: AS = *psa*A - *trn*S: CD = *trn*C - *trn*D: HK = *trn*H - *trn*K: KK = *trn*K_1 - *trn*K_2

Haplotype	AS:*Hinf*I band I	AS:*Hinf*I band II	AS:*Hinf*I band III	CD:*Hinf*I band I	CD:*Hinf*I band II	CD:*Hinf*I band III	HK:*Hae*III band I	KK:*Hinf*I band I
A	1	1	2	1	2	3	2	1
B	1	1	2	1	2	3	2	0
C	1	1	2	2	2	1	2	0
D	1	1	2	1	2	1	2	0
E	3	1	2	1	2	3	2	0
F	3	1	2	1	1	2	2	0
G	3	1	2	?	1	0	2	0
H	3	1	2	?	1	0	1	0
I	2	2	2	2	2	1	2	0
J	2	2	2	2	2	1	3	0
K	1	0	1	1	2	3	2	0
L	1	0	1	1	2	3	1	0

Haplotypes A - L defined as particular associations of these restriction patterns, as shown in Figure 4:3. Polymorphisms are scored in order of decreasing molecular weight, with the absence of a restriction fragment represented by 0. Uncertainty of band molecular weight represented by ?

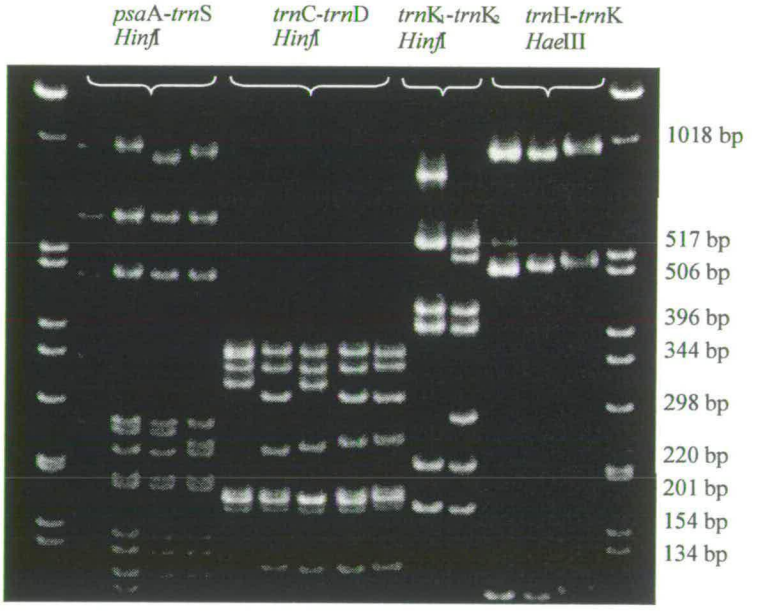


Figure 4:3. Polymorphism detected in the chloroplast genome of British *Euphrasia*, revealed by RFLP analysis of four chloroplast regions (*psa*A - *trn*S, *trn*C - *trn*D, *trn*K_1 - *trn*K_2, *trn*H - *trn*K; Table 4:3). For each primer/enzyme combination, polymorphic bands were scored in order of decreasing molecular size (I - III). The estimated sizes of the scored polymorphic bands detailed in Table 4.3 are: AS/*Hinf*I band I 950, 920, 875 bp; AS/*Hinf*I band II 255, 240 bp; AS/*Hinf*I band III 140, 135 bp; CD/*Hinf*I band I 365, 360 bp; CD/*Hinf*I band II 320, 300 bp; CD/*Hinf*I band III 240, 235, 230 bp; HK/*Hinf*I band I 515, 510, 505 bp; KK/*Hae*III band I 790 bp. Lanes 1 and 16: 1kb ladder (Gibco-BRL Life Technologies).

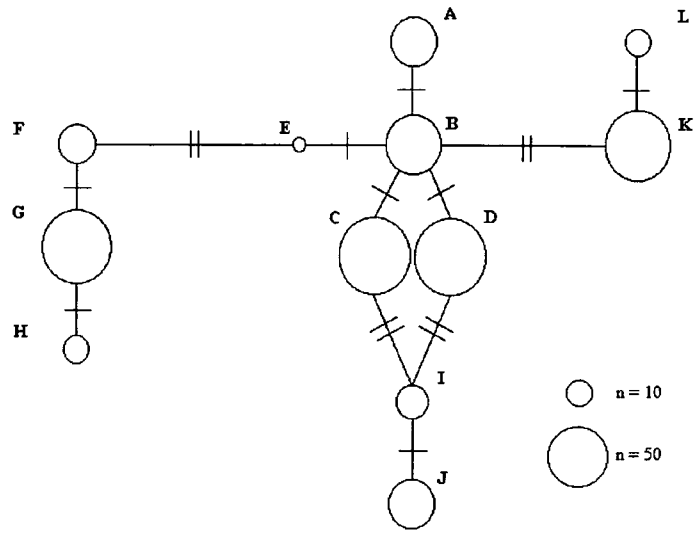
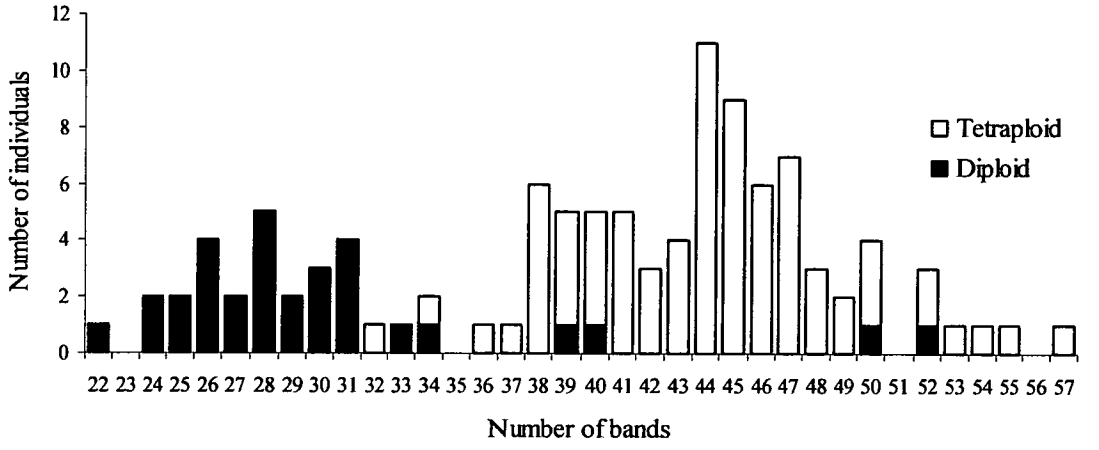


Figure 4:4. Relationships between chloroplast haplotypes, represented as a minimum spanning network. Haplotypes A to L are described Table 4:3. Mutational steps are indicated by single cross line. The areas of the circles represents number of individuals with each haplotype. n = number of individuals.

Four diploid individuals contained a higher number of AFLP bands than other diploid individuals (Figure 4:5). These individuals were from two populations (D16: *Euphrasia vigursii*; D5: *Euphrasia officinalis* subsp. *rostkoviana*). These two populations contained chloroplast haplotypes in common with tetraploid groups (25% in population D16; 20% in population D5). Out of the 84 bands present in the diploid group, these two populations contained 25 bands not found in other diploid populations. Of these, 22 were present in the tetraploid group.

Analysis of molecular variance for both chloroplast and AFLP markers showed that variation was not significantly partitioned between geographic regions, but was significantly partitioned between the ploidy groups of each region (Table 4:4). This partitioning of variation was repeated across two regions with equal numbers of individuals in each ploidy group (Table 4.5). On grouping populations according to ploidy level across all regions, approximately 30% of both chloroplast and AFLP marker variation was partitioned between the ploidy groups and the remaining 70% of the variation within ploidy groups (Table 4:6).

i)



ii)

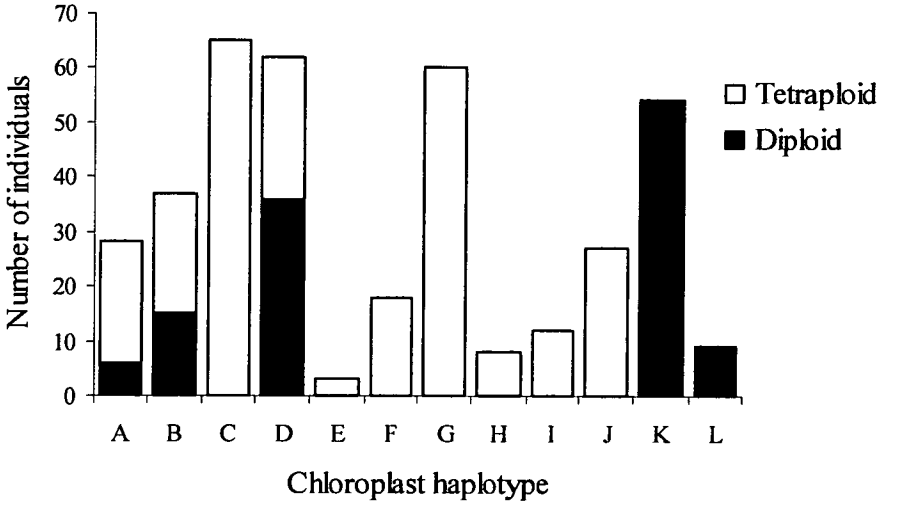


Figure 4:5. Distribution of molecular markers across individuals and ploidal groups. i) number of AFLP bands per individual; ii) chloroplast haplotypes. Haplotypes A to L are described in Table 4:3.

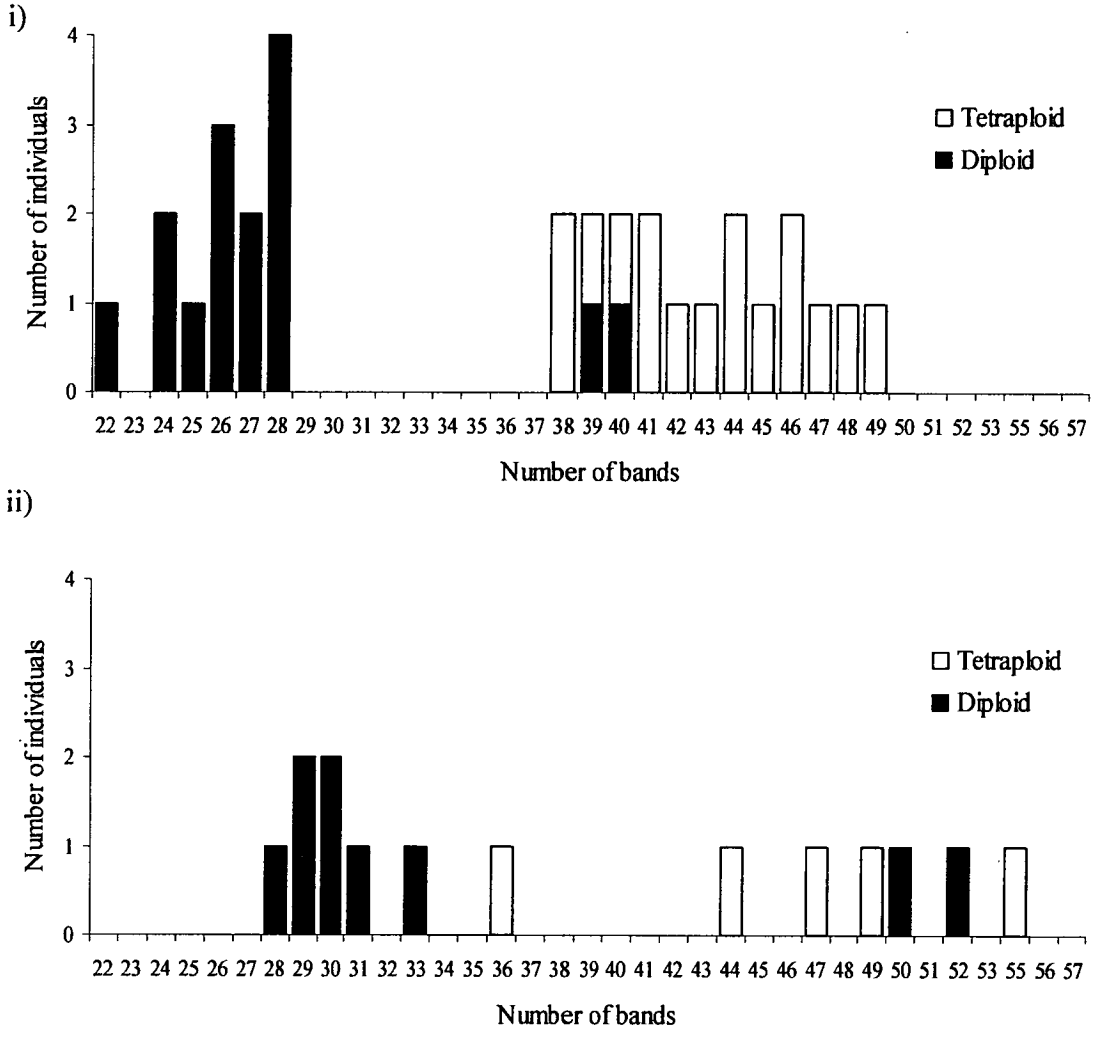


Figure 4:6. Number of AFLP bands per individual, grouped according to ploidy level. i) south-west England: ii) Wales.

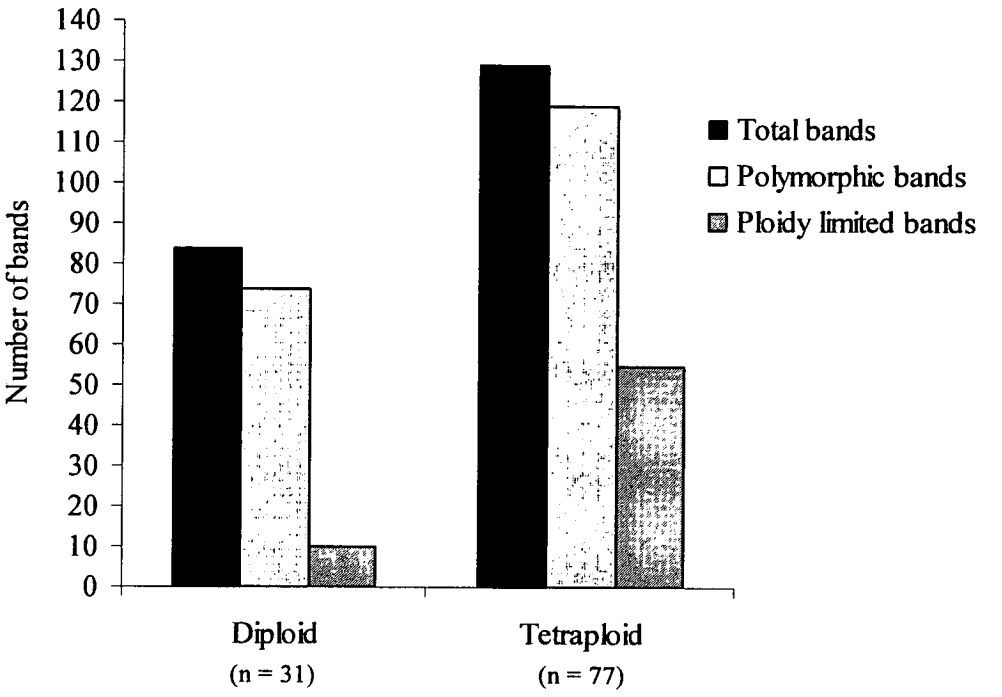


Figure 4:7. Number of total, polymorphic and ploidy limited AFLP bands, grouped according to ploidy group. n = number of individuals.

Table 4:4. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Individuals were grouped according to region and ploidy level within each region. d.f. = degrees of freedom; SSD = sums of square deviations (NS. not significant; **p < 0.001).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among regions	6	106.680	-0.20524	^{NS}-15.38
Among ploidy groups within regions	4	64.725	0.69000	**51.69
Within ploidy groups	372	316.216	0.85004	63.68
Total	382	487.621	1.33481	100.00
AFLP genotypes				
Among regions	6	302.418	-1.67785	^{NS} -11.94
Among ploidy groups within regions	3	172.634	5.87464	**41.79
Within ploidy groups	98	966.383	9.86106	70.15
Total	107	1441.435	14.05785	100.00

Table 4:5. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes for two regions (south-west England and Wales). Individuals were grouped according to region and ploidy level within each region. d.f. = degrees of freedom; SSD = sums of square deviations. (NS. not significant; **p < 0.001).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among regions	1	20.990	-0.06260	^{NS} -5.01
Among ploidy groups within regions	2	43.192	0.59991	**48.05
Within ploidy groups	150	106.695	0.71130	56.97
Total	153	170.877	1.24860	100.00
AFLP genotypes				
Among regions	1	34.827	-1.74001	^{NS} -13.05
Among ploidy groups within regions	2	152.109	5.89654	**44.23
Within ploidy groups	42	385.411	9.17646	68.83
Total	45	572.348	13.33298	100.00

Table 4:6. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to ploidy level (diploid or tetraploid). d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast Haplotypes				
Among ploidy groups	1	81.463	0.45264	**29.28
Among populations within ploidy groups	70	359.759	0.94398	**61.07
Within populations	311	46.400	0.14920	9.65
Total	382	487.621	1.54582	100.00
AFLP genotypes				
Among ploidy groups	1	246.975	3.79444	**30.60
Among populations within ploidy groups	46	966.794	7.69002	**46.47
Within populations	60	227.667	3.79444	22.93
Total	107	1441.435	16.54847	100.00

4.4. Discussion

4.4.1. Differentiation of ploidy groups

Molecular markers were significantly partitioned between ploidy levels at both local and widespread regional levels. The high numbers of unique haplotypes and bands in both groups supports the presence of two isolated gene pools in Britain. This isolation is likely to be related to meiotic pairing abnormalities in the hybrid zygotes produced by inter-ploidal crosses (Yeo, 1954). This allows the sympatric co-existence of populations of both ploidy levels without merging of the two gene pools.

4.4.2. Is the barrier between ploidy levels absolute?

Anecdotal field evidence of hybrid swarms (Yeo, 1956) and production of fertile hybrids from crosses between diploid and tetraploid parents (Yeo, 1954; Yeo, 1959) suggests that gene flow can occur between ploidy groups. The distribution of a number of the molecular markers within both ploidy groups supports this inter-ploidal gene flow. Of course, the three chloroplast haplotypes common to both ploidy groups may also have resulted from common ancestry and not necessarily gene flow between groups. However, this explanation seems unlikely in view of the observation that two putative diploid populations (populations D16; *Euphrasia vigursii* and D5; *Euphrasia officinalis* subsp. *rostkoviana*) have AFLP band numbers per individual that are similar to those of the tetraploids. Several bands in both these populations are not present in other diploid individuals, but almost all (22/25) of these bands occur in the tetraploid group, suggesting their origination from that group. Given the high number of bands involved it is unlikely that they resulted from common ancestry within the two gene pools.

As yet, the chromosomes have not been counted in either of these populations, so the high number of AFLP bands may indicate that these populations are tetraploid and not diploid, as assumed from their morphology. Both of these populations (D16 and D5) possess long glandular hairs, a character that is taken as a good indicator of diploid taxa (Yeo, 1978a). Long glandular hairs are also present in the tetraploid *Euphrasia arctica* (Yeo, 1978). Abundant glandular hairs are especially common in two Scottish varieties *notata* and *reayensis*. These varieties have previously been

regarded as examples of hybridisation between diploid *Euphrasia officinalis* subsp. *rostkoviana* and tetraploid *Euphrasia arctica* subsp. *borealis* (= *Euphrasia brevipila*) (Townsend, 1897). More recent classifications have treated them as varieties of the tetraploid species (Pugsley, 1930; Yeo, 1978a). Cytological investigations of two populations of variety *notata* showed 22 bivalents and one univalent at meiosis (Yeo, 1954). This supports the hypothesis that they resulted from hybridisation between the two ploidy levels.

Both the two anomalous populations (D16: *Euphrasia vigursii*; D5 *Euphrasia officinalis* subsp. *rostkoviana*) were also genotyped with microsatellite loci (Chapter 2). Each individual had a maximum of two alleles per locus, which were shown to segregate within each population. This, in contrast to the AFLP data, suggests that both populations are diploid and so supports hybridisation and gene flow between ploidy groups. Alternatively, all the microsatellite loci may have been present on homologous chromosomes in a tetraploid individual. In this case they may show regular Mendelian inheritance. Direct chromosome counts from both these populations is therefore still required to confirm their ploidy status.

4.4.3. Is there evidence that the tetraploids are allopolyploid?

Among the tetraploid group I found seven chloroplast haplotypes and 55 AFLP bands not found in the diploid group. In contrast, the diploid group contained two chloroplast haplotypes and 10 AFLP bands not found in the tetraploids. It is unlikely that the high number of molecular markers exclusive to the tetraploids represents either an under-sampling of the diploid group in Britain or their fixation in the tetraploid group and concurrent loss in the diploid group (Cook *et al.*, 1998). These bands are therefore likely to have originated from another diploid group, supporting previous evidence of an allopolyploid origin of the tetraploid taxa (Yeo, 1959; Yeo, 1976). To understand the present diversity within the tetraploid group in Britain, it would be informative to identify this other putative diploid group. Good candidates for this group occur within the other European series *Alpinae* (Yeo, 1978a; Greilhuber *et al.*, 1984; Vitek, 1985) and series *Parviflorae* (Ehrendorfer and Vitek, 1984; Vitek, 1986). Previous crossing experiments between the distinctive large flowered taxon, *Euphrasia alpina* and *Euphrasia officinalis* subsp. *rostkoviana*,

produced marked meiotic chromosomal abnormalities with the production of a few artificial hybrids. In addition, these hybrids were morphologically similar to the widespread common tetraploid species: *Euphrasia stricta*, *Euphrasia arctica* and *Euphrasia nemorosa* (Yeo, 1970b; Yeo, 1976).

If the tetraploid taxa originated from allopolyploidisation between diploid parents with marked chromosomal differences, then it would be expected that they show non Mendelian segregation of some molecular markers at meiosis, with resulting fixed heterozygosity (Soltis and Soltis, 1993). This has been shown in a previous molecular study, using isozyme markers, in two populations of *Euphrasia heslop-harrisonii* on Skye, Scotland (Oliver, 1999). Using co-dominant markers, more extensive sampling is required to see if this is applicable across a wider range of tetraploid taxa.

4.4.4. Evolutionary significance of polyploids in *Euphrasia*

The high molecular diversity of tetraploid groups in comparison with diploids is correlated with its ability to withstand broader environmental conditions and occupy new environments (Thompson and Lumaret, 1992; Chapter 6). The role of polyploidy in the ecological diversification in British *Euphrasia* has not been formally tested but British tetraploid taxa occupy a broader range of niches than British diploid *Euphrasia* (Silverside, 1998). Occupation of a broader range of habitats may lead to high morphological diversity. This diversity may be further increased by recurrent hybridisation between tetraploid populations from independent origins. By bringing together differing genetic combinations, this may result in genomic reshuffling and the production of new chromosomal and gene arrangements (Soltis and Soltis, 1995).

The high number of unique chloroplast haplotypes found within the tetraploid group indirectly supports the evidence for multiple origins of the tetraploid group. These haplotypes fall into three main groups, with at least two mutational steps between each (Figure 4:4). This may represent at least three separate origins of tetraploid lineages with subsequent accumulation of further chloroplast mutations within each. However, a single origin of the tetraploid group, with repeated introgression of

chloroplast haplotypes from the diploid taxa, may be given as an alternative hypothesis for the diversity of chloroplast haplotypes found within the tetraploid group.

4.4.5. Broad scale population structure

Neither variation of chloroplast PCR-RFLP and AFLP bands were significantly partitioned between geographic regionals, either locally or across Britain. Limited gene flow between regions cannot explain therefore the generation of diversity in British *Euphrasia*. This contrasts with analysis of chloroplast haplotype distribution, with high G_{ST} and low h_S values found across all populations, indicating limited gene flow between local populations, and geographical structuring at least at the population scale. This supports previous observations that *Euphrasia* seeds do not seem to be adapted to long distance wind or animal dispersal (Barker, 1982) and mostly fall close to the parent plant (Molau, 1993). This structuring does not occur on larger geographical scales across regions studied. This suggests that locally restricted gene flow coupled with occasional long distance gene flow has been sufficient to prevent partitioning of variation.

Relatively rapid spread over large geographical distances of *Euphrasia* taxa have been recorded within the last 100 years. The spread of *Euphrasia stricta* since its introduction into North America gives a clear example (Downie and McNeil, 1988). Dispersal over these long distances may have been aided by human activity. This taxon may have spread along transportation routes across North America and with transportation of hay across Sweden (Karlsson, 1984). Given the similar extensive human activities in Britain, comparable rates of migration between regions are likely to have occurred. However, migration of *Euphrasia* into Britain probably occurred within the last 12000 years, after the retreat of the last glacial ice sheet (Hewitt, 1996; Brochmann *et al.*, 2003). Gene flow between regions may therefore be presently low, but colonisation may have been too recent for significant structuring to develop since the last post-glacial migration. This study cannot help to determine whether gene flow currently occurs between regions.

4.4.6. Summary

Analysis of the distribution of molecular markers within Britain has provided indirect evidence in support of past multiple allopolyploidisation events occurring within *Euphrasia*. Though further work is required, it does indicate that the present diversity seen in British *Euphrasia* may have been the result of migration of a number of lineages throughout Britain, combined with a variable degree of mixing between them. Low hybrid fertility from chromosomal abnormalities during meiosis has kept the diploid lineage predominantly distinct from the tetraploid lineages, with approximately 30% of the overall genetic variation presently occurring between these two groups. Mixing of genes within and between lineages has possibly resulted in the generation of the remaining high diversity that is presently seen in British *Euphrasia*, with 70% of the overall genetic variation occurring within both ploidy groups. Using the same molecular approach, the next two chapters shall examine the partitioning of variation within these two ploidy groups. The next chapter will explore variation in the diploid group, examining whether this variation can be partitioned according to the current taxonomic groups described.

CHAPTER 5: Partitioning of genetic variation within the diploid group of British *Euphrasia*. Is there an association with current taxonomic species delimitation?

Abstract: Three diploid *Euphrasia* species are currently recognised in Britain. The more widespread species *Euphrasia officinalis* is subdivided into three subspecies. The other two species, *Euphrasia vigursii* and *Euphrasia rivularis* are rare endemic species with high conservation status within the UK Biodiversity Action Plan. Taxonomic delimitations within this group are supported by the molecular study of British diploid *Euphrasia* described in this chapter. Significant structuring of AFLP marker bands was found among taxa. This structuring was seen in the discrete phenetic clustering of taxa within principal coordinate analyses and the significant partitioning of variation among taxa within analysis of molecular variance. Molecular evidence for endemic species is supportive of, but not conclusive, for their formation by inter-ploidal hybridisation, with incongruence between chloroplast and nuclear genomes. Chloroplast haplotypes that are more common within tetraploid taxa, are also found in diploid *Euphrasia rivularis*, but this contrasts with the low number of tetraploid AFLP bands found in the diploid endemic taxa.

5.1. Introduction

Traditionally, partitioning of variation within genera has relied on the ability to distinguish distinct groups, based on the recognition of differing morphological characters (Karp *et al.*, 1996). In many cases, taxon limits are unambiguous and there is a clear consensus as to the most appropriate taxonomic treatment for a group. However in critical groups morphological characters alone may be inadequate. The formation of taxonomic categories may also be affected by differing weighting of individual morphological characters. The use of molecular markers, generating large numbers of characters, has increasingly been used to overcome some of these problems (Karp *et al.*, 1996). With their apparent near neutrality and equal phenetic weighting (Weising *et al.*, 1995), they can be used to complement other taxonomic methods, improving the identification of divisions within a group. This in turn may lead to the development of more appropriate conservation management strategies, maximising the retention of diversity within a group, under the economic limitations that often prevail.

The advantages of this approach have been demonstrated in the taxonomically difficult *Aconitum* group. Here RAPD analysis showed that *Aconitum noveboracense* Gray, the first American plant to have a recovery plan, was not distinct from the more common taxon *Aconitum columbianum* Nutt. (Cole and Kuchenreuther, 2001). In contrast, other molecular studies have supported the taxonomic status given to species in morphologically difficult groups. These include the support of species from *Orobanche* in Israel (Paran *et al.*, 1997) and arctic *Draba* in Norway (Scheen *et al.*, 2002).

The genus *Euphrasia* represents a taxonomically complex group where species recognition balances the need to describe the present morphological diversity whilst limiting the number of artificial groups produced (Yeo, 1978a). This is particularly marked in Britain, where the current taxonomic treatment has many rare endemic species, currently recognised as having national conservation importance (Anon, 1995b). British *Euphrasia* have been broadly divided into two morphologically distinct groups, differing in ploidy (Yeo, 1978a). Molecular analyses, using chloroplast PCR-RFLP and AFLP techniques, have shown significant partitioning of variation between these diploid and tetraploid groups within Britain and so support this taxonomic division (Chapter 4).

Diploid taxa, generally possessing longer glandular hairs and broader capsules, have been placed in a single series, *Euphrasia* within the subsection *Euphrasia*, separate from tetraploids (Pugsley, 1930; Yeo, 1978a). Both ploidy groups have been further subdivided into a number of taxa, many of which have been given species status. The aim of this chapter is to examine this intra-ploidal taxonomic partitioning in more detail. By studying the diploid species I wish to determine whether the morphological groupings that have been applied reflect the underlying partitioning of genetic variation. With two of these diploid taxa currently recognised as high conservation priority endemic species in the UK Biodiversity Action Plans (Anon, 1995b), understanding this partitioning of diversity and its relationship to current *Euphrasia* taxonomy will be important when considering future conservation management strategies.

Currently three diploid *Euphrasia* species are recognised in Britain, *Euphrasia officinalis*, *Euphrasia vigursii* and *Euphrasia rivularis* (Preston *et al.*, 2002; Figure 5:1). The commonest species, *Euphrasia officinalis*, tends to be a large flowered plant occurring in damp meadows and pastures (Silverside, 1990b). Marked morphological variation occurs across its extensive European geographical range. This has led to subdivision into subspecies (Yeo, 1978a; Silverside, 1991d). Three of these subspecies occur in Britain (Preston *et al.*, 2002). *Euphrasia officinalis* subsp. *rostkoviana* is subspecies of damp fertile meadows, locally common in upland areas of Wales, north-west England and southern Scotland (Silverside, 1990b). It is a late seasonal plant, tending to flower at a higher node, with more branches than the early seasonal variant *Euphrasia officinalis* subsp. *monticola.* This latter subspecies is less common, occurring in a few wetter upland areas, especially in north-west England (Silverside, 1990b). *Euphrasia officinalis* subsp. *anglica* is a smaller flowered plant, with a more capitate appearance (Pugsley 1929; Yeo, 1978a). It occurs in a restricted range in grazed pastures and heathland in southern and eastern England and is considered to be endemic to the British Isles (Preston *et al.*, 2002). However, similar plants have been described in Normandy, France (Pugsley, 1930; Yeo, 1978a). The morphological range of *Euphrasia officinalis* in Britain currently encompasses a number of previous records, initially classified under other taxa. These include plants previously assigned to *Euphrasia rostkoviana* subsp. *campestris* (Townsend, 1897), *Euphrasia officinalis* subsp. *officinalis* (Bucknall, 1917) and *Euphrasia hirtella* (Pugsley, 1919).

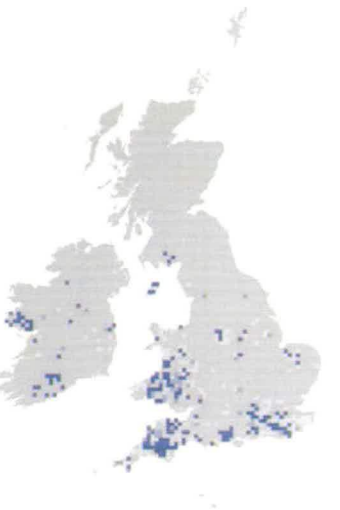
The two other British diploid taxa are endemic and classified as Biodiversity Action Plan species with high conservation status (Anon, 1995b). *Euphrasia vigursii* (Davey, 1907; Yeo, 1956) is restricted to *Agrostis curtsii* - *Ulex gallii* heathland in south-west England (Preston *et al.*, 2002). Morphological characteristics, shared with both *Euphrasia officinalis* and tetraploid *Euphrasia micrantha*, has led to the hypothesis that it originates from an inter-ploidal hybrid origin (Silverside, 1990b).





Euphrasia officinalis* subsp. *rostkoviana
Population: Wales – D3
Date collected: 17th July 2001





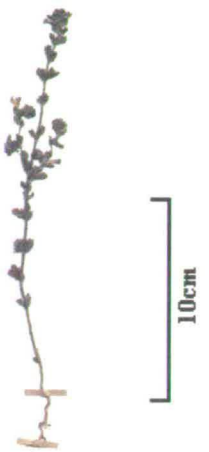
Euphrasia officinalis* subsp. *anglica
Population: south-west England – D1
Date collected: 21st July 2000

Figure 5:1. British *Euphrasia* species, collected within the diploid series *Euphrasia*, section *Euphrasia*. Population numbers described in Table 5:1. Distribution maps from Preston *et al.* (2002). Dark blue = records 1987 - 1999; medium blue = records 1970 - 1986; pale blue = records pre 1970.





Euphrasia rivularis
Population: north-west England – D10
Population: Wales – D7
Date collected: 28th June 2000; 15th Aug. 2000





Euphrasia vigursii
Population: south-west England – D16
Date collected: 18th July 2000

Figure 5:1. *Continued.*

The inter-ploidal hybrid origin of *Euphrasia vigursii* is supported by chromosomal counts of a triploid individual, morphologically classified as a hybrid between *Euphrasia officinalis* subsp. *anglica* and *Euphrasia micrantha* (Yeo, 1954). Such triploids have been proposed to form the bridge between diploid and tetraploid plants, and to lead to the formation of novel diploid offspring, by backcrossing with diploid plants (Yeo, 1956).

The third species, *Euphrasia rivularis* (Pugsley, 1929), also combines the characters of diploid *Euphrasia officinalis* and tetraploid *Euphrasia micrantha*, with further possible adaptive modifications to a montane flush habitat (Silverside, 1990b). It has a disjunct distribution in Britain (Preston *et al.*, 2002), with morphologically similar populations occurring in both north-west England and Wales (Figure 5:1).

Cultivation experiments have provided evidence that the morphological differences between the diploid taxa described above are not simply due to differing environmental conditions. This has been reported for both *Euphrasia officinalis* subsp. *rostkoviana* (Karlsson, 1982; Zopfi, 1998a) and *Euphrasia officinalis* subsp. *anglica* (Yeo, 1959; Yeo, 1962). As these taxa are usually allopatric, populations combining morphological characters from differing taxa are less frequent than the taxonomic gradations that occur among the tetraploid taxa (Preston *et al.*, 2002). Gene flow is, however, considered to occur freely when populations are sympatric. This has resulted in the recognition of hybrid populations, including *Euphrasia officinalis* subsp. *rostkoviana* x subsp. *monticola*, *Euphrasia officinalis* subsp. *rostkoviana* x subsp. *anglica*, *Euphrasia officinalis* subsp. *rostkoviana* or subsp. *monticola* x *E. rivularis* and *Euphrasia officinalis* subsp. *anglica* x *E. vigursii* (Stace, 1997). Phenotypic variation is therefore not discontinuous between taxa. This raises the question whether the currently defined taxonomic groups reflect significant partitioning of genetic variation. Under a null hypothesis, gene flow has been sufficient within the diploid group such that genetic variation is not related to defined morphological groups. Marker variation among taxa will therefore be no greater than within taxa and there will be no unique taxonomic markers.

By estimating the distribution of variable markers within and between taxa, one can test this null hypothesis. Rejection of the null hypothesis will imply some congruence between genetic and morphological variation. In addition, under the hypothesis of tetraploid parentage, the two endemic diploid species should contain markers present in the tetraploid parent, but not in *Euphrasia officinalis*. By employing a hierarchical sampling strategy across the range of diploid taxa within Britain, I therefore specifically wish to determine:

1. The distribution of molecular marker variation within and between taxonomic groups.
2. Quantitative estimates of partitioning of genetic variation within and between taxonomic groups.
3. The importance of geographical structuring and/or taxonomic divisions in determining the distribution of these markers.
4. The presence of tetraploid markers within both endemic species.
5. The presence of unique molecular markers within each taxonomic group.

5.2. Materials and Methods

5.2.1. Materials

16 populations of diploid *Euphrasia* taxa were sampled from four regions within Britain (Table 5:1). Taxa included widespread *Euphrasia officinalis* subsp. *rostkoviana*, the more restricted *Euphrasia officinalis* subsp. *anglica* and the two endemic taxa *Euphrasia vigursii* and *Euphrasia rivularis* (Figure 5:1; Figure 5:2). As only one individual of *Euphrasia officinalis* subsp. *monticola* was found, this taxon was excluded from the study. Following the widespread sampling strategy, as outlined in Chapter 1, 7 to 13 individuals were collected from each population. This was performed using the same sampling technique as described in Chapter 4.

5.2.2. DNA extraction and molecular marker analyses

DNA extraction was performed as described in Chapter 4. Diversity was assessed in both nuclear and chloroplast genomes. Presence or absence of dominant bands were estimated in two to three individuals per population, using AFLPs, generated from

Table 5:1. Sampling of diploid *Euphrasia* taxa from four British regions for chloroplast PCR-RFLP and AFLP analysis. N (cp) = number of individuals sampled in each population for chloroplast PCR-RFLP analyses; N (AFLP) = number of individuals sampled in each population for AFLP analyses.

Number	Taxon	Population	Grid reference	N (cp)	N (AFLP)
South-west England					
D1	*Euphrasia officinalis* subsp. *anglica*	Kit Hill	SX/374.715	5	3
D11	*Euphrasia vigursii*	Wicca Pool	SW/465.403	5	3
D12	*Euphrasia vigursii*	Carvannel Downs	SW/641.446	9	-
D13	*Euphrasia vigursii*	Porthtowan	SW/688.477	10	3
D14	*Euphrasia vigursii*	Ventogimps Moor	SW/781.511	10	-
D15	*Euphrasia vigursii*	Tregrethona Downs	SW/959.628	10	3
D16	*Euphrasia vigursii*	Treslea Downs	SX/134.683	8	3
Total				57	15
East England					
D2	*Euphrasia officinalis* subsp. *anglica*	Box Hill	TQ/170.510	5	2
Total				5	2
Wales					
D3	*Euphrasia officinalis* subsp. *rostkoviana*	Gower	SS/539.928	5	2
D4	*Euphrasia officinalis* subsp. *rostkoviana*	Caeae Llety-cyli	SN/603.535	5	2
D5	*Euphrasia officinalis* subsp. *rostkoviana*	Devil's Bridge	SN/751.735	5	2
D6	*Euphrasia rivularis*	Moel Siabod	SH/711.549	9	2
D7	*Euphrasia rivularis*	Snowdon	SH/605.558	10	1
D8	*Euphrasia rivularis*	Cwm Glas	SH/616.565	9	-
Total				43	9
North-west England					
D9	*Euphrasia rivularis*	Stickle Tarn	NY/290.083	5	2
D10	*Euphrasia rivularis*	Honiston Pass	NY/226.134	10	3
Total				15	5

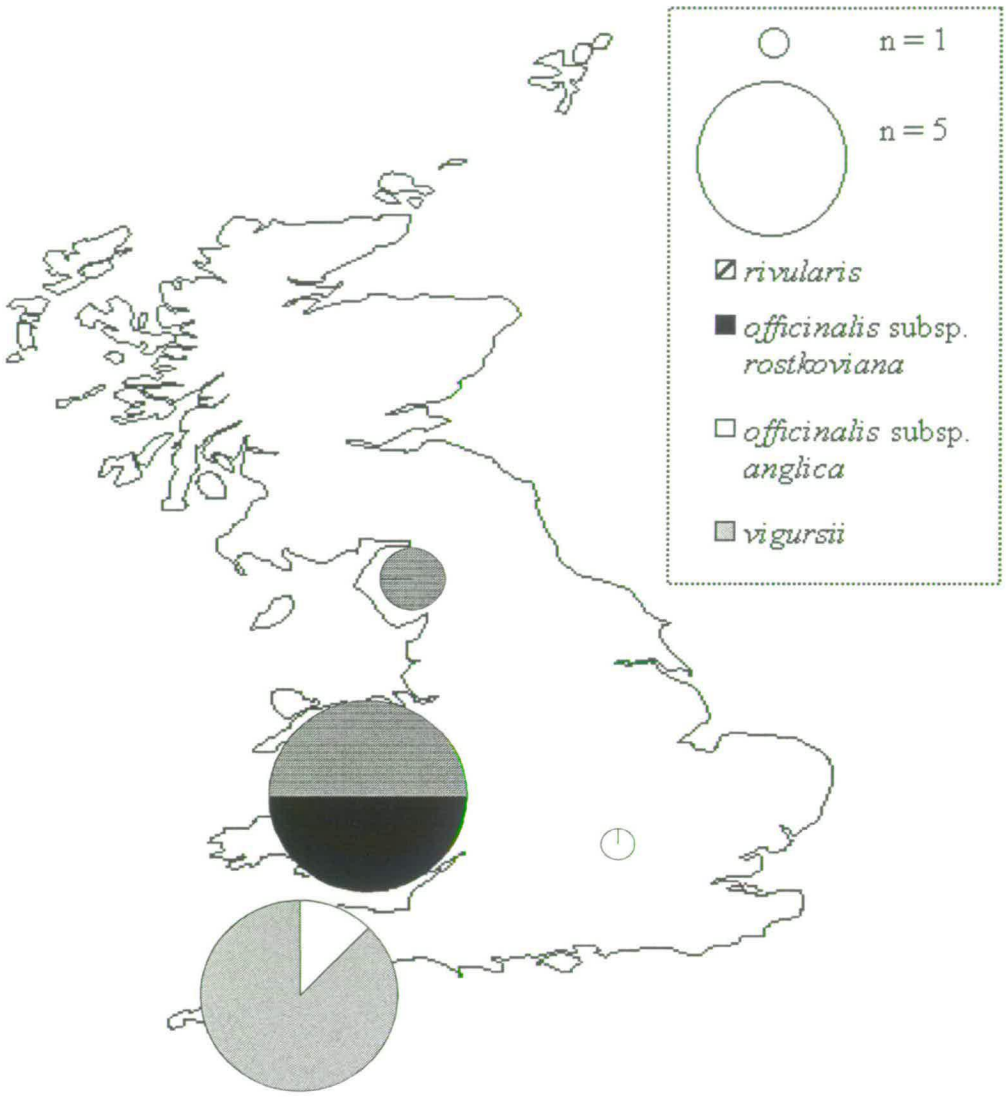


Figure 5:2. Distribution of diploid sites within Britain, grouped according to region. The areas of the circles represents number of populations within each region. n = number of populations.

two primer pair combinations. Chloroplast haplotypes were estimated in 4 to 10 individuals per population, using chloroplast PCR-RFLPs, by the digestion of four amplified chloroplast regions with two restriction enzymes. Both techniques were performed and scored as described in Chapter 4.

5.2.3. Data analysis

Two populations; D16: *Euphrasia vigursii* and D5: *Euphrasia officinalis* subsp. *rostkoviana*, had greater than expected numbers of AFLP bands (Chapter 4). This

high band number may reflect a tetraploid and not a diploid chromosome complement. Due to uncertainty over their ploidal level (Chapter 4) they were excluded from further analysis. Chloroplast and AFLP Excel macros (Chapter 2) were used to define chloroplast haplotypes from the chloroplast restriction fragment data and determine presence of unique AFLP bands within taxonomic groups. They were also used to format both sets of molecular data for relevant population software packages.

5.2.3.1. Population Genetic analyses

Diversity of chloroplast haplotypes within and between populations was estimated by calculation of total diversity (h_T), average intrapopulation diversity (h_S), and level of differentiation among populations (G_{ST}), following the methods of Pons and Petit (1995), using HAPLODIV.

5.2.3.2. Phenetic analyses

A phenetic approach was used to investigate AFLP band distribution. This approach was taken because of the low number of individuals sampled per population and the significant levels of inbreeding detected (Chapter 3), so that I cannot estimate allele frequencies from dominant AFLP markers using the assumption of Hardy-Weinberg equilibrium (Travis *et al.*, 1996).

The total number and total unique number of chloroplast haplotypes and AFLP bands within each taxon were calculated. A null hypothesis assuming a random distribution of chloroplast haplotypes among taxa, and comparable number of unique AFLP bands among widespread and endemic species, was tested with chi-squared contingency analysis, using the statistical package, MINITAB®.

A presence/absence AFLP data matrix was used to estimate pairwise genetic similarity between all individuals using Jaccard's similarity coefficient ($S(ij) = a/(a+b+c)$) (Jaccard, 1912). $S(ij)$ measures genetic similarity between individuals i and j. a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. A pairwise genetic distance matrix was calculated by $D(ij) = 1 -$

S(ij). The matrices were generated using the software package R, version 4 (Casgrain and Legendre, 2001) which was also used to perform multivariate analyses. The ordination technique, principal coordinate analysis (Gower, 1966), was used to provide a visual representation of genetic similarity between individuals from a range of taxa.

Genetic variation was partitioned by analysis of molecular variance (AMOVA), using the software program ARLEQUIN (Excoffier *et al.*, 1992; Schneider *et al.*, 2000), based on pairwise Euclidean distance matrices for both chloroplast haplotypes and AFLP data. The significance of variation at each hierarchial level was tested using a non-parametric permutation procedure (1000 randomisations) (Excoffier *et al.*, 1992). To examine variation among taxa, the genetic variation was partitioned between three hierarchial levels: among taxa, among populations within taxa and within populations. This was repeated to include i) grouping of two subspecies of *Euphrasia officinalis* into one taxon; ii) splitting of *Euphrasia rivularis* into the two geographical groups, Wales and north-west England. iii) splitting of *Euphrasia vigursii* into inland and coastal populations. Examination of variation among regions was performed by similar nested AMOVA with the hierarchial levels being among regions, among populations within regions and within populations.

5.3. Results

5.3.1. Chloroplast PCR-RFLP haplotype and AFLP band distribution

Across all diploid taxa there were five chloroplast haplotypes (Haplotypes A, B, D, L, K; Table 5:2; Figure 5:3). Overall each population was predominantly fixed for one chloroplast haplotype with 89.8% of the variation partitioned between populations, G_{ST} = 0.898 (h_T = 0.749, h_S = 0.076). The distribution of chloroplast haplotypes was significantly structured across taxa (χ^2 = 75.67, d.f. = 3, $p < 0.000$) (Figure 5:4). *Euphrasia vigursii* predominantly contained haplotype K, in common with both subspecies of *Euphrasia officinalis*. One population (D15: Tregonetha Downs; Table 5:1) was fixed for haplotype L. This haplotype differed from the former haplotype by one five base pair indel (Table 5:2). *Euphrasia rivularis*

Table 5:2. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*: AS = *psa*A - *trn*S: CD = *trn*C - *trn*D: HK = *trn*H - *trn*K: KK = *trn*K_1 - *trn*K_2 (Taken from Chapter 4).

Haplotype	AS:*Hinf*I band I	AS:*Hinf*I band II	AS:*Hinf*I band III	CD:*Hinf*I band I	CD:*Hinf*I band II	CD:*Hinf*I band III	HK:*Hae*III band I	KK:*Hinf*I band I
A	1	1	2	1	2	3	2	1
B	1	1	2	1	2	3	2	0
C	1	1	2	2	2	1	2	0
D	1	1	2	1	2	1	2	0
E	3	1	2	1	2	3	2	0
F	3	1	2	1	1	2	2	0
G	3	1	2	?	1	0	2	0
H	3	1	2	?	1	0	1	0
I	2	2	2	2	2	1	2	0
J	2	2	2	2	2	1	3	0
K	1	0	1	1	2	3	2	0
L	1	0	1	1	2	3	1	0

Haplotypes A - L defined as particular associations of these restriction patterns. Polymorphisms are scored in order of decreasing molecular weight, with the absence of a restriction fragment represented by 0. Uncertainty of band molecular weight represented by ?

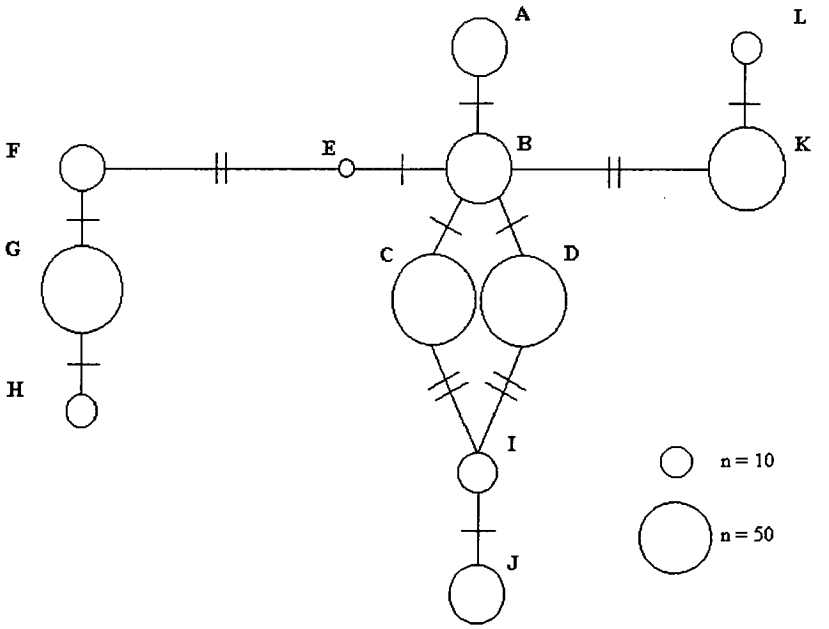


Figure 5:3. Relationships between chloroplast haplotypes, represented as a minimum spanning network. Haplotypes A to L are described in Table 5:2. Mutational steps are indicated by single cross line. The areas of the circles represents number of individuals with each haplotype. n = number of individuals (Taken from Chapter 4).

possessed haplotypes B and D (Figure 5:4). These two haplotypes were generally not shared by either subspecies of *Euphrasia officinalis* but have been found to occur in tetraploid taxa (Chapter 6). The disjunct populations of *Euphrasia rivularis* differed in frequencies of chloroplast haplotypes. Populations from north-west England were fixed for haplotype B, whereas Welsh populations were predominantly fixed for haplotype D, with 3 out of 31 individuals possessing haplotype B. These two haplotypes differed by one 10 base pair indel (Table 5:2).

The two AFLP primer pairs produced 58 scorable bands. Of these bands, 48 were polymorphic and 10 were monomorphic. The total number of AFLP bands in each taxon ranged from 36 (*Euphrasia vigursii*) to 43 (*Euphrasia rivularis*). Approximately 50% of these showed intra-taxon variation. Each taxon contained few private bands (Figure 5:5), mainly present in single individuals. Two further bands were unique to the two endemic species, *Euphrasia vigursii* and *Euphrasia rivularis*, at low frequency. Combining these two endemic species resulted in the presence of eight AFLP bands that were absent from *Euphrasia officinalis*. This number is not significantly different from the number of unique bands present in *Euphrasia officinalis* ($\chi^2 = 0.049$, d.f. = 1, $p = 0.825$). The populations of *Euphrasia rivularis* in north-west England contained nine AFLP bands not present in either of the populations in Wales. The Welsh populations contained an additional four AFLP bands not present in either of north-west England populations. The majority of these AFLP bands were present in only a few individuals within either taxa. This number of unique intra-specific bands compared with 21 unique intra-specific bands present within *Euphrasia officinalis* (10 unique AFLP bands within *Euphrasia officinalis* subsp. *rostkoviana* and 11 unique AFLP bands within *Euphrasia officinalis* subsp. *anglica*). The majority of these AFLP bands were also present in only a few individuals within either taxa.

Principal coordinate analysis of AFLP data discriminated all three taxa and the two disjunct regions of *Euphrasia rivularis* along the first axis, explaining 21.27% of the total variation. The two subspecies of *Euphrasia officinalis* and two geographical areas of *Euphrasia vigursii* were both discriminated along the second axis, which

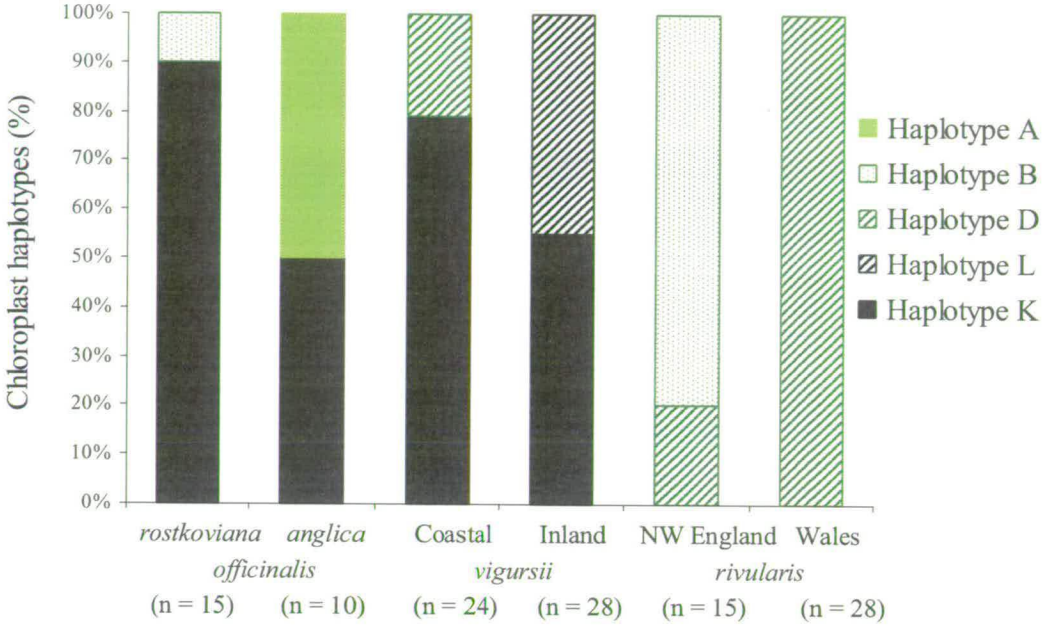


Figure 5:4. Percentage of chloroplast haplotypes within each taxa. n = number of individuals. Haplotypes are described in Table 5:2.

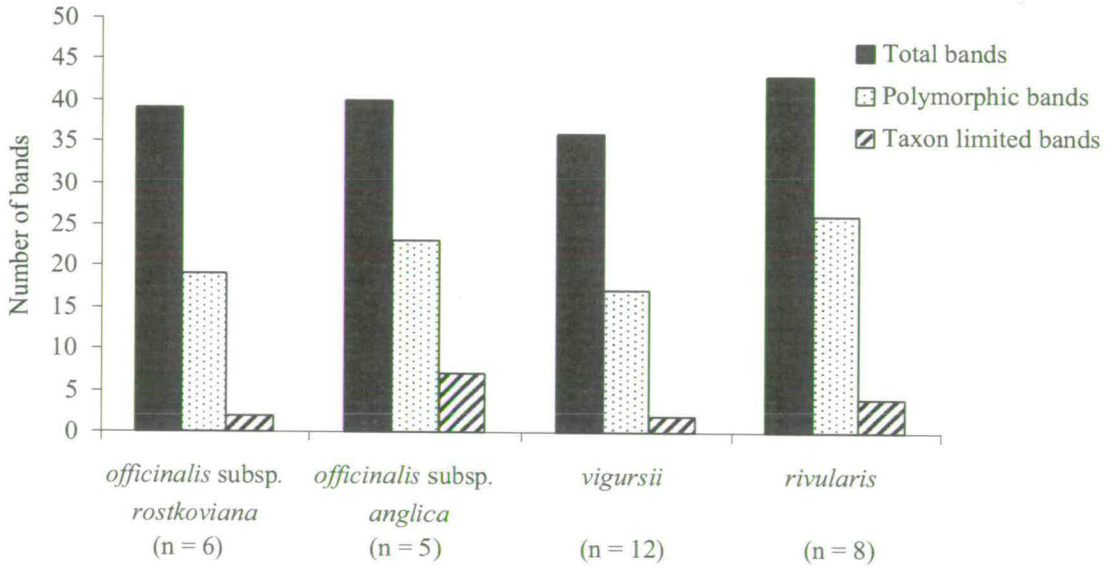


Figure 5:5. Number of total, polymorphic and taxon limited AFLP bands, grouped according to taxa. n = number of individuals.

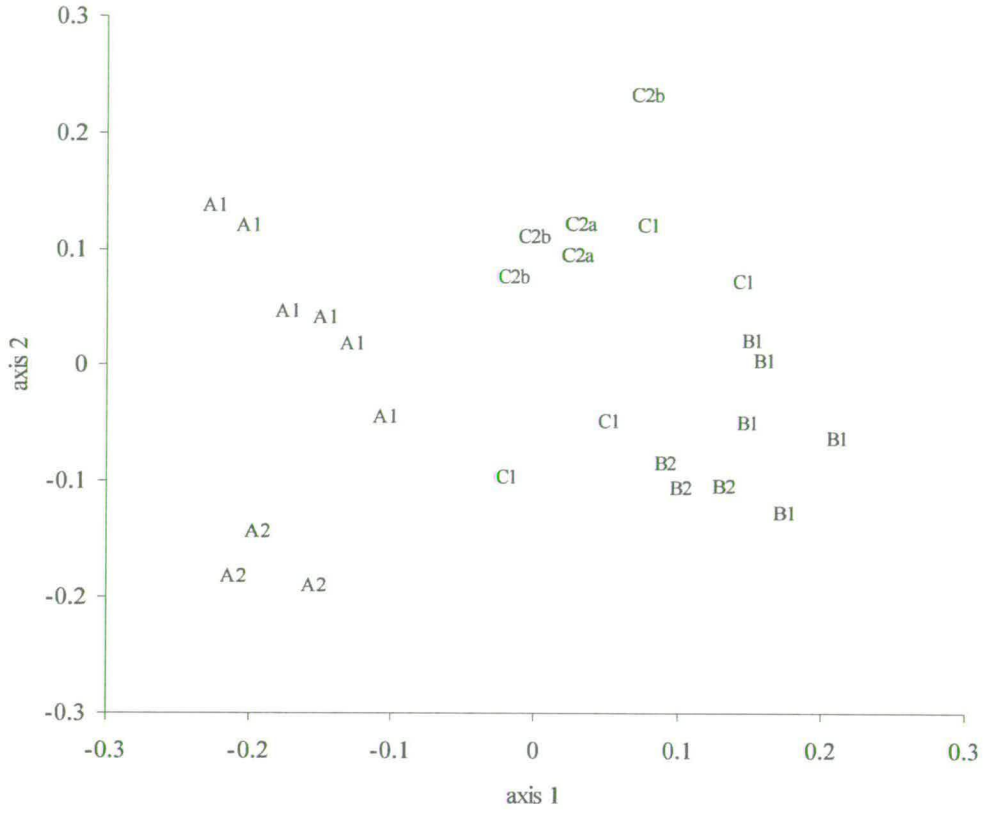


Figure 5:6. Principal coordinate analysis of genetic distance between individuals from four diploid taxa, constructed from 58 AFLP bands. First axis explained 21.27% and second axis 13.72% of the total variation. Taxa are represented by different letters, infra-taxonomic divisions by numbers. A1 = *vigursii* (coastal); A2 = *vigursii* (inland); B1 = *rivularis* (NW England); B2 = *rivularis* (Wales); C1 = *officinalis* subsp. *rostkoviana*; C2a = *officinalis* subsp. *anglica* (E England); C2b = *officinalis* subsp. *anglica* (SW England).

explained a further 13.72% of the variation (Figure 5:6). Analysis of molecular variance showed clear separation among taxa, with 56.86% of chloroplast and 20.35% of AFLP marker variation being partitioned between taxa (Table 5:3). Combining the two subspecies of *Euphrasia officinalis* resulted in 2.8% less variation partitioned between taxa for AFLP bands (variation between taxonomic groups = 17.55%, $p = 0.004$). Separating disjunct *Euphrasia rivularis* into north-west England and Wales groups increased the variation partitioned between taxa for AFLP bands by 2.14% (variation between taxonomic groups = 22.49%, $p < 0.000$). Separating populations of *Euphrasia vigursii* into inland and coastal areas increased

the variation partitioned between taxa for AFLP bands by 1.32% (variation between taxonomic groups = 21.67%, $p < 0.002$). Analysis of molecular variance also showed clear separation among regions, with 47.34% of chloroplast and 18.92% of AFLP marker variation being partitioned between regions (Table 5:4). This partitioning of variation by regional grouping was slightly less than that described by taxonomic grouping.

5.4. Discussion

Molecular markers were not uniformly distributed within the diploid series in Britain. Taxonomic discrimation was most clearly demonstrated by the use of AFLP marker bands. Analysis of these bands showed taxonomic discrimination along the 1st axis of the principal coordinate analysis, with significant amount of variation partitioned among the taxonomic groups. Both endemic species *Euphrasia vigursii* and *Euphrasia rivularis* were genetically distinct. The two subspecies of *Euphrasia officinalis* were genetically more similar to one another than either of these two endemic taxa, with 3% of the total AFLP marker variation partitioned between these two subspecies compared with 18% of total AFLP marker variation partitioned between all taxa.

Taxonomic discrimination was not, however, supported using the microsatellite markers, described in Chapter 3. Principal coordinate analysis of four microsatellite loci, from seven diploid population, showed no taxonomic discrimination along the 1st axis (results not shown). However, the first axis accounted for only 4% of the variation in the data set, and subsequent axes accounted for similarly low levels of variability indicating that the signal to noise ration was very low. With low numbers of loci and high levels of allelic diversity (Chapter 2; Chapter 3), these microsatellite loci are unlikely to provide useful taxonomic signal amongst these taxa.

AFLP band profiles discriminated individuals of *Euphrasia rivularis* according to region. Variation between these two regions within *Euphrasia rivularis* accounted for 2% of the total variation across all taxa. In addition, these two regions had markedly different frequencies of chloroplast haplotypes and possessed a relatively

Table 5:3. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to 4 taxonomic groups. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among taxa	3	51.987	0.62260	**56.86
Among populations within taxa	10	33.806	0.42288	**38.62
Within populations	93	4.600	0.04946	4.52
Total	106	90.393	1.09494	100.00
AFLP genotypes				
Among taxa	3	53.085	1.36995	**20.35
Among populations within taxa	7	61.261	2.59408	**38.54
Within populations	15	41.500	2.76667	41.11
Total	25	155.846	6.73070	100.00

Table 5:4. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to regions. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001; *p < 0.05).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among region	3	46.071	0.50442	*47.34
Among populations within region	10	39.722	0.51170	**48.02
Within populations	93	4.600	0.04946	4.64
Total	106	90.393	1.06558	100.00
AFLP genotypes				
Among region	3	49.682	1.28495	*18.92
Among populations within region	7	64.664	2.73820	**40.33
Within populations	15	41.500	2.76667	40.75
Total	25	155.846	6.78981	100.00

high number of unshared AFLP bands. Both molecular markers therefore show that the two regions of *Euphrasia rivularis* are genetically distinct from one another. Within the examined diploid group, this distinctiveness is relatively large. The marked difference in chloroplast haplotype frequencies and mutually exclusive clustering for AFLPs is more suggestive of separate origins within the two different regions, rather than divergence from a common origin.

The inland and coastal populations of *Euphrasia vigursii* were discriminated along the 2nd axis of the principal coordinate analysis. As calculated using AMOVA, 1% of the total variation was partitioned among these two areas. Within species divisions were therefore present in all three taxa. These divisions range from being given subspecies status to those just considered as geographical separated populations. All of these divisions, however explained a similar amount of partitioning of AFLP marker variation, ranging from 1% to 3% of the total variation. Therefore, while intra-specific differentiation is broadly equivalent within these taxa as measured by AFLPs it does not, however, correspond with equivalent levels of morphological differentiation.

The sampling of each taxon showed a marked geographical bias, reflecting the restricted geographical range of many of the diploid British *Euphrasia*. Given the high degree of spatially restricted taxa within the British diploid group, this sampling artefact was unavoidable. An alternative explanation of geographical, rather than taxonomic, isolation being the important source of genetic differentiation cannot therefore be ruled out. The division of variation accounted for by region was, however, slightly lower than that accounted for by taxonomic grouping. This implies that partitioning of genetic variation was governed at least to some extent by taxonomic and not geographical separation, although further clarification is required. The importance of a taxonomic over a geographical explanation for the partitioning of genetic variation receives some support by the clustering of geographically disparate populations of *Euphrasia officinalis* subsp. *anglica* in the principal coordinate analysis (Figure 5:6). In this respect, additional sampling of the more widespread taxon, *Euphrasia officinalis* subsp. *rostkoviana* would be informative.

The most convincing support for the hypothesis of the origin of the diploid endemic taxa from hybridisation between diploid and tetraploid taxa is seen in the chloroplast haplotypes of *Euphrasia rivularis*. The two chloroplast haplotypes B and D were frequent in this endemic taxon but rare in the proposed parental diploid taxon, *Euphrasia officinalis* subsp. *rostkoviana* (Figure 5:4). Though absent from the proposed parental tetraploid taxon, *Euphrasia micrantha,* the haplotypes were also found in a number of other tetraploid taxa, including *Euphrasia scottica*, *Euphrasia confusa* and *Euphrasia tetraquetra* (Chapter 6). A tetraploid origin of the assumed maternally inherited chloroplast genome would agree with the findings from previous artificial inter-ploidy fertilisation experiments. Yeo (1966) found that fertilisation of ovules in inter-ploidy crosses with a tetraploid female parent, though less frequent, resulted in better development of the seed contents in comparison to crosses where the female parent was diploid.

AFLP genotyping distinguished eight taxon-specific bands in either *Euphrasia vigursii* or *Euphrasia rivularis* but absent from *Euphrasia officinalis*. Many of these unique AFLP bands were, however, present only in single individuals. Given the marked genetic difference between the two ploidy groups, with the presence of a high number of unique tetraploid bands (Chapter 4), under the hypothesis of inter-ploidy hybrid origin, a higher number of these tetraploid bands may be expected to occur in the endemic diploid taxa. Hybridisation between taxa may result in unequal movement of markers into the hybrid offspring. For example, Buerkle and Reiseberg (2001) found unequal but consistent introgression of unique *Helianthus petiolaris* Hort. ex DC. RAPD markers into *Helianthus annuus* L. This disproportionate movement of dominant bands across a reproductive barrier may reflect chromosomal divergence between species (Reiseberg *et al.*, 1995).

Within tetraploid *Euphrasia* there is some evidence for allopolyploidy. This includes bivalent formation in tetraploids (Yeo, 1954) and additional AFLP marker bands in British tetraploid taxa, not found within the British diploid group (Chapter 4). This supports significant divergence between the two pairs of tetraploid chromosomes (Yeo, 1968). A possible explanation of the absence of unique tetraploid bands in the

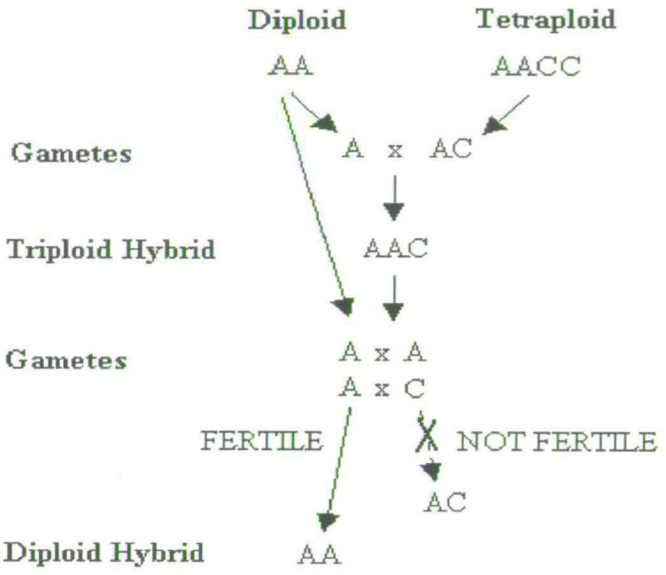


Figure 5:7. Schematic diagram of hypothesed formation of diploid endemic species via inter-ploidal hybridisation. Letter A and C represent different genomes. In cases where normal hybrid gametes are formed from a triploid hybrid, homologous paired AA next generation plants are fertile, whereas non-homologous paired AC next generation plants are not. Hence AFLP bands present in genome C are not passed into the fertile diploid hybrid AA.

endemic diploid taxa may therefore be that chromosomes from the diploid parent can pair at meiosis with complementary chromosomes in the tetraploid parent but not with the non-complementary chromosomes. As a result, AFLP marker bands on the non-complementary set of chromosomes in the tetraploid parent are not passed into the diploid hybrid. Evidence of inter-ploidal hybridisation from the nuclear genome is therefore equivocal (Figure 5:7).

Cultivation and reciprocal transplant experiments performed on *Euphrasia officinalis* subsp. *rostkoviana*, across a number of differing grassland habitats in the Swiss Alps, have shown that adaptations to differing habitats, with morphological modification, can evolve within a few decades (Zopfi, 1998b). The morphological divergence between *Euphrasia officinalis* and the two British diploid endemic taxa is though more marked (Silverside, 1990b). The hypothesis that these endemic diploid species

arose entirely from *Euphrasia officinalis*, with no involvement of tetraploid parentage, requires the emergence of a significant degree of new morphological traits and adaptation to the strikingly different ecological habitats. The possibility that this resulted from variation in a few expressed genes with major phenotypic effects cannot be ruled out but, given the morphological similarity of the endemic taxa, such as *Euphrasia vigursii*, to inter-ploidal hybrids, a better explanation of this degree of morphological differentiation comes from the input of additional genetic material by inter-ploidal hybridisation. Further work is still required to help gain a better understanding of the relative genetic parental contributions to these endemic taxa and the role that ecological divergence may have in their formation.

The variation of molecular markers scored by chloroplast PCR-RFLP and AFLP techniques is congruent with structuring recognised by phenotypic variation. The molecular data therefore support the use of morphological characters to define taxonomic groups and identify major partitioning of variation within the diploid series in Britain. This study therefore supports the present approach of a species-based conservation strategy for diploid endemic species, defined under the Biodiversity Action Plan process (Anon, 1995b). Conservation of these species is especially important given the present threat imposed by habitat loss, seen most clearly in the case of *Euphrasia vigursii* in the decline of their lowland heathland habitat in south-west England (Wigginton, 1999).

CHAPTER 6: Partitioning of genetic variation within the tetraploid group of British *Euphrasia*. Is there an association with current taxonomic delimitation?

Abstract: 15 tetraploid *Euphrasia* species, from three series, are currently recognised in Britain. Four of these species are rare endemic species with high conservation status within the UK Biodiversity Action Plans. To assess whether the current taxonomic framework best reflects underlying patterns of variation, two approaches were undertaken. i) A broadscale taxonomic assessment was undertaken by the analysis of small numbers of individuals of all species. ii) An intensive sampling of paired populations of two species was undertaken to establish whether geography or taxonomy was the best predictor of genetic relationships. Both approaches used AFLP and chloroplast PCR-RFLP molecular markers. Despite some structuring of both AFLP and chloroplast PCR-RFLP markers within the group, with significant levels of variation partitioned among species, no overall clear taxonomic discrimination was demonstrated along the 1st axes of the principal coordinate analyses, using either widespread or more intensive sampling strategies. Taxonomic groupings at the series level were also not supported by AFLP markers, but grouping of chloroplast haplotypes suggests distinct separation between the combined series *Boreales* and *Nemorosae* and the series *Parviflorae*. A hypothesis of at least three separate polyploid events in the formation of the tetraploid *Euphrasia* group in Britain can explain this distinction. From this molecular study it is evident that the current simple species-based approach to conservation of this complex group fails to reflect the partitioning of genetic variation within the group and that a new approach is required.

6.1. Introduction

Molecular analyses, using chloroplast PCR-RFLP and AFLP techniques have shown that the partitioning of variation within the British diploid *Euphrasia* series *Euphrasia* matches the taxonomic divisions within the group, as described by morphological criteria (Chapter 5). Similar congruence between genetic and morphological discontinuities has been shown in other taxonomically complex groups (Zouhair *et al.*, 2000; Román *et al.*, 2003). The use of genetic fingerprinting techniques at higher taxonomic levels has also been informative in other plant genera. These have included the support of the three main sections within genus *Juniperus* L. (Adams and Demeke, 1993), support for two sections within

Cochlearia L. (Koch *et al.*, 1996) and examination of taxa within two sections of *Rubus* subgenus *Rubus* (Kraft *et al.*, 1996).

Morphological discontinuities within the tetraploid group of British *Euphrasia* are similarly recognised at differing taxonomic levels. 15 species are currently recognised within three series within the subsection *Euphrasia* (Yeo, 1978a; Figure 6:1). The aim of this chapter is to examine whether any partitioning of genetic variation corresponds to presently described taxonomy, at both species and series levels, within the tetraploid group in Britain. With four tetraploid species currently recognised under the Biodiversity Action Plan process (Anon, 1995b), the understanding of this partitioning and its relationship to current *Euphrasia* taxonomy will be important when considering future conservation management strategies.

6.1.1. Taxonomy of tetraploid *Euphrasia*

Tetraploid species in Britain are grouped within three series of subsection *Euphrasia*; *Boreales*, *Nemorosae* and *Parviflorae*. The three series are discriminated by the combination of a number of overlapping morphological quantitative traits These traits consist of differences in length of cauline internodes, presence or absence of glandular hairs, length of stalk of glandular hairs, shape of upper cauline and lower floral leaves and teeth, node of first flowering, size of upper lip of corolla, length and shape of capsule and the appearance of capsular margin (Yeo, 1978a). Species from two other series have previously been recorded but these are not currently recognised as having widespread British distributions. *Euphrasia stricta*, within the series *Majoriflorae*, is presently restricted to Guernsey (Pugsley, 1922; Yeo, 1962; Yeo, 1968) and *Euphrasia salisbergensis* var. *hibernica*, within the subsection *Angustifoliae*, has been recorded from a single locality in Yorkshire but this determination has since been refuted (Yeo, 1975; Sledge, 1975).

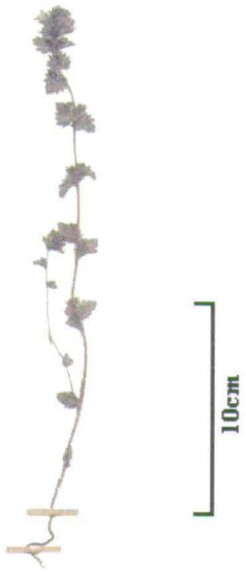
6.1.2. Series *Boreales*

Series *Boreales* contains only one species, *Euphrasia arctica* (Yeo, 1978a). This large flowered robust species, occurring in damp meadows and pastures, has a widespread distribution throughout Britain but is more common in the north (Silverside, 1990a; Figure 6:1). Marked morphological variation occurs across its

extensive European geographical range, leading to taxonomic subdivision at the subspecies level. Two of these subspecies occur in Britain (Preston *et al.*, 2002). *Euphrasia arctica* subsp. *borealis* is a later seasonal variant plant, tending to flower at a higher node than the earlier larger flowered subspecies *Euphrasia arctica* subsp. *arctica* (Yeo, 1978a). Within Britain, this species incorporates the morphological variation previously described within *Euphrasia suecica* (Bucknall, 1917; Pugsley, 1930), glandular *Euphrasia breviplia* and eglandular *Euphrasia borealis* (Yeo, 1970a).

6.1.3. Series *Nemorosae*

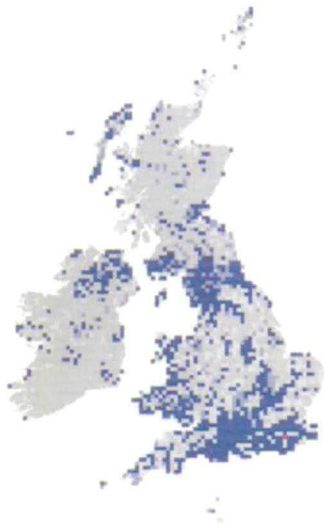
The series *Nemorosae* includes a group of taxa that are later seasonal variants compared to *Euphrasia arctica*. The species of this series also tend to be more branched with their indumentum rarely glandular (Pugsley, 1930). Series *Nemorosae* includes the species *Euphrasia tetraquetra*, *Euphrasia nemorosa*, *Euphrasia pseudokerneri* (Pugsley, 1929) and *Euphrasia confusa* (Pugsley, 1922). *Euphrasia nemorosa* is widely distributed throughout Europe. In Britain this medium flowered species occurs in grassland and lowland heathland but is restricted to coastal calcareous soils in the north and west (Silverside, 1990a; Figure 6:1). It incorporates the morphological variation, previously described within the hirsute species, *Euphrasia curta* (Drabble, 1916; Pugsley 1930). The other species within this series have a more restricted distribution, generally confined to British Isles. The small flowered species, *Euphrasia confusa* has the most widespread distribution, occurring in well drained, grazed, poorly acid to calcareous turf of heathland and moorland, generally at higher altitudes (Silverside, 1990a; Figure 6:1). The small flowered *Euphrasia tetraquetra* is generally restricted to short turf of cliffs and dunes and the large flowered late seasonal variant *Euphrasia pseudokerneri* is restricted to calcareous grassland and damp fen (Preston *et al.*, 2002; Figure 6:1). These latter two species also occur outside Britain, in north-west France and west Ireland, respectively (Yeo, 1978a). The morphological range within the series *Nemorosae* also currently encompasses a previous record in north Scotland initially classified under the central European species, *Euphrasia caerulea* (Bucknall, 1917; Pugsley, 1930).





Euphrasia arctica* subsp. *borealis
Series: *Boreales*
Population: north-west England – T3
Date collected: 27th June 2000





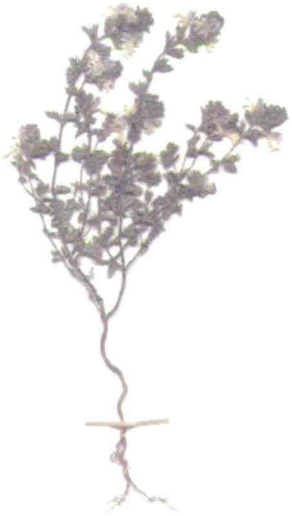
Euphrasia nemorosa
Series: *Nemorosae*
Population: south-west England – T68
Date collected: 18th July 2000

Figure 6:1. British *Euphrasia* species, collected within the tetraploid series *Boreales*, *Nemorosae* and *Parviflorae*, section *Euphrasia*. Population numbers described in Table 6:1. Distribution maps from Preston *et al.* (2002). Dark blue = records 1987 - 1999; medium blue = records 1970 - 1986; pale blue = records pre 1970; red = introduced record.





Euphrasia tetraquetra
Series: *Nemorosae*
Population: south-west England – T66
Date collected: 17th July 2000





Euphrasia pseudokerneri
Series: *Nemorosae*
Population: east England – T55
Date collected: 27th August 2002

Figure 6:1. *Continued.*





Euphrasia confusa
Series: *Nemorosae*
Population: south-west England – T16
Date collected: 21st July 2000





Euphrasia frigida
Series: *Parviflorae*
Population: mid Scotland – T29
Date collected: 21st July 2002

Figure 6:1. *Continued.*





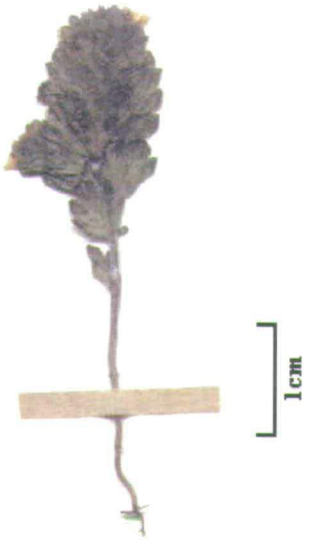
Euphrasia ostenfeldii
Series: *Parviflorae*
Population: north Scotland – T52
Date collected: 7th August 2001





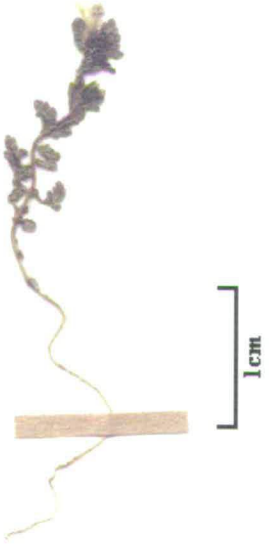
Euphrasia marshallii
Series: *Parviflorae*
Population: north Scotland – T38
Date collected: 9th August 2001

Figure 6:1. *Continued.*





Euphrasia foulaensis
Series: *Parviflorae*
Population: north-west Scotland – T24
Date collected: 5th August 2001





Euphrasia cambrica
Series: *Parviflorae*
Population: Wales – T11
Date collected: 15th August 2000

Figure 6:1. *Continued.*





Euphrasia campbelliae
Series: *Parviflorae*
Population: north-west Scotland – T13
Date collected: 3rd August 2001



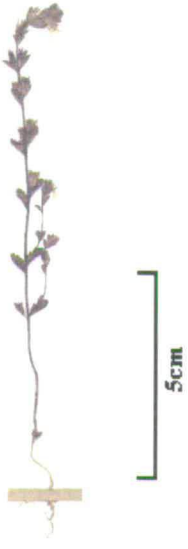
"Euphrasia fharaidensis"
Series: "*Parviflorae*"
Population: north Scotland – T19
Date collected: 7th August 2001

Figure 6:1. *Continued.*





Euphrasia micrantha
Series: *Parviflorae*
Population: mid Scotland – T42
Date collected: 12th August 2001





Euphrasia scottica
Series: *Parviflorae*
Population: north-west Scotland – T62
Date collected: 3rd August 2001

Figure 6:1. *Continued.*





Euphrasia heslop-harrisonii
Series: *Parviflorae*
Population: north-west Scotland – T36
Date collected: 4th August 2001

Figure 6:1. *Continued.*

6.1.4. Series *Parviflorae*

The series *Parviflorae* holds the highest diversity of British tetraploid species, containing 10 out of the 15 currently described species. It includes generally small flowered species of diverse habit, with distributions ranging from widespread European to restricted British endemics (Yeo, 1978a). The widespread European species include *Euphrasia frigida* occurring on wet basic montane ledges, *Euphrasia micrantha* a species of dry heathland and *Euphrasia scottica* occurring in wet moorland and acidic hill flushes (Silverside, 1991a; Figure 6:1). Yellow small flowered populations in Exmoor, initially classified under another widespread European species of montane grassland, *Euphrasia minima* (Hiern, 1909), are currently encompassed within *Euphrasia confusa*, series *Nemorosae* (Pugsley, 1919; Stuart Thompson, 1919).

The distribution of *Euphrasia frigida*, *Euphrasia micrantha* and *Euphrasia scottica* extends northwards from mainland Britain, with *Euphrasia frigida* and *Euphrasia scottica* reaching Iceland (Pugsley, 1933; Yeo, 1978a). A further two species within series *Parviflorae* have a similar distribution, extending north from their southern British range. These small flowered species are the hirsute *Euphrasia ostenfeldii* (Yeo, 1971), occurring on dry limestone ledges and cliffs and *Euphrasia foulaensis* occurring on grazed damp cliff top turf (Silverside, 1991b; Figure 6:1).

The remaining five species within the series *Parviflorae* are endemic to Britain, four of which are classified as Biodiversity Action Plan species with high conservation status (Anon, 1995b). Most of these species are small flowered, with *Euphrasia marshallii* (Pugsley, 1929; Pugsley, 1936) and *Euphrasia rotundifolia* (Pugsley 1929) having the largest flower size. These two hirsute species are restricted to damp basic cliff top turf along the north Scottish coast (Silverside, 1991b; Figure 6:1). *Euphrasia campbelliae* (Pugsley, 1940) and *Euphrasia heslop-harrisonii* (Pugsley, 1945) also have markedly restricted northern British distributions. *Euphrasia campbelliae* occurs in coastal heathy turf of north-west Scotland and *Euphrasia heslop-harrisonii* in grazed estuarine saltmarshes along the north-west and north Scottish coast (Silverside, 1991b; Silverside, 1991c; Figure 6:1). The smallest

flowered species *Euphrasia cambrica* (Pugsley, 1929; Pugsley, 1936) is restricted to poor montane grazed turf of north Wales (Silverside, 1991c; Figure 6:1).

6.1.5. Other tetraploid taxa

The morphological range of tetraploid species in Britain have previously included a number of other distinct taxa, that are currently not recognised as species. All these taxa are restricted to Scotland, including *Euphrasia septentrionalis* (Druce and Lumb, 1921; Druce, 1930), *Euphrasia atroviolacea* (Druce and Lumb, 1923; Druce, 1930), *Euphrasia lumbii* (Druce, 1930), *Euphrasia rhumica* (Pugsley, 1945) and *Euphrasia eurocarpa* (Pugsley, 1945). The morphological variation within the species *Euphrasia kerneri* (Townsend, 1897) is currently encompassed within *Euphrasia pseudokerneri* (Pugsley, 1930). Since the publication of the current series-level taxonomy within the subsection *Euphrasia* (Yeo, 1978a), a further taxon has been recognised and is awaiting formal description and species recognition. This small flowered taxon, provisionally named "*Euphrasia fharaidensis*" is endemic to Britain, restricted to moderately base rich coastal flushes in north Scotland (Silverside, 1991c; Silverside, 1998).

6.1.6. Morphological variation within the tetraploid group

Species and series within the tetraploid group are narrowly separated morphologically by a combination of continuous overlapping character states. Morphometric analysis, in seven species in Ireland, has demonstrated that it is possible to identify morphologically similar species by the combination of six characters (Bobear, 1969). The morphological complexity is at its greatest in Britain, with western and northern areas holding one of the highest diversities of *Euphrasia* found in Europe (Yeo, 1978a).

Cultivation experiments have provided evidence that morphological differences between the tetraploid species and series described above are not due to differing environmental conditions. This has been reported for 8 of the 15 species, including all three British tetraploid series. The species examined to date are: *Euphrasia arctica* subsp. *borealis*, *Euphrasia nemorosa*, *Euphrasia tetraquetra*, *Euphrasia pseudokerneri*, *Euphrasia confusa*, *Euphrasia frigida*, *Euphrasia micrantha* and

Euphrasia scottica (Yeo, 1959; Yeo, 1962; Bobear, 1969). Tetraploid species commonly occur sympatrically and hybridisation as been invoked to explain populations combining gradations of morphological characters from two or more species. This gradation may occur between species of the same or differing series (Stace, 1997). Phenotypic variation is therefore not completely discontinuous between series or species. Given this pattern of variation, is the hypothesis that currently defined series or taxonomic groups reflects significant partitioning of genetic variation correct? Under the null hypothesis, gene flow has been sufficient within the tetraploid group such that genetic variation is not related to defined morphological groups at either the series or species level. Under this null hypothesis, marker variation among series or species will therefore be no greater than variation within series or species and there will be an absence of unique taxonomic markers in either of the morphological groupings.

By estimating the distribution of variable markers within and between series and species I will test this null hypothesis. Rejection of the null hypothesis will imply significant congruence between genetic and morphological variation. I therefore specifically wish to determine:

1. The distribution of molecular marker variation within and between groups at both the series and the species level.
2. Quantitative estimates of partitioning of genetic variation within and between groups at both the series and the species level.
3. The importance of geographical structuring and/or taxonomic divisions in determining the distribution of molecular genetic markers.
4. The presence of unique molecular markers within each group at both the series and the species level.

6.2. Materials and Methods

Partitioning of genetic variation within the tetraploid group was performed using two differing sampling strategies. The first strategy focused sampling at the species level, and aimed to adequately represent partitioning of variation across all tetraploid species within British *Euphrasia*. With the high numbers of species involved, this

resulted in low within species sampling. The second sampling strategy was aimed to complement the first widespread strategy with more intensive sampling focused on two species.

The tetraploid group of British *Euphrasia* are taxonomically complex with a high proportion of populations showing character combinations from differing species. Both sampling strategies concentrated on collecting material from populations determined to morphologically represent one of the currently recognised *Euphrasia* species in Britain, avoiding sampling from populations determined as likely to have been the result of hybrid origin or not currently recognised at taxonomic level. In taking this approach it is accepted that a significant proportion of the total genetic variation in the tetraploid group was not sampled. It was felt, however that given the complexity of the tetraploid *Euphrasia* group in Britain this approach was the most appropriate for an initial molecular study into the group. The results from this study may subsequently be used to incorporate these intermediate populations into future examinations of partitioning of genetic variation within British *Euphrasia*.

6.2.1. Widespread sampling

Widespread sampling involved sampling plants from 56 populations of 15 currently recognised *Euphrasia* species from all three tetraploid series within seven regions of Britain (Table 6:1; Figure 6:2). Species included the widespread *Euphrasia arctica* subsp. *borealis*, *Euphrasia nemorosa*, *Euphrasia confusa*, *Euphrasia tetraquetra*, *Euphrasia ostenfeldii*, *Euphrasia scottica*, *Euphrasia micrantha*, *Euphrasia frigida*, more restricted *Euphrasia marshallii*, *Euphrasia foulaensis*, *Euphrasia pseudo-kerneri* and four endemic species *Euphrasia campbelliae*, *Euphrasia heslop-harrisonii*, *Euphrasia cambrica* and "*Euphrasia fharaidensis*" (Table 6:1; Figure 6:2). Two species were excluded from the study. Populations of *Euphrasia arctica* subsp. *arctica*, occur only in Orkney and Shetland, which were not visited in the time allocated for fieldwork and too few individuals of *Euphrasia rotundifolia* were found. For each population 3 to 8 individuals were collected using the sampling technique as described in Chapter 4.

Table 6:1. Sampling of tetraploid *Euphrasia* within seven British regions, for chloroplast PCR-RFLP and AFLP analysis. Species allocated to series as defined by Yeo (1978a), with the addition of "*Euphrasia fharaidensis*" to series *Parviflorae*. N (cp) = number of individuals sampled in each population for chloroplast PCR-RFLP analyses; N (AFLP) = number of individuals sampled in each population for AFLP analyses.

Number	Series	Species	Population	Grid reference	N (cp)	N (AFLP)
South-west England						
T15	*Nemorosae*	*Euphrasia confusa*	Bunny Hill	SX/119.678	4	3
T16	*Nemorosae*	*Euphrasia confusa*	Kit Hill	SX/374.715	4	3
T40	*Parviflorae*	*Euphrasia micrantha*	Wheal Busy	SW/742.423	5	3
T41	*Parviflorae*	*Euphrasia micrantha*	Chapel Porth	SW/697.494	5	2
T65	*Nemorosae*	*Euphrasia tetraquetra*	Wicca Pool	SW/465.403	4	3
T66	*Nemorosae*	*Euphrasia tetraquetra*	Porthtowan	SW/689.479	4	2
Total					26	16
Wales						
T1	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Rhos Common	SN/791.078	5	-
T2	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Craig Branddu	SN/715.458	5	-
T11	*Parviflorae*	*Euphrasia cambrica*	Snowdon	SH/605.558	3	1
T43	*Nemorosae*	*Euphrasia nemorosa*	Cae Biaen Dyffryn	SN/606.447	5	-
T50	*Parviflorae*	*Euphrasia ostenfeldii*	Snowdon	SH/602.556	5	2
T67	*Nemorosae*	*Euphrasia tetraquetra*	Mewslade	SS/419.873	5	3
Total					28	6
East England						
T44	*Nemorosae*	*Euphrasia nemorosa*	Haslingfield	TL/408.517	5	-
T54	*Nemorosae*	*Euphrasia pseudokerneri*	Therfield Heath	TL/330.395	5	-
T55	*Nemorosae*	*Euphrasia pseudokerneri*	Fleam Dyke	TL/545.545	5	-
T56	*Nemorosae*	*Euphrasia pseudokerneri*	Devil's Ditch	TL/617.616	5	-
Total					20	0
North-west England						
T3	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Rosthwaite	NY/255.159	4	-
T57	*Parviflorae*	*Euphrasia scottica*	Honiston Pass	NY/230.136	5	1
Total					9	1

Table 6:1. *Continued.*

Number	Series	Species	Population	Grid reference	N (cp)	N (AFLP)
Mid Scotland						
T4	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Straloch	NO/035.636	3	-
T5	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Tulloch Moor	NH/960.161	4	-
T29	*Parviflorae*	*Euphrasia frigida*	Creag an Lochain	NN/592.411	5	-
T42	*Parviflorae*	*Euphrasia micrantha*	Tulloch Moor	NH/962.167	5	3
T59	*Parviflorae*	*Euphrasia scottica*	Dalwhinnie	NN/642.850	5	-
Total					22	3
North-west Scotland						
T7	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Siabost	NB/271.473	5	2
T12	*Parviflorae*	*Euphrasia campbelliae*	Reinigeadal	NB/218.017	5	2
T13	*Parviflorae*	*Euphrasia campbelliae*	Mealasta	NA/994.235	5	2
T14	*Parviflorae*	*Euphrasia campbelliae*	Islibhig	NA/993.280	5	2
T23	*Parviflorae*	*Euphrasia foulaensis*	Mealasta	NA/993.240	4	3
T24	*Parviflorae*	*Euphrasia foulaensis*	Mangurstadh	NB/005.315	5	3
T30	*Parviflorae*	*Euphrasia frigida*	Bealach na ba	NG/780.417	5	-
T33	*Parviflorae*	*Euphrasia heslop-harrisonii*	Ardtoe	NM/627.712	5	-
T35	*Parviflorae*	*Euphrasia heslop-harrisonii*	Ardnish	NG/674.238	5	-
T36	*Parviflorae*	*Euphrasia heslop-harrisonii*	Seilebost	NG/083.668	5	2
T46	*Nemorosae*	*Euphrasia nemorosa*	Dail Beag	NB/227.458	5	-
T51	*Parviflorae*	*Euphrasia ostenfeldii*	Storr, Skye	NG/510.540	5	3
T62	*Parviflorae*	*Euphrasia scottica*	Carlabhagh	NB/190.413	5	3
T63	*Parviflorae*	*Euphrasia scottica*	Dail Beag	NB/226.462	5	-
Total					69	22
North Scotland						
T8	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Betty Hill	NC/700.621	3	2
T9	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Durness	NC/405.669	5	2
T10	*Boreales*	*Euphrasia arctica* subsp. *borealis*	Reay	NC/658.653	5	2
T17	*Nemorosae*	*Euphrasia confusa*	Inchnadamp	NC/267.213.	5	3
T18	*Parviflorae*	"*Euphrasia fharaidensis*"	Inchnadamp	NC/272.210	5	3
T19	*Parviflorae*	"*Euphrasia fharaidensis*"	Rhiconich	NC/253.523	4	2

Table 6:1. *Continued.*

Number	Series	Species	Population	Grid reference	N (cp)	N (AFLP)
T22	*Parviflorae*	"*Euphrasia fharaidensis*"	Fharaid Head	NC/387.713	5	3
T25	*Parviflorae*	*Euphrasia foulaensis*	Scourie Bay	NC/144.449	5	-
T27	*Parviflorae*	*Euphrasia foulaensis*	Coldbackie	NC/611.598	4	-
T37	*Parviflorae*	*Euphrasia marshallii*	Betty Hill	NC/605.627	5	2
T38	*Parviflorae*	*Euphrasia marshallii*	Melness	NC/577.653	5	2
T39	*Parviflorae*	*Euphrasia marshallii*	Strathy Point	NC/827.694	5	1
T47	*Nemorosae*	*Euphrasia nemorosa*	Betty Hill	NC/702.608	5	2
T48	*Nemorosae*	*Euphrasia nemorosa*	Fharaid Head	NC/392.687	5	2
T49	*Nemorosae*	*Euphrasia nemorosa*	Dunnett Bay	ND/223.700	3	1
T52	*Parviflorae*	*Euphrasia ostenfeldii*	Inchnadamp	NC/272.210	5	1
T53	*Parviflorae*	*Euphrasia ostenfeldii*	An-t-sron	NC/443.580	5	1
T60	*Parviflorae*	*Euphrasia scottica*	Newton	NC/153.308	5	-
T64	*Parviflorae*	*Euphrasia scottica*	Lochan Hackel	NC/572.533	5	-
Total					89	29

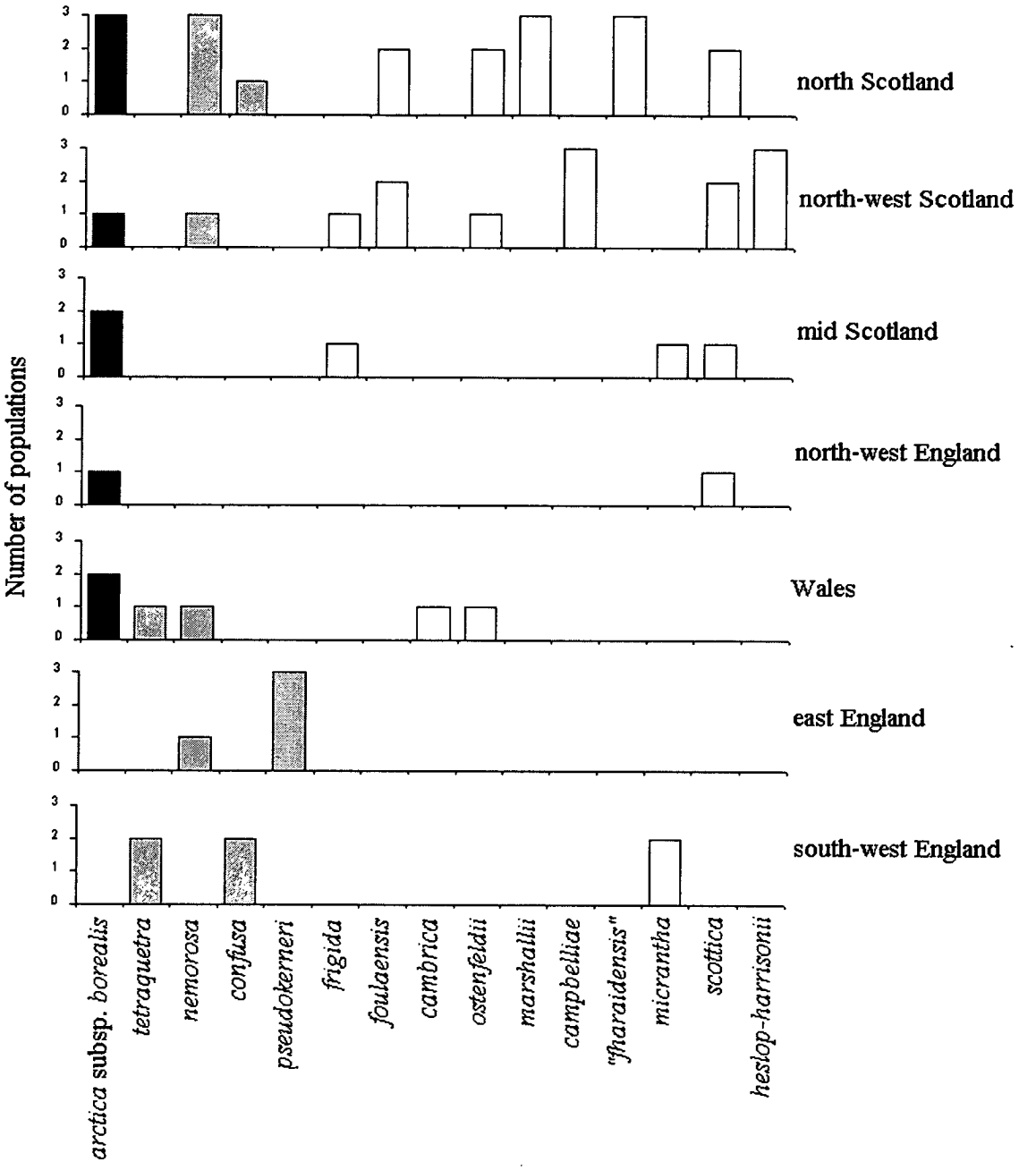


Figure 6:2. Number of populations of each tetraploid species sampled from seven regions within Britain. Shading indicates different series (black = *Boreales*, grey = *Nemorosae* and white = *Parviflorae*). Species allocated to series as defined by Yeo (1978a), with the addition of "*Euphrasia fharaidensis*" to series *Parviflorae*.

6.2.2. Paired sampling of two species

More detailed sampling was conducted within the two tetraploid species, *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa* from the series *Boreales* and *Nemorosae*, respectively. Within each of four geographic areas, in Scotland, paired samples of the two species were collected (Figure 6:3; Table 6:2). The populations comprising each pair were located as close to one another as possible. The close proximity of morphologically distinct populations allowed the testing of the null hypothesis that two species represent separate gene pools, with populations of each being genetically more related to more geographically distant, morphologically similar populations, than to sympatric populations of the other species. Under the alternate hypothesis of significant gene flow, paired populations would represent a single gene pool, more genetically related to each other than to morphologically similar populations, in other geographical areas. Within each population 6 to 16 individuals were collected using the sampling technique as described in Chapter 4 (Table 6:2). One population of *Euphrasia arctica* subsp. *borealis* (T10; Thurso) was determined as the variant *reayensis*.

6.2.3. DNA extraction and molecular marker analyses

DNA extraction was performed as described in Chapter 4. Diversity was assessed in both nuclear and chloroplast genomes. Presence or absence of dominant AFLP bands, generated from two primer pair combinations, was scored in 1 to 3 individuals per population for the widespread sampling strategy and in 6 to 16 individuals per population in the paired sampling strategy (Table 6:1; Table 6:2). Chloroplast haplotypes were scored in 3 to 5 individuals per population in both sampling strategies, using chloroplast PCR-RFLP, by the digestion of four amplified chloroplast regions with two restriction enzymes. Both techniques were performed and scored as described in Chapter 4.

6.2.4. Data analysis

Chloroplast and AFLP Excel macros (Chapter 2) were used to define chloroplast haplotypes from the chloroplast restriction fragment data and determine presence of unique AFLP bands within taxonomic groups. They were also used to format both sets of molecular data for relevant population software packages.



Euphrasia arctica* subsp. *borealis
Population: Lewis – T7
Date collected: 5th August 2001



Euphrasia nemorosa
Population: Lewis – T46
Date collected: 3rd August 2001

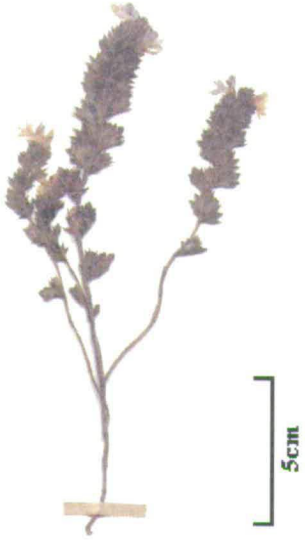


Euphrasia arctica* subsp. *borealis
Population: Durness – T9
Date collected: 8th August 2001



Euphrasia nemorosa
Population: Durness – T48
Date collected: 8th August 2001

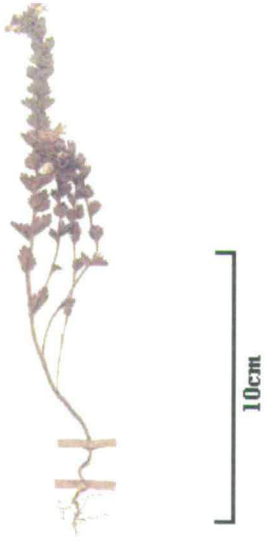
Figure 6:3. Paired populations of *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*, from across north-west and north Scotland regions. Population numbers described in Table 6:2.



Euphrasia arctica* subsp. *borealis
Population: Betty Hill – T8
Date collected: 11th August 2001



Euphrasia nemorosa
Population: Betty Hill – T47
Date collected: 11th August 2001



Euphrasia arctica* var. *reayensis
Population: Thurso – T10
Date collected: 10th August 2001



Euphrasia nemorosa
Population: Thurso – T49
Date collected: 10th August 2001

Figure 6:3. *Continued*

Table 6:2. Paired sampling of two tetraploid species within north-west and north Scotland regions, for chloroplast PCR-RFLP and AFLP analysis. N (cp) = number of individuals sampled in each population for chloroplast PCR-RFLP analyses; N (AFLP) = number of individuals sampled in each population for AFLP analyses.

Number	Species	Location	Grid reference	N(cp)	N(AFLP)
North-west Scotland					
Lewis					
T7	*Euphrasia arctica* subsp. *borealis*	Siabost	NB/271.473	5	14
T46	*Euphrasia nemorosa*	Dail Beag	NB/227.458	5	14
North Scotland					
Durness					
T9	*Euphrasia arctica* subsp. *borealis*	Durness	NC/405.669	5	12
T48	*Euphrasia nemorosa*	Fhariad Head	NC/392.687	5	6
Betty Hill					
T8	*Euphrasia arctica* subsp. *borealis*	Betty Hill	NC/700.621	3	14
T47	*Euphrasia nemorosa*	Betty Hill	NC/702.608	5	11
Thurso					
T10	*Euphrasia arctica* var. *reayensis*	Reay	NC/958.653	5	9
T49	*Euphrasia nemorosa*	Dunnett Bay	ND/223.700	3	16

6.2.5. Widespread taxonomic analyses

Euphrasia cambrica (Population T11) had 36 AFLP bands within the individual tested. This number was comparable to that found in diploid species (mean number of AFLP bands = 39.50 ± 2.89) and lower than expected in tetraploid species (mean number of AFLP bands = 65.33 ± 9.75). No chromosomal counts have been performed in this species, to date. Therefore, though it is assumed to be tetraploid, this has not been confirmed. Consequently, this population was excluded from further analyses. Its exclusion had no discernable effects on the inter-relationships of other individuals in the analyses. Partitioning of genetic variation was analysed by grouping populations by i) species, ii) series iii) geographic region. The allocations of populations to each of the currently recognised series were as described in Yeo's (1978a) monograph. In addition, "*Euphrasia fharaidensis*" was allocated to the series *Parviflorae* (Table 6:1).

Overall diversity of chloroplast haplotypes within and between populations was estimated by calculating total diversity (h_T), average intrapopulation diversity (h_S), and the level of differentiation among populations (G_{ST}), following the methods described by Pons and Petit (1995). This was performed using the computer software program HAPLODIV. The total number and total unique number of chloroplast haplotypes within each of the groups were calculated. The null hypothesis of random distribution of chloroplast haplotypes among series and regions was tested with chi-squared contingency analysis, using the statistical package, MINITAB®. To ensure adequate numbers in each category for statistical analysis, chloroplast haplotypes were combined into three groups according to their location within the minimum spanning network of chloroplast haplotypes. These groups were defined as containing haplotypes separated by single mutational steps in the minimum spanning network, in comparison to two or more mutational steps between the haplotype groups. The groups were haplotypes A to E, F to H and I to J (Table 6:3; Figure 6:4). Also species grouped according to series and regions were also grouped according to country (Scotland versus England and Wales).

Table 6:3. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*: AS = *psa*A - *trn*S: CD = *trn*C - *trn*D: HK = *trn*H - *trn*K: KK = *trn*K_1 - *trn*K_2 (Taken from Chapter 4).

Haplotype	AS:*Hinf*I band I	AS:*Hinf*I band II	AS:*Hinf*I band III	CD:*Hinf*I band I	CD:*Hinf*I band II	CD:*Hinf*I band III	HK:*Hae*III band I	KK:*Hinf*I band I
A	1	1	2	1	2	3	2	1
B	1	1	2	1	2	3	2	0
C	1	1	2	2	2	1	2	0
D	1	1	2	1	2	1	2	0
E	3	1	2	1	2	3	2	0
F	3	1	2	1	1	2	2	0
G	3	1	2	?	1	0	2	0
H	3	1	2	?	1	0	1	0
I	2	2	2	2	2	1	2	0
J	2	2	2	2	2	1	3	0
K	1	0	1	1	2	3	2	0
L	1	0	1	1	2	3	1	0

Haplotypes A - L defined as particular associations of these restriction patterns. Polymorphisms are scored in order of decreasing molecular weight, with the absence of a restriction fragment represented by 0. Uncertainty of band molecular weight represented by ?

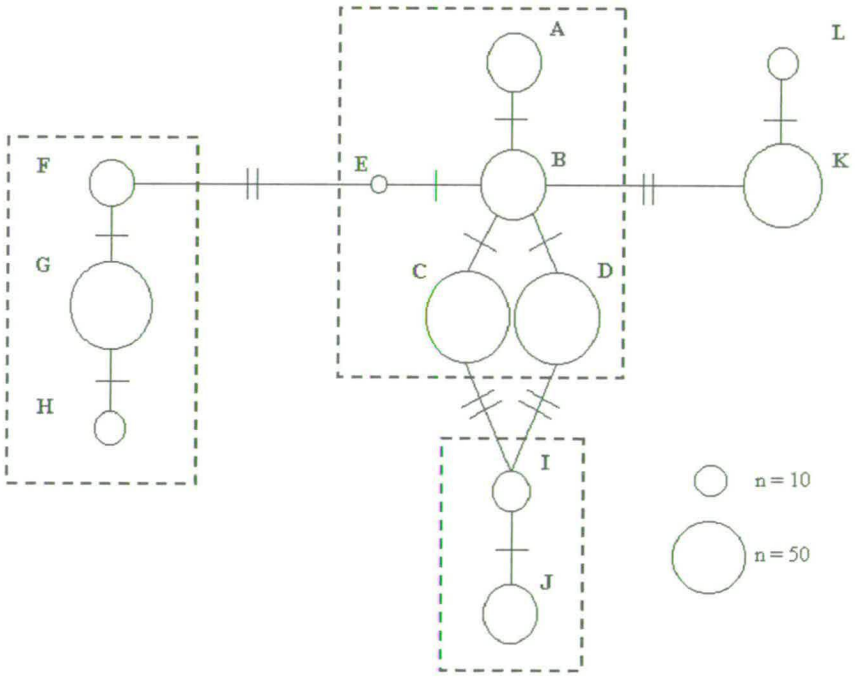


Figure 6:4. Haplotype groups defined according to relationships between chloroplast haplotypes, represented as a minimum spanning network. The three tetraploid groups are delimitated by boxes (haplotypes A to E, F to H and I to J). Haplotypes are described in Table 6:3. Mutational steps are indicated by single cross line. The areas of the circles represents number of individuals with each haplotype. n = number of individuals (Taken from Chapter 4).

With low number of individuals sampled per population and significant levels of inbreeding within many populations (Chapter 3), the assumption of Hardy-Weinberg equilibrium necessary for the estimation of allele frequencies from dominant AFLP markers was not fulfilled (Lynch and Milligan, 1994). A phenetic approach was therefore used to investigate AFLP band distribution. AFLP band variation was analysed by species, series or geographic region. The total number and total number of unique AFLP bands within each of these groups were calculated. The absence/presence AFLP data matrix was used to estimate pairwise genetic similarity between all individuals using Jaccard's similarity coefficient (Jaccard, 1912). From this a pairwise genetic distance matrix was calculated, as described in Chapter 4 and the matrix was used to perform multivariate analysis using the software package R (Casgrain and Legendre, 2001). The ordination technique, principal coordinate analysis (Gower, 1966), was used to provide a visual representation of genetic similarity between individuals from a range of species, series or geographical regions.

Estimation of partitioning of genetic variation was performed using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). Pairwise Euclidean distance matrices were calculated for both chloroplast haplotypes and presence/absence AFLP data, using the equations of Excoffier *et al.* (1992). These matrices were used to perform AMOVA, using the software program ARLEQUIN (Schneider *et al.*, 2000). The significance of variation at each hierarchial level was tested using a non-parametric permutation procedure (1000 randomisations) (Excoffier *et al.*, 1992). For each of the three different grouping methods, the genetic variation was partitioned among three hierarchial levels: among groups (species, series or geographical regions), among populations within groups and within populations.

6.2.6. Paired population analyses

Similar analyses of chloroplast and AFLP band distribution were carried out for the data set derived from paired sampling of *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*. These included estimation of overall chloroplast haplotype diversity within and between populations, grouping of chloroplast haplotypes and AFLP bands according to either species or regional divisions, and calculation of the

total number, and total unique number, of chloroplast haplotypes and AFLP bands within each of these divisions. A similar phenetic approach was used to investigate AFLP band distribution. A visual representation of genetic similarity between individuals was obtained from principal coordinate analysis and partitioning of genetic variation was examined by analysis of molecular variance. Variation among species was examined by partitioning of genetic variation among three hierarchial levels: among species, among populations within species and within populations. Examination of variation among geographical areas was performed by similar nested AMOVA with the hierarchial levels among areas, among populations within areas and within populations.

6.3. Results

6.3.1. Widespread sampling of tetraploid species

Across all tetraploid species there were 10 chloroplast haplotypes (haplotypes A to J; Figure 6:4). Overall, each population was predominantly fixed for one chloroplast haplotype with 85.9% of the variation partitioned between populations, $G_{ST} = 0.859$ ($h_T = 0.856$, $h_S = 0.152$). The distribution of chloroplast haplotypes was significantly structured across series and regions grouped by country ($\chi^2 = 86.177$, d.f. = 4, $p < 0.000$; $\chi^2 = 14.751$, d.f. = 2, $p = 0.001$, respectively) (Figure 6:5; Figure 6:6). 8 out of the 10 haplotypes were widely distributed throughout all regions sampled within Britain. Only haplotypes E and H showed a geographically restricted distribution. Haplotype E was restricted to north-west Scotland and haplotype H to north-west and north Scotland. Testing the null hypothesis of random distribution of haplotypes among species was not possible due to low within species sampling. Three haplotypes were distributed widely across species and series (Haplotype A, B, G; Figure 6:4; Figure 6:7). The other seven haplotypes were predominately distributed in either *Boreales* and *Nemorosae* series or the *Parviflorae* series. Haplotypes I and J were restricted to *Boreales* and *Nemorosae* series. Haplotypes F and H predominantly occurred in these two series, though they also occurred in *Euphrasia foulaensis* and *Euphrasia marshallii* respectively, within the series *Parviflorae*.

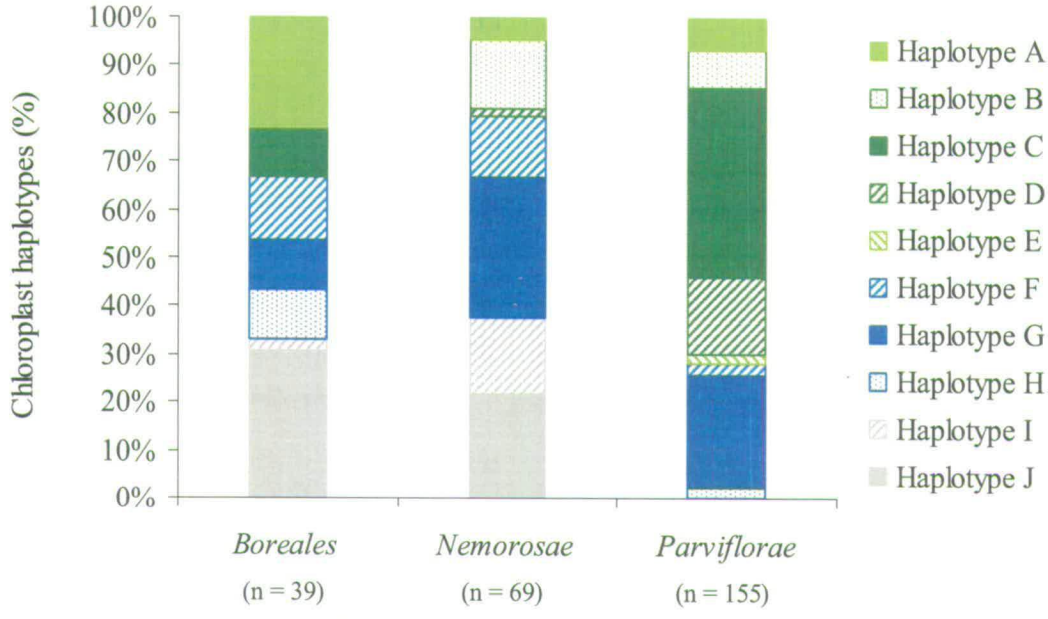


Figure 6:5. Percentage of chloroplast haplotypes within each of Yeo's series. n = number of individuals. Haplotypes are described in Table 6:3.

Haplotypes C and D predominantly occurred in *Parviflorae* series, though they also occurred in *Euphrasia arctica* subsp. *borealis* and *Euphrasia confusa* of the series *Boreales* and *Nemorosae* respectively. Haplotype D was fixed in samples of the two endemic species *Euphrasia campbelliae* and *Euphrasia cambrica*, as well as occurring in "*Euphrasia fharaidensis*", *Euphrasia scottica* and *Euphrasia confusa*. Haplotype E was only found in *Euphrasia heslop-harrisonii* in the *Parviflorae* series.

The two AFLP primer pairs produced 129 scorable bands. Of these bands, 119 were polymorphic and 10 were monomorphic. The total number of AFLP bands in each species ranged from 47 (*Euphrasia heslop-harrisonii*) to 79 (*Euphrasia arctica* subsp. *borealis*) (Figure 6:8). Approximately 60 - 70% of these showed intra-specific polymorphism. A lower percentage of polymorphic bands were found in *Euphrasia foulaensis*, *Euphrasia campbelliae*, *Euphrasia scottica* and *Euphrasia heslop-harrisonii*.

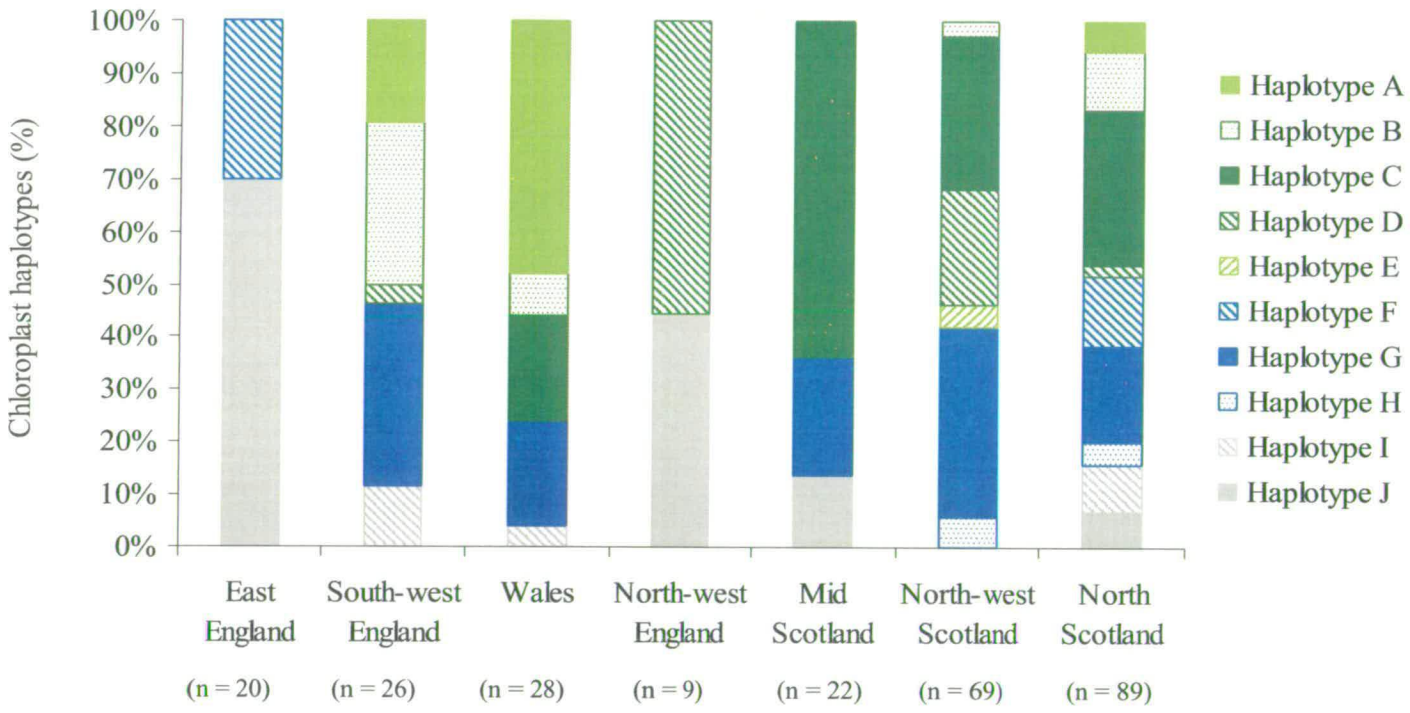


Figure 6:6. Percentage of chloroplast haplotypes within each region. n = number of individuals. Haplotypes are described in Table 6:3.

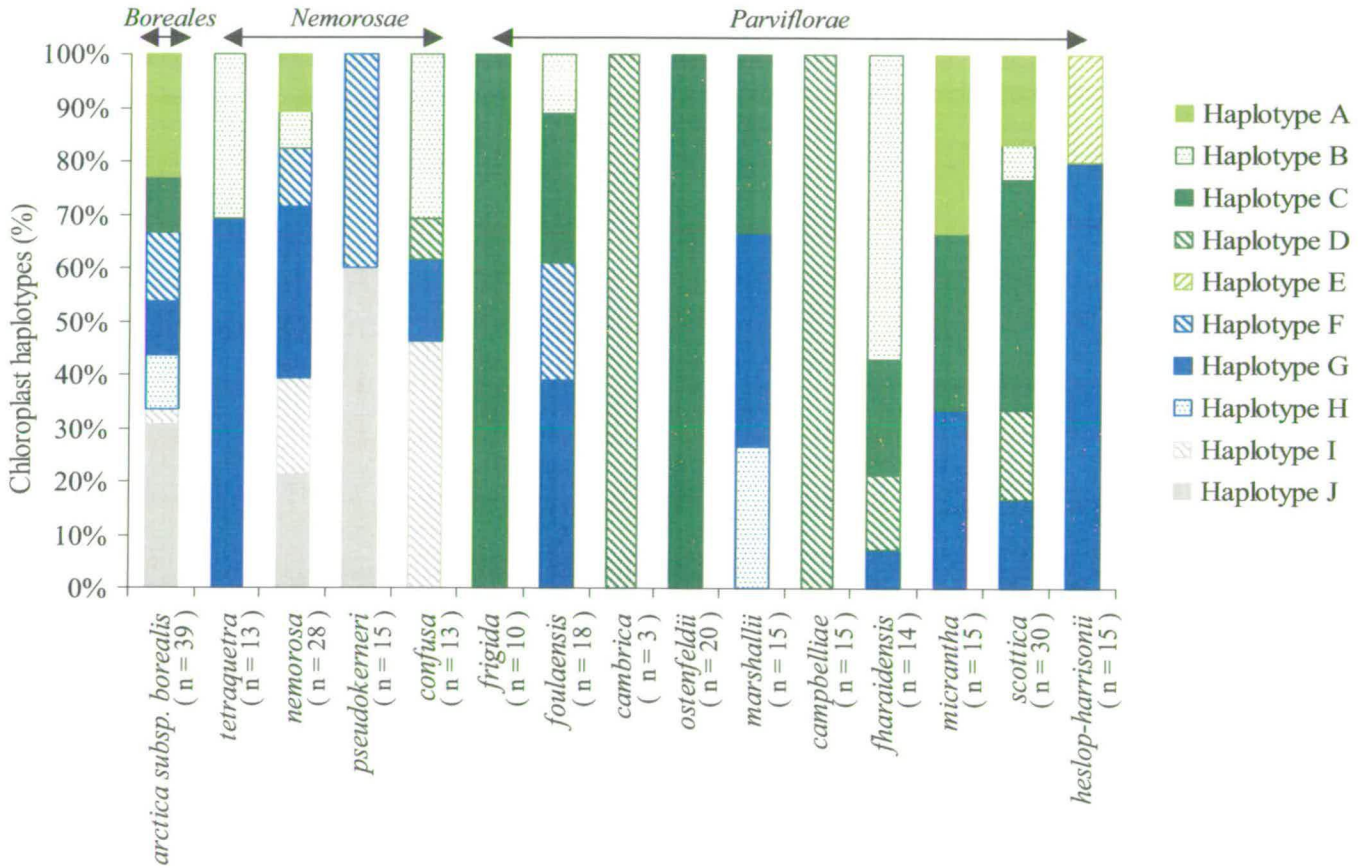


Figure 6:7. Percentage of chloroplast haplotypes within each species. n = number of individuals. Haplotypes are described in Table 6:3.

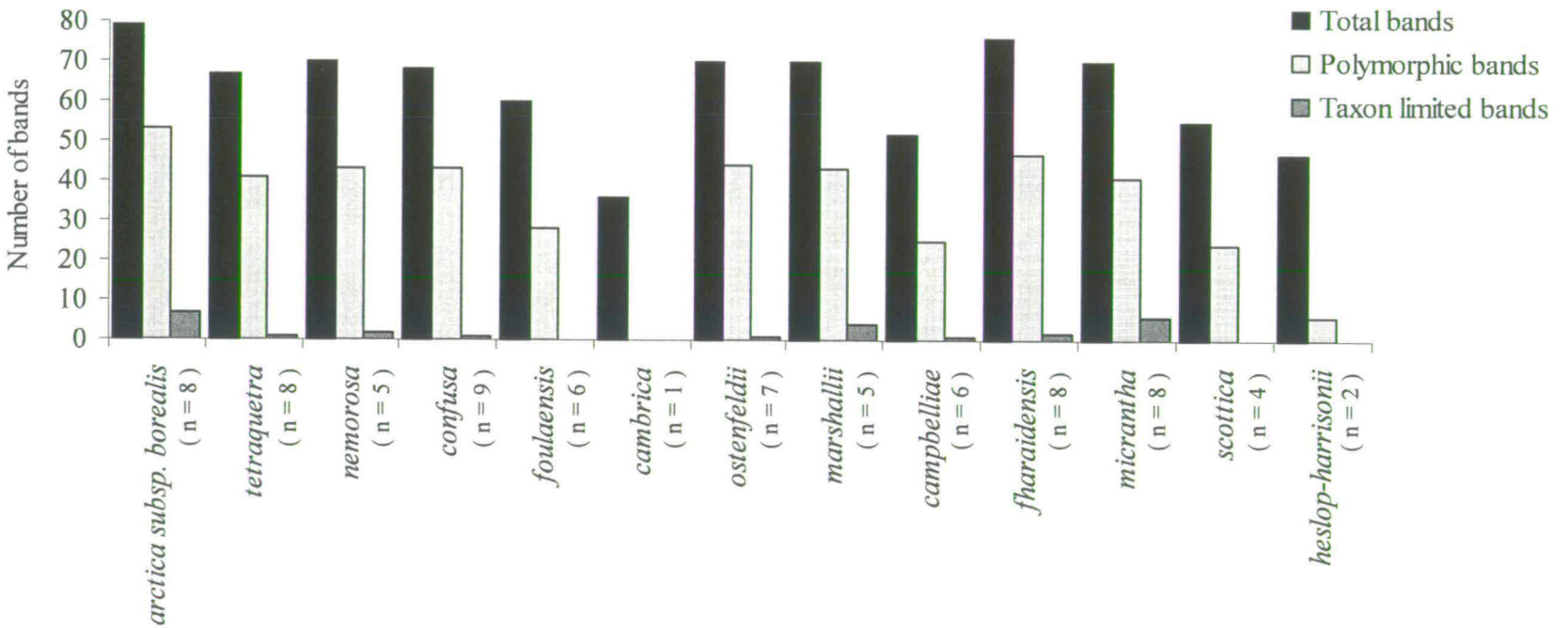


Figure 6:8. Number of total, polymorphic and species limited AFLP bands, grouped according to species. n = number of individuals.

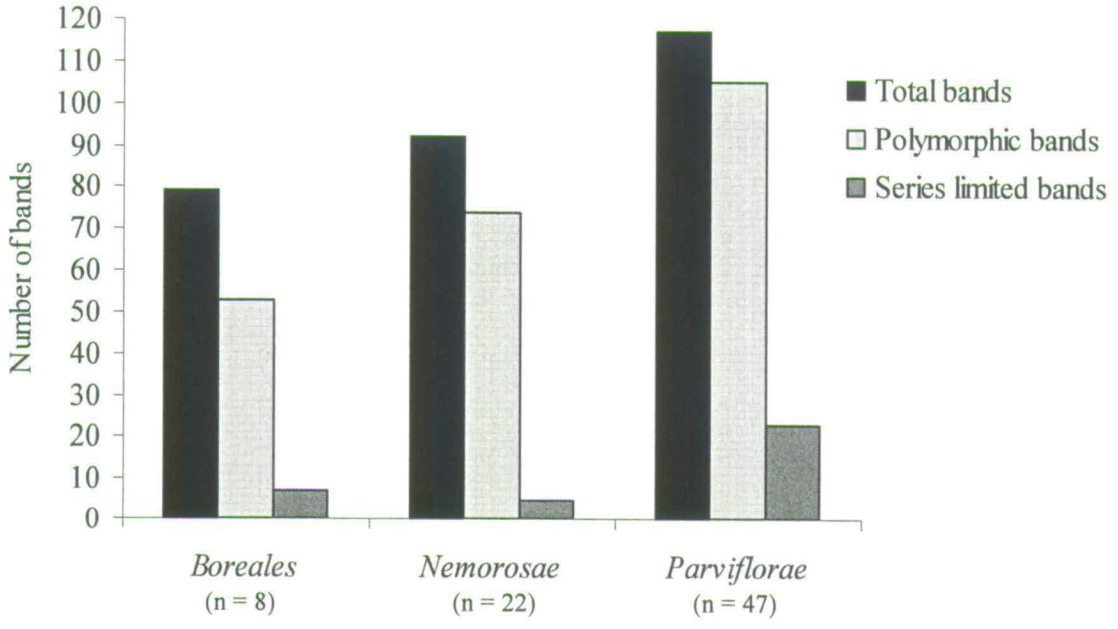


Figure 6:9. Number of total, polymorphic and series limited AFLP bands, grouped according to Yeo's series. n = number of individuals.

Many species contained species-limited bands, generally only 1 or 2 of bands, mainly present in one individual only. More species-limited bands were present in *Euphrasia arctica* subsp. *borealis*, *Euphrasia marshallii* and *Euphrasia micrantha*. In this latter species a unique band was present in all eight individuals, from three populations. All three series contained series-limited bands, with the series *Parviflorae* containing the highest number (Figure 6:9). Out of all the regions sampled, north Scotland and south-west England contained the highest number of region-limited bands (Figure 6:10).

Neither species, series nor regional groupings were discriminated along the 1st or 2nd axes of principal coordinate analysis (Figure 6:11; Figure 6:12; Figure 6:13). These two axes each explained approximately 8% of the total variation in each instance.

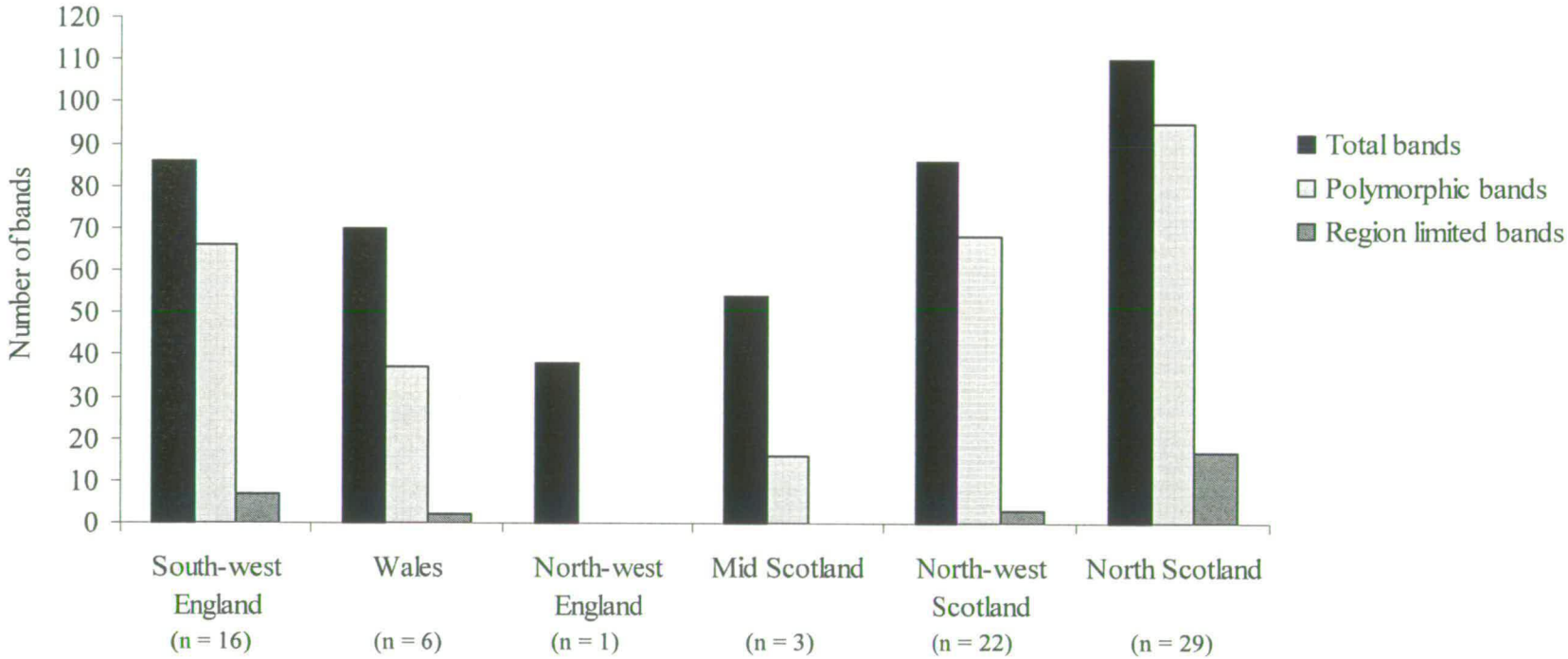


Figure 6:10. Number of total, polymorphic and region limited AFLP bands, grouped according to region. n = number of individuals.

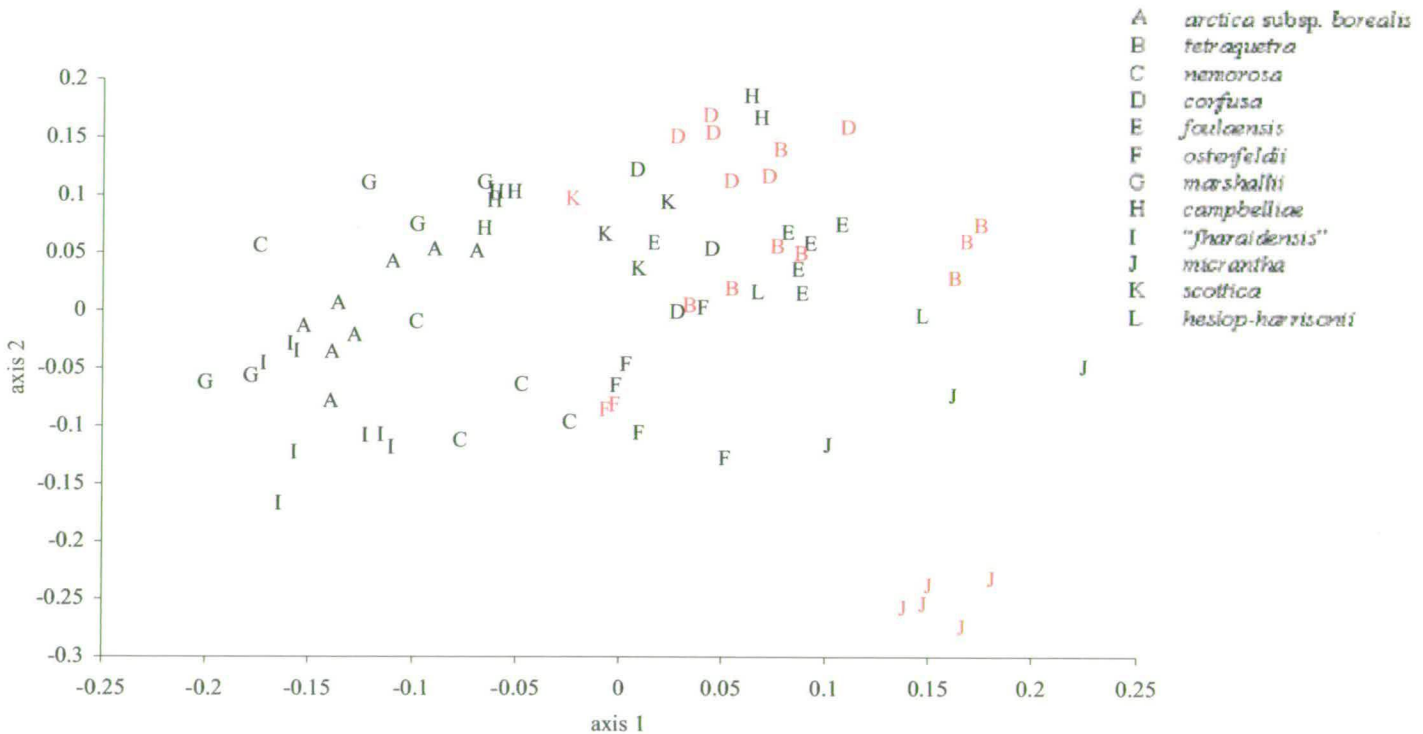


Figure 6:11. Principal coordinate analysis of genetic distance between indiviudals from 12 tetraploid species, constructed from 129 bands. First axis explained 8.24% and second axis 8.09% of the total variation. Species are represented by different letters; geographical regions represented by different colour (black = Scotland; red = England and Wales)

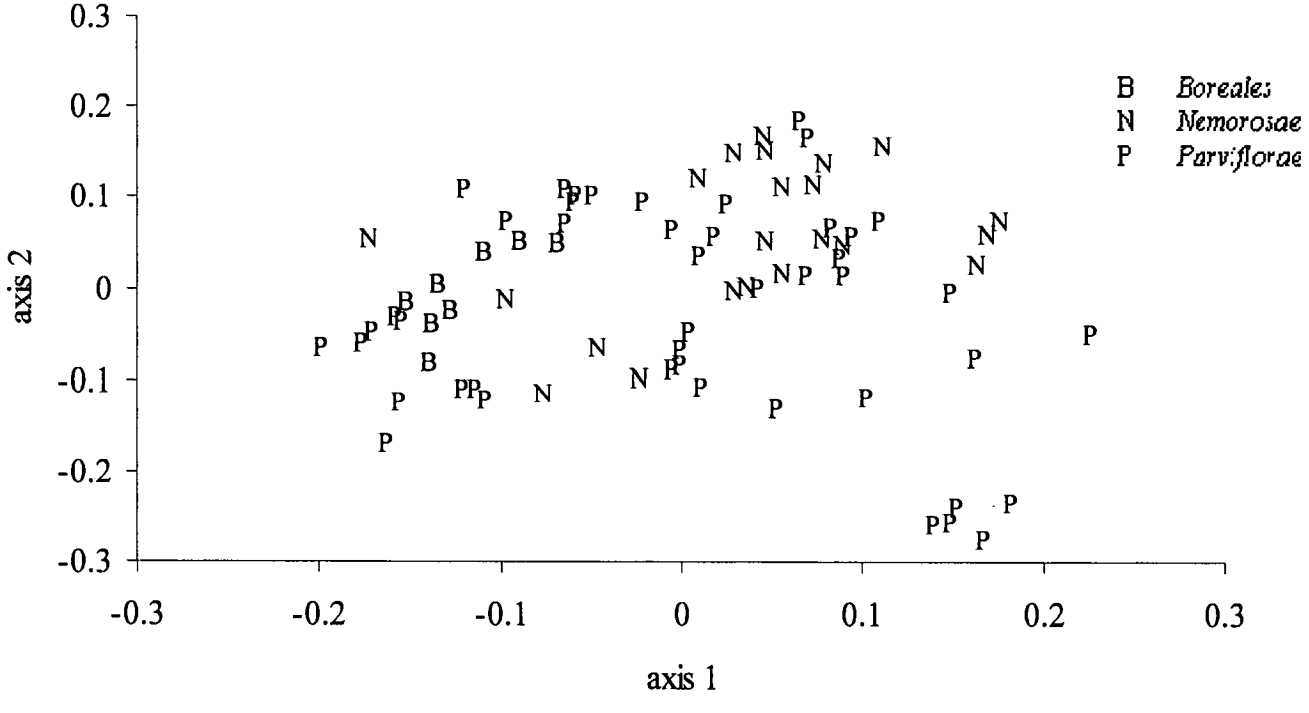


Figure 6:12. Principal coordinate analysis of genetic distance between individuals from three series, constructed from 129 AFLP bands. First axis explained 8.24% and second axis 8.09% of the total variation. Series are represented by different letters.

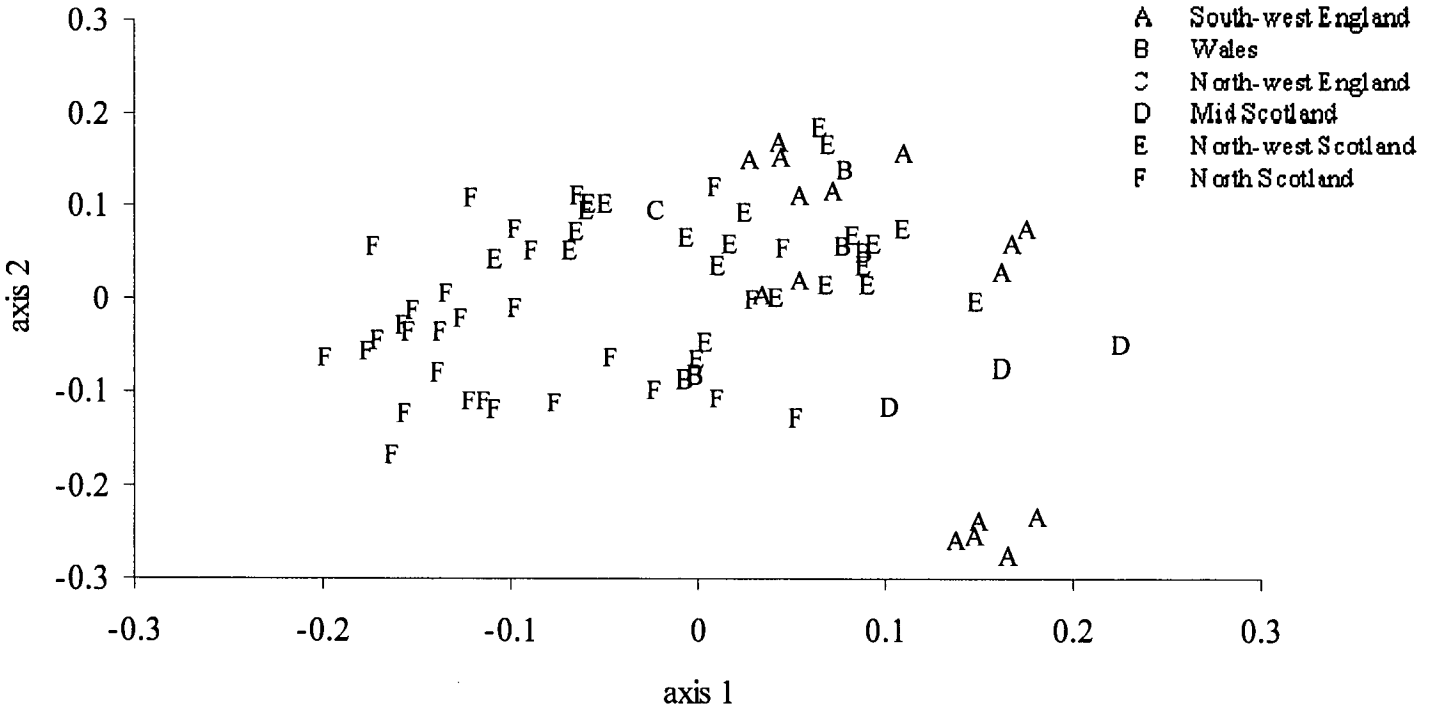


Figure 6:13. Principal coordinate analysis of genetic distance between individuals from six regions, constructed from 129 AFLP bands. First axis explained 8.24% and second axis 8.09% of the total variation. Regions are represented by different letters.

The genetic distances between individuals were generally smaller within than between species, with each species occurring in distinctive but overlapping phenetic space. This was most clearly demonstrated by the clustering of individuals within *Euphrasia confusa*, *Euphrasia ostenfeldii*, *Euphrasia scottica* and *Euphrasia micrantha* (Figure 6:11). Despite the clear separation of the Scottish population, *Euphrasia micrantha* was the most distinctive species, occupying a discrete non-overlapping phenetic space along the first two axes. Phenetic space of populations of *Euphrasia tetraquetra*, *Euphrasia marshallii*, *Euphrasia campbelliae*, "*Euphrasia fharaidensis*" and individuals of *Euphrasia heslop-harrisonii* were discontinuous.

Analysis of molecular variance showed significant partitioning of genetic variation among species, explaining 11.27% of chloroplast and 18.95% of AFLP marker variation (Table 6:4). Grouping by either series or regions explained significant but less variation than species grouping. Partitioning of variation among series explaining 8.12% and 3.09% (Table 6:5) and by region 7.69% and 8.43% of total chloroplast and AFLP marker variation respectively (Table 6:6).

Table 6:4. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to 15 species. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001; *p < 0.05).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among species	13	100.548	0.15133	*11.27
Among populations within species	41	199.018	0.99014	**73.77
Within populations	205	41.150	0.20073	17.96
Total	259	340.715	1.34220	100.00
AFLP genotypes				
Among species	11	380.869	2.46020	**18.95
Among populations within species	22	392.943	6.33252	**48.79
Within populations	42	175.833	4.18651	32.26
Total	75	949.645	12.97923	100.00

Table 6:5. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to Yeo's series. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001; *p < 0.05).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among series	2	26.789	0.11205	*8.12
Among populations within series	52	272.776	1.06680	**77.33
Within populations	205	41.150	0.20073	14.55
Total	259	340.715	1.37959	100.00
AFLP genotypes				
Among series	2	63.731	0.41028	*3.09
Among populations within series	31	710.080	8.41880	**64.73
Within populations	42	175.833	4.18651	32.19
Total	75	949.645	13.00659	100.00

Table 6:6. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to regions. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001; *p < 0.05).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among region	6	52.085	0.10412	*7.69
Among populations within region	48	247.481	1.04872	**77.48
Within populations	205	41.150	0.20073	14.83
Total	259	340.715	1.35357	100.00
AFLP genotypes				
Among region	5	172.272	1.10466	**8.43
Among populations within region	28	601.539	7.82013	**59.64
Within populations	42	175.833	4.18651	31.93
Total	75	949.645	13.11130	100.00

6.3.2. Paired population sampling of two tetraploid species

Within all eight populations five chloroplast haplotypes were found (Haplotypes F to J; Figure 6:4). Overall each population was predominantly fixed for one chloroplast haplotype with 88.1% of the variation partitioned between populations, $G_{ST} = 0.881$ ($h_T = 0.844$, $h_S = 0.100$). The distribution of chloroplast haplotypes were significantly structured among areas but not among species ($\chi^2 = 8.018$, d.f = 1, p = 0.005; $\chi^2 = 0.131$, d.f. = 1, p = 0.717, respectively) (Figure 6:14; Figure 6:15). Haplotype F only occurred in the Thurso area, haplotype H only occurred in Lewis and haplotypes I and J predominately occurred in Durness.

The two AFLP primer pairs produced 133 scorable bands. Of these 124 were polymorphic and nine monomorphic. Both species contained a similar number of AFLP bands, with approximately 80 - 90% polymorphic and 6 - 7% species limited (Figure 6:16). Most of these bands occurred in less than five individuals, though *Euphrasia nemorosa* contained one species limited band, present in 50% of its individuals. Each area contained approximately 100 AFLP bands of which less than 5% were area limited (Figure 6:17). Two area limited bands in Lewis occurred in approximately 50% of individuals.

The two species were not discriminated along the 1st or 2nd axes of principal coordinate analysis (Figure 6:18). These two axes explained 13.28% and 8.23% of the total variation, respectively. This was supported by analysis of molecular variance which showed no significant partitioning of genetic variation between species (Table 6:7). Lewis was separated from mainland Scotland areas along the first axis of the principal coordinate analysis. The north Scotland areas clustered together, with Durness occupying a discrete phenetic space. The remaining two areas, Betty Hill and Thurso were not discriminated from one another (Figure 6:19). The discrimination of Lewis and Durness by principal coordinate analysis was reflected by analysis of molecular variance, where 73.84% of chloroplast and 17.75% of total AFLP marker variation was significantly partitioned among four areas (Table 6:8).

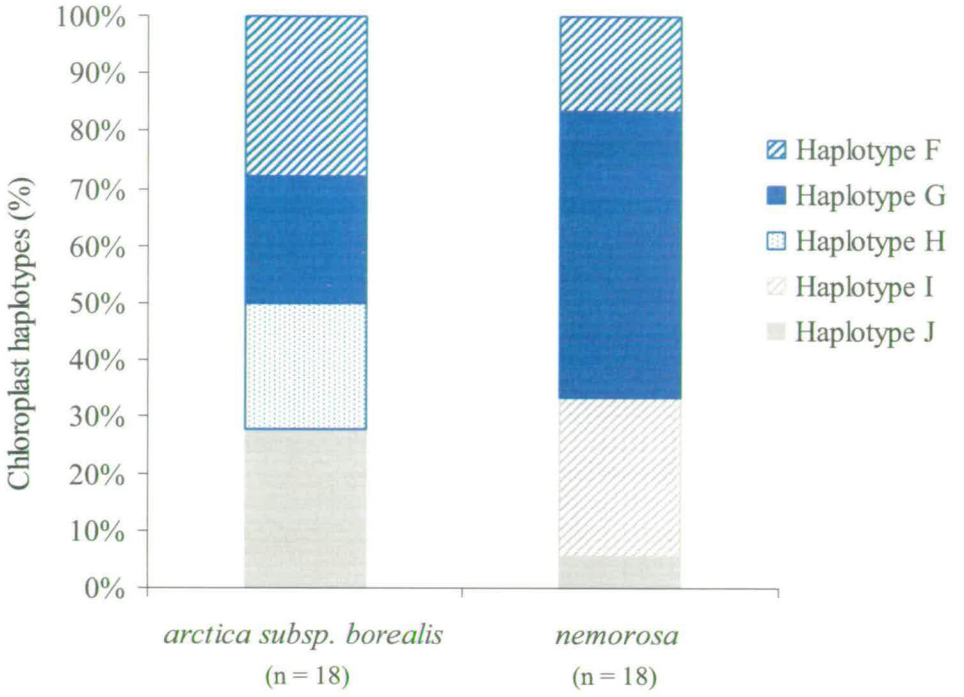


Figure 6:14. Percentage of chloroplast haplotypes within *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*. n = number of individuals. Haplotypes are described in Table 6:3.

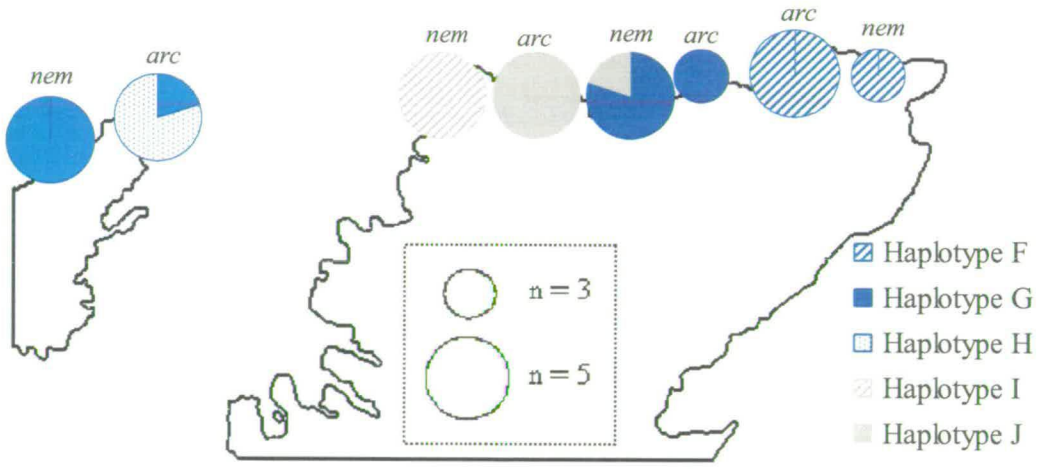


Figure 6:15. Distribution of chloroplast haplotypes, grouped according to species within geographical area, across north-west and north Scotland regions. The areas of the circles represents number of individuals in each population. n = number of individuals. *arc* = *Euphrasia arctica* subsp. *borealis*; *nem* = *Euphrasia nemorosa*. Haplotypes are described in Table 6:3.

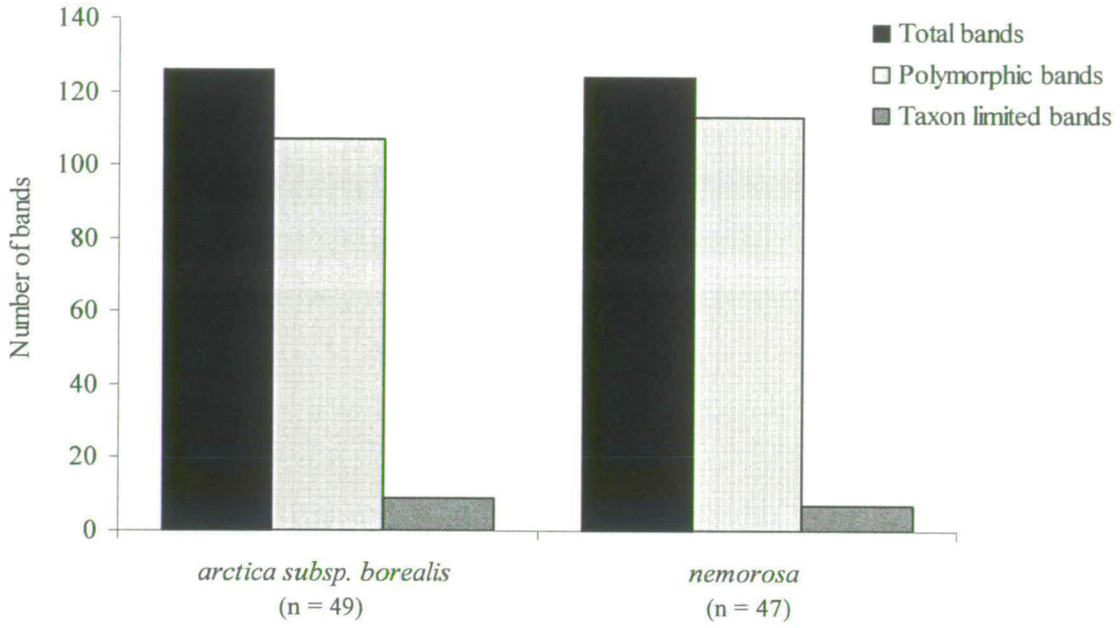


Figure 6:16. Number of total, polymorphic and species limited AFLP bands for *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa* sampled from four different geographic areas. n = number of individuals.

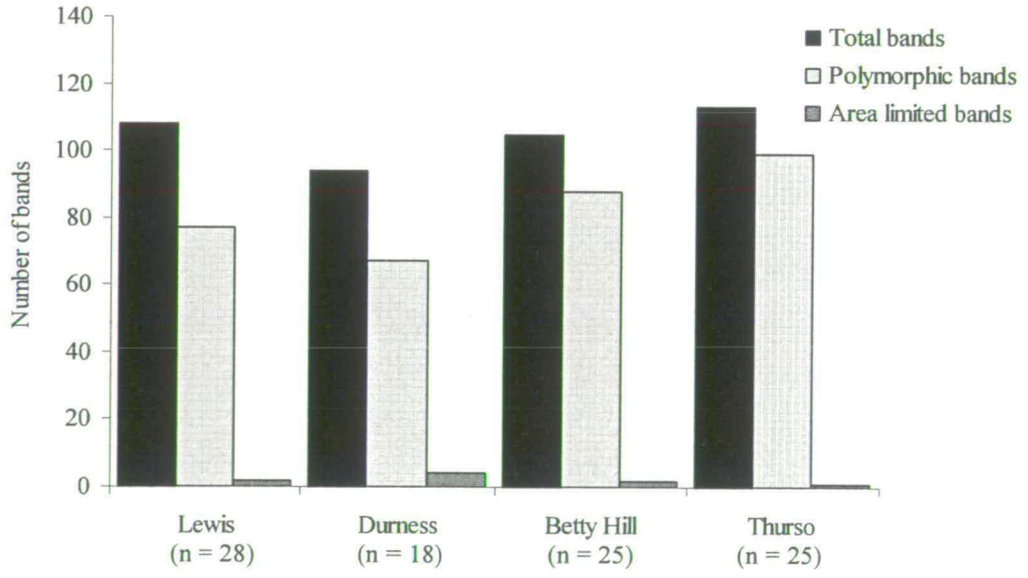


Figure 6:17. Number of total, polymorphic and area limited AFLP bands, grouped according area. n = number of individuals.

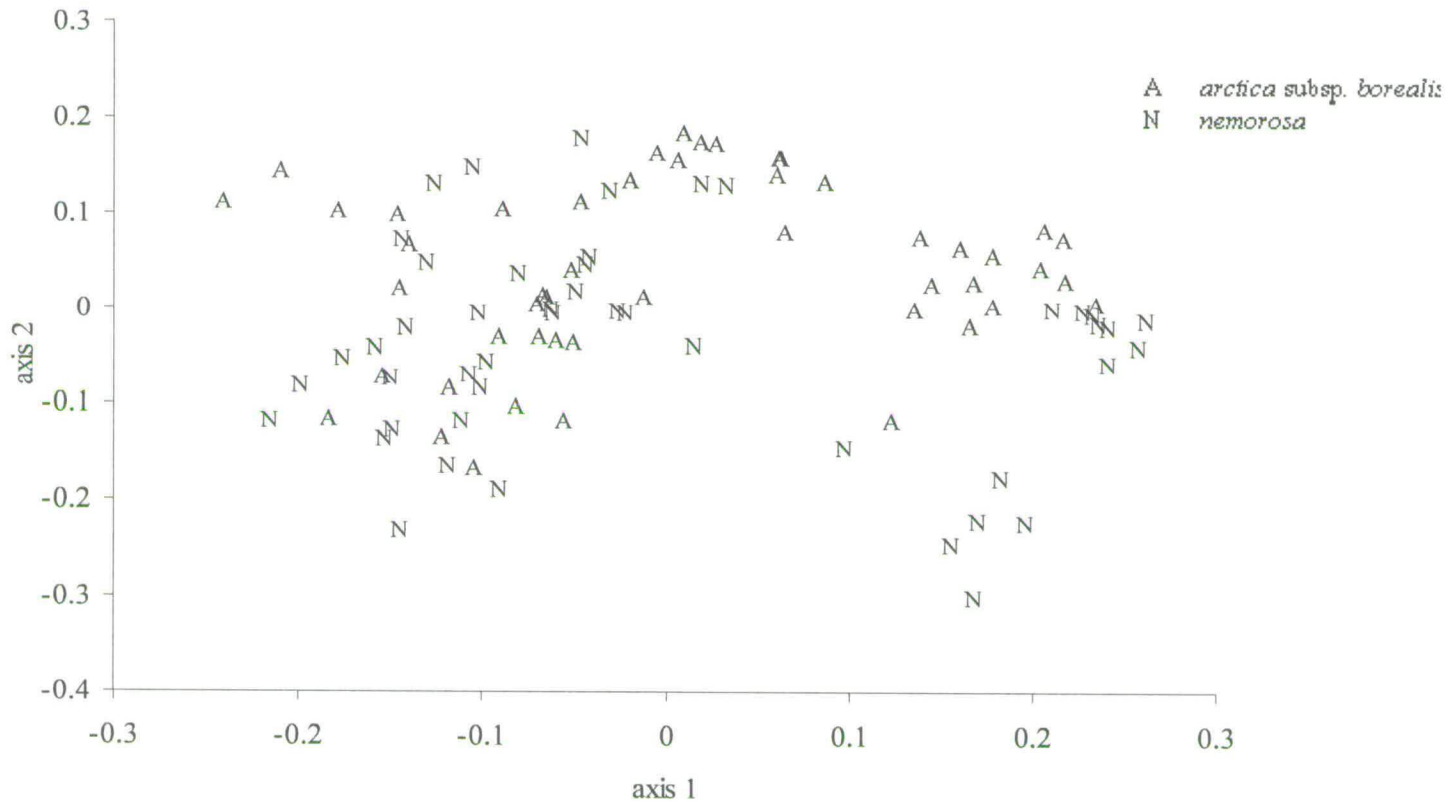


Figure 6:18. Principal coordinate analysis of genetic distance between individuals from two tetraploid species across four areas, within north-west and north Scotland regions, constructed from 133 AFLP bands. First axis explained 13.28% and second axis 8.23% of the total variation. Species are represented by different letters.

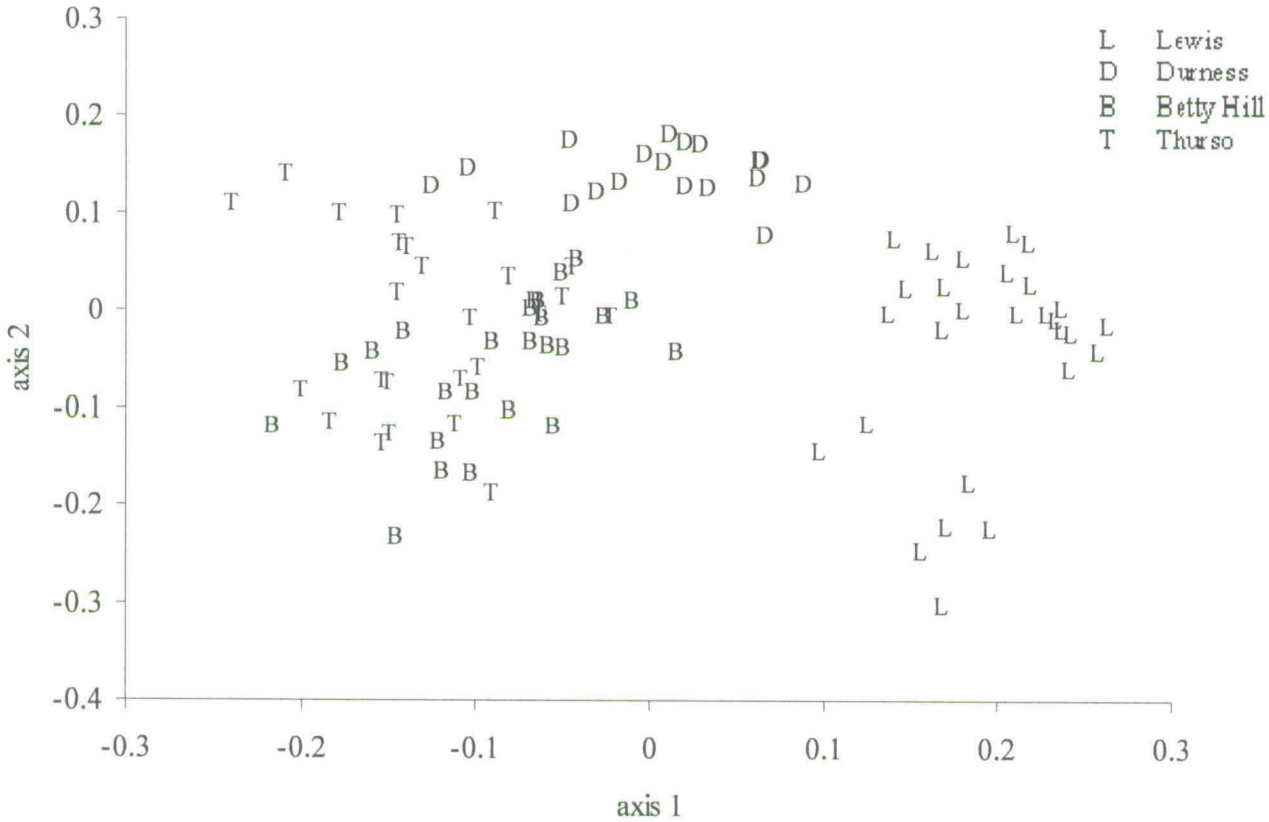


Figure 6:19. Principal coordinate analysis of genetic distance between individuals from two tetraploid species across four areas, within north-west and north Scotland regions, constructed from 133 AFLP bands. First axis explained 13.28% and second axis 8.23% of the total variation. Regions are represented by different letters.

Table 6:7. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to the two species; *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001; NS. not significant).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among species	1	1.500	-0.26543	^{NS} -21.86
Among populations within species	6	35.922	1.30851	**107.74
Within populations	28	4.800	0.17143	14.12
Total	35	42.222	1.21451	
AFLP genotypes				
Among species	1	50.854	-0.65227	^{NS}-4.45
Among populations within species	6	457.595	5.67483	**38.72
Within populations	88	847.728	9.63328	65.73
Total	95	1356.77	14.65584	100.00

Table 6:8. Analysis of molecular variance (AMOVA) for chloroplast PCR-RFLP haplotypes and AFLP genotypes. Populations were grouped according to four areas in north-west and north Scotland. d.f. = degrees of freedom; SSD = sums of square deviations. (**p < 0.001; *p < 0.05).

	d.f.	SSD	Variance Components	% Total Variation
Chloroplast haplotypes				
Among areas	3	32.947	1.09479	*73.84
Among populations within areas	4	4.475	0.21653	**14.60
Within populations	28	4.800	0.17143	11.56
Total	35	42.222	1.48275	100.00
AFLP genotypes				
Among area	3	334.008	2.71925	*17.75
Among populations within areas	4	174.441	2.9682	**19.36
Within populations	88	847.728	9.63328	62.89
Total	95	1356.177	15.31734	100.00

6.4. Discussion

6.4.1. Taxonomic grouping at the species level

Molecular marker distributions were structured within the tetraploid group, with a small but significant amount of variation partitioned among species. The amount of partitioning of genetic variation in the broadscale analysis, accounting for 11% of the overall chloroplast haplotype and 19% of the overall AFLP marker variation, represented a similar degree to that found among diploid species (Chapter 5). Unlike the diploid group, however, no overall clear taxonomic discrimination was demonstrated along the 1st axis of the principal coordinate analyses in either of the widespread or more intensive sampling strategies used. In these principal coordinate analyses only *Euphrasia micrantha* was genetically distinct from other species. The remaining species generally showed greater similarity with individuals of the same species compared to individuals of differing species, but while this is a general trend, the species do not fall out into discrete, mutally exclusive clusters. This structuring also needs to be placed within the context of the overall sampling strategy. By concentrating on morphologically discrete groups and excluding populations with intermediate characters, the extent of differentiation between species has been artificially increased. Therefore the true extent of differentiation between species will be even lower than that shown by the overlapping phenetic spaces seen within the principal coordinate analyses. The non-uniform distribution of molecular markers were therefore clearly not associated with clear cut delimitation of tetraploid taxa at the species level.

In the more intensive study of the two species *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*, variation in the AFLP markers were partitioned geographically and not taxonomically. A similar geographical partitioning of variation was seen with principal component analysis of 58 morphological characters among the same two species in North America (Downie *et al.*, 1988). In areas of extensive hybridisation the use of limited number of morphological characters may therefore be a poor indicator of partitioning of genetic variation. This inaccuracy may be more marked in the case of chloroplast variation where uniparental inheritance results in

lower effective population size (Ennos *et al.*, 1999). This is reflected in the more intensive sampling of *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa* where 73% of chloroplast haplotype variation was partitioned among geographical areas in comparison to the partitioning of only 18% of AFLP marker variation (Table 6:8)

Therefore, within the tetraploid group of *Euphrasia* in Britain molecular evidence does not support the current species-level taxonomy. Molecular markers though not randomly distributed within the tetraploid group, do not reveal clear genetic discontinuities that support the presence of morphologically and genetically discrete entities. There are also many "stray samples" on the principal coordinate plots, so that this approach does not form the basis for a working predictive taxonomy that may be used in a consistent fashion. An alternative approach to achieve a robust and predictive taxonomy needs to be sought at higher taxonomic levels, as advocated by Karlsson (1976).

6.4.2. Taxonomic grouping at the series level

Taxonomic grouping at the series level was even less well supported than grouping at the species level by the AFLP markers. Only 3% of the AFLP marker variation was partitioned among series, compared with 19% among species. The series were also not discriminated along the 1st axis of the principal coordinate analysis. However there was greater support for taxonomic discrimination at this level from the distribution of chloroplast haplotypes. Chloroplast haplotypes, though similar in the amount of partitioning of variation among series as among species (8% compared with 11%, respectively) and not associated with unique haplotypes to any one series, were not uniformly distributed among series in the tetraploid group. This is more clearly seen when considering the distribution of groups of related haplotypes rather than the distribution of individual haplotypes.

The 10 chloroplast haplotypes found within the tetraploid group may be partitioned into three groups according to their relationships to one another in the minimum spanning network (Figure 6:4). Two haplotype groups (haplotypes F to G and haplotypes I and J) were restricted to the series *Boreales* and *Nemorosae*,

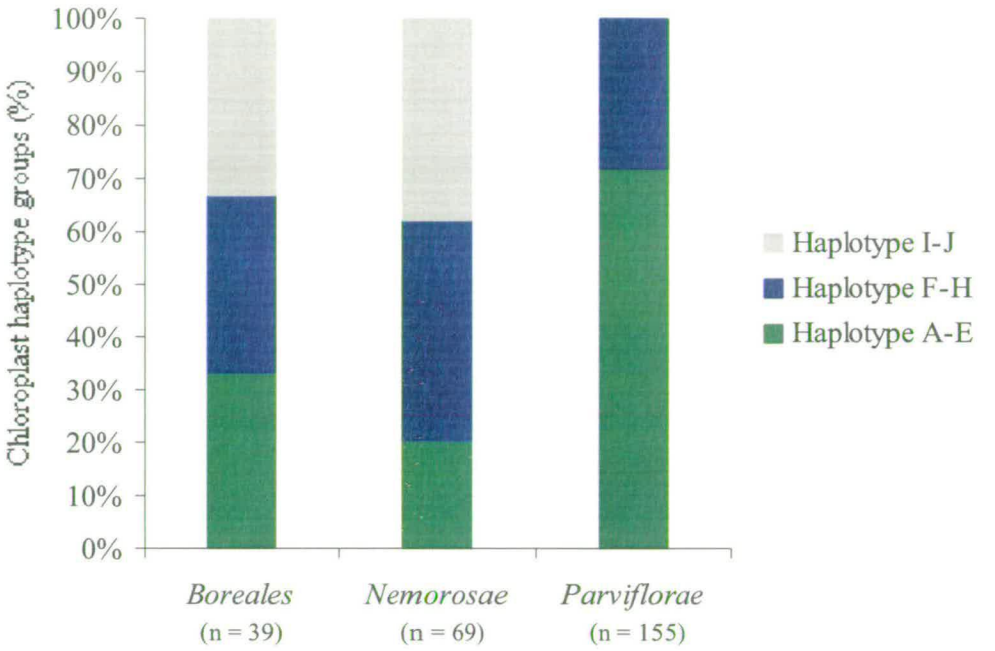


Figure 6:20. Percentage of chloroplast haplotype groups within each of Yeo's series. n = number of individuals. Haplotype groups are described in Figure 6:4.

whilst a third haplotype group (haplotypes A to E) predominantly occurred in the series *Parviflorae* (Figure 6:20). This suggests the possibility that at least three polyploid events have occurred in the formation of the tetraploid group. At least two polyploid events led to the combined formation of the series *Boreales* and *Nemorosae* and a separate third polyploid event to the formation of series *Parviflorae*.

However, with 3 out of the 10 haplotypes widely distributed across the series, the distribution of chloroplast haplotypes among the combined series *Boreales* and *Nemorosae* and the more distinct series *Parviflorae* is not absolute. The distribution of these widespread haplotypes may be explained by gene flow among the two distinct groups of series, blurring the group's boundaries. An alternate hypothesis to the formation of multiple groups, however, may also be a single polyploid event, followed by divergence of chloroplast haplotypes and subsequent sorting of an

ancestral population. Further work is required to help determine between these two opposing hypotheses.

6.4.3. Grouping at the regional level

Neither type of molecular marker was uniformly distributed among regions within Britain. In the widespread sampling strategy no clear regional differentiation was detected. The significant amount of variation partitioned among regions, accounting for 8% of both overall chloroplast haplotype and AFLP marker variation, was less than that accounted for by partitioning among species or series and was not associated with discrimination along the 1st axis of the principal coordinate analysis. Given the restricted distribution of many of the tetraploid species (Figure 6:1), an independent comparison of geographical versus taxonomic delimitation may not be possible. The more intensive study of *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa* did, however, show geographical rather than taxonomic structuring of both nuclear and chloroplast DNA markers.

6.4.4. Summary

The use of molecular markers produced by chloroplast PCR-RFLP and AFLP techniques have been useful in quantifying genetic variation within the tetraploid group. In this respect, this study supports previous findings of the usefulness of molecular markers when applied across a range of taxonomic levels within plant genera (Adams and Demeke, 1993). In contrast to the analyses between ploidy levels and within the diploid group, the structuring of genetic variation within the tetraploid group is not congruent with structuring recognised by phenotypic variation. Incongruence between genetic and morphological discontinuities at the species level has been noted previously in other plant genera. For example *Festuca pratensis* Huds. was found to be more closely related to species within the genus *Lolium* (Tourn.) L. than to species within the genus *Festuca* L. (Stammers *et al.*, 1995).

In the tetraploid group of *Euphrasia* an interplay of several factors may result in structuring for some species but not for other species. It is evident that a simple species approach to this complex group may be fraught with problems and the present species-based conservation strategy is not sufficient to conserve the variation

within this tetraploid group (Anon, 1995b). Based on the findings of this study a different approach is therefore required. The use of molecular markers, especially chloroplast PCR-RFLP, have begun to elucidate the biological processes that occurred in the generation of diversity with the tetraploid group in Britain. These processes should be taken into account when considering future conservation strategies within British *Euphrasia*.

Increased understanding of the processes generating diversity in British *Euphrasia* requires the integration of the understanding of genetic variation and breeding system variation, placed within an ecological context. The next two chapters shall discuss the results obtained across both the ploidy groups in this integrated context. Firstly a preliminary study in the association between molecular markers and habitat diversity within the tetraploid group shall be described. Then a more overall description of the integrated processes involved in the generation of diversity in British *Euphrasia* will be given.

CHAPTER 7: A pilot study into ecological specialisation within tetraploid British *Euphrasia*

Abstract: A preliminary study into the ecological requirements of 10 tetraploid *Euphrasia* taxa in Britain has been undertaken. Using the distribution of three chloroplast haplotype groups, these taxa are grouped into two main haplotype-taxonomic groups (Group I: chloroplast groups F to H, I and J; Group II: chloroplast group A to E). Estimation of four mean Ellenberg environmental indicator values, from recorded associated species, suggest significant differences in the ecological requirements of these two haplotype-taxonomic groups. These differences relate to the general fertility, pH and calcium content of the soil. Within one of these groups marked variation in moisture conditions among taxa is also described. One species, *Euphrasia scottica*, shares wet habitat conditions with two tetraploid endemic species. Its importance in the formation of these endemic species is suggested and the overall importance of ecological specialisation in the diversification of British *Euphrasia* highlighted. More detailed ecological studies in British *Euphrasia* are recommended.

7.1. Introduction

Ecological specialisation is one of the important mechanisms by which evolving taxa generate and maintain their distinctiveness. Within *Euphrasia* this may have resulted in the evolution of the high number of endemic taxa, especially within hotspot areas of diversity in the oceanic climate zone of western and northern Britain (Preston *et al.*, 2002). Non-uniform distribution of chloroplast haplotypes suggests the presence of two distinct haplotype-taxonomic groups (HTG) within the tetraploid *Euphrasia* group in Britain (Chapter 6). The combined series *Boreales* and *Nemorosae* share the chloroplast haplotypes F to H and I to J. This is not a monophyletic assemblage of haplotypes, but rather two distinct groups of haplotypes that are scattered among individuals and species within both these series. The more distinct series, *Parviflorae*, is typified by an assemblage of haplotypes A to E (Table 7:1; Figure 7:1). Unique variation found within and among these two main haplotype-taxonomic groups may allow a wide range of ecological specialisation and so may play an important role in generating the high diversity seen in British *Euphrasia*. This diversity may be further promoted by the combination of distinct genotypes found within these two differing groups, with the production of novel hybrids capable of

Table 7:1. Chloroplast haplotypes detected by RFLP analysis of chloroplast regions in British *Euphrasia*: AS = *psa*A - *trn*S: CD = *trn*C - *trn*D: HK = *trn*H - *trn*K: KK = *trn*K_1 - *trn*K_2 (Taken from Chapter 4).

Haplotype	AS:*Hinf*I band I	band II	band III	CD:*Hinf*I band I	band II	band III	HK:*Hae*III band I	KK:*Hinf*I band I
A	1	1	2	1	2	3	2	1
B	1	1	2	1	2	3	2	0
C	1	1	2	2	2	1	2	0
D	1	1	2	1	2	1	2	0
E	3	1	2	1	2	3	2	0
F	3	1	2	1	1	2	2	0
G	3	1	2	?	1	0	2	0
H	3	1	2	?	1	0	1	0
I	2	2	2	2	2	1	2	0
J	2	2	2	2	2	1	3	0
K	1	0	1	1	2	3	2	0
L	1	0	1	1	2	3	1	0

Haplotypes A - L defined as particular associations of these restriction patterns. Polymorphisms are scored in order of decreasing molecular weight, with the absence of a restriction fragment represented by 0. Uncertainty of band molecular weight represented by ?

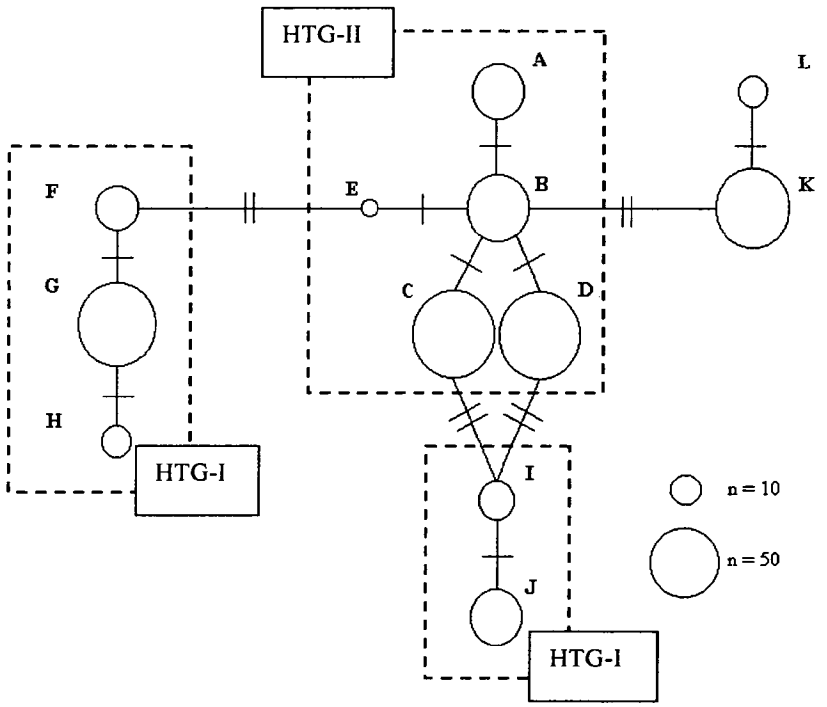


Figure 7:1. Haplotype-taxonomic groups defined according to relationships between chloroplast haplotypes, represented as a minimum spanning network. The two haplotype-taxonomic groups are indicated by boxes (HTG-I: haplotypes F to H and I to J, and HTG-II: haplotypes A to E). Haplotypes are described in Table 7:1. Mutational steps are indicated by single cross line. The areas of the circles represents number of individuals with each haplotype. HTG = haplotype-taxonomic group; n = number of individuals (Taken from Chapter 4).

surviving and adapting to new habitats.

The object of this chapter is to study the ecological requirements of these two main haplotype-taxonomic groups. Ecological studies in this genus have not previously been performed in Britain. In Ireland, ecological specialisation was found for all the nine species studied, each one occupying a distinct habitat, differing in soil pH, sward height and associated species (Bobear, 1969). This specialisation also extends to taxonomic grouping below the species level. For example, two variants of *Euphrasia stricta* occupied differing habitats within hay meadow plant communities in Gotland, Sweden, being separated on the basis of differences in moisture and nutrient levels (Karlsson, 1984). This was not, however, associated with the loss of hybrid fertility between these two variants (Karlsson, 1986).

Both of these studies on ecological specialisation of *Euphrasia* taxa used differences in associated species as an indicator of habitat differentiation. In studying hay meadow plant communities in Sweden, Karlsson (1984) used these associated species to calculate mean Ellenberg numbers for each of the variants of *Euphrasia stricta*. The estimation of these Ellenberg indicators values allowed a relatively quick, easy, sensitive and integrated measure of the environment due to their strong correlation with environmental variables (Ellenberg, 1979; Dzwonko, 2001).

Any environmental differences between the two haplotype-taxonomic groups within the tetraploid group in Britain should be more marked than those found between individual species and variants. As an initial investigation into these differences, Ellenberg indicator values should therefore indicate the presence of environmental differences between these groups. Under a null hypothesis that there are no ecological differences among these two main haplotype-taxonomic groups, no significance differences should be found among the groups for mean Ellenberg indicator values. The alternative hypothesis of significant environmental differences among the two haplotype-taxonomic groups can therefore be tested against this null hypothesis. The finding of significant environmental differences may then be used to strengthen the case for more detailed ecological studies on British *Euphrasia*.

7.2. Materials and Methods

Associated plant species were recorded in 37 populations, from 10 tetraploid taxa in Britain (Table 7:2). Species were recorded as either present or absent, by one observer (Alan Silverside), for all sites visits during the collection of plant material for molecular marker analysis. Records were taken from the perceived local habitat of each population (Alan Silverside pers. comm.; Oliver, 1999).

7.3. Data analysis

The distribution of chloroplast haplotype clusters in each taxon was used to partition taxa to either of two haplotype-taxonomic groups. Taxa with predominantly chloroplast haplotype groups F to H and I and J were allocated to HTG-I and taxa with predominantly chloroplast haplotype group A to E, to HTG-II. These approximately correspond to the series *Boreales* and *Nemorosae* and the more distinct series *Parviflorae*, respectively (Figure 7:2; Table 7:2). There was, of course, an element of subjectivity in this allocation of samples to groups, but in the absence of a clear cut divide, it reflected a pragmatic approach to the division of the group.

Mean Ellenberg values for each taxon were calculated in relation to four major environmental factors using the Ellenberg values within the software package ComKey V. 0.22 (Legg, unpublished). The four Ellenberg values were relative light flux during summer (light value), soil moisture and water supply (moisture value), soil pH and calcium content (reaction value), and ammonia and nitrate supply (nitrogen value) (Persson, 1981). The nitrogen value has been shown to be an indicator of general fertility rather than specific nitrogen concentration (Hill and Carey, 1997). Two Ellenberg estimates of latitudinal zones and altitudinal belts (temperature value) and degree of continentality of the general climate with special emphasis on minimum and maximum temperatures (continentality value) have been shown not to be applicable to British plant communities (Hill *et al.*, 2000) and so were excluded from the analysis (Preston and Hill, 1997). Tests for normality and

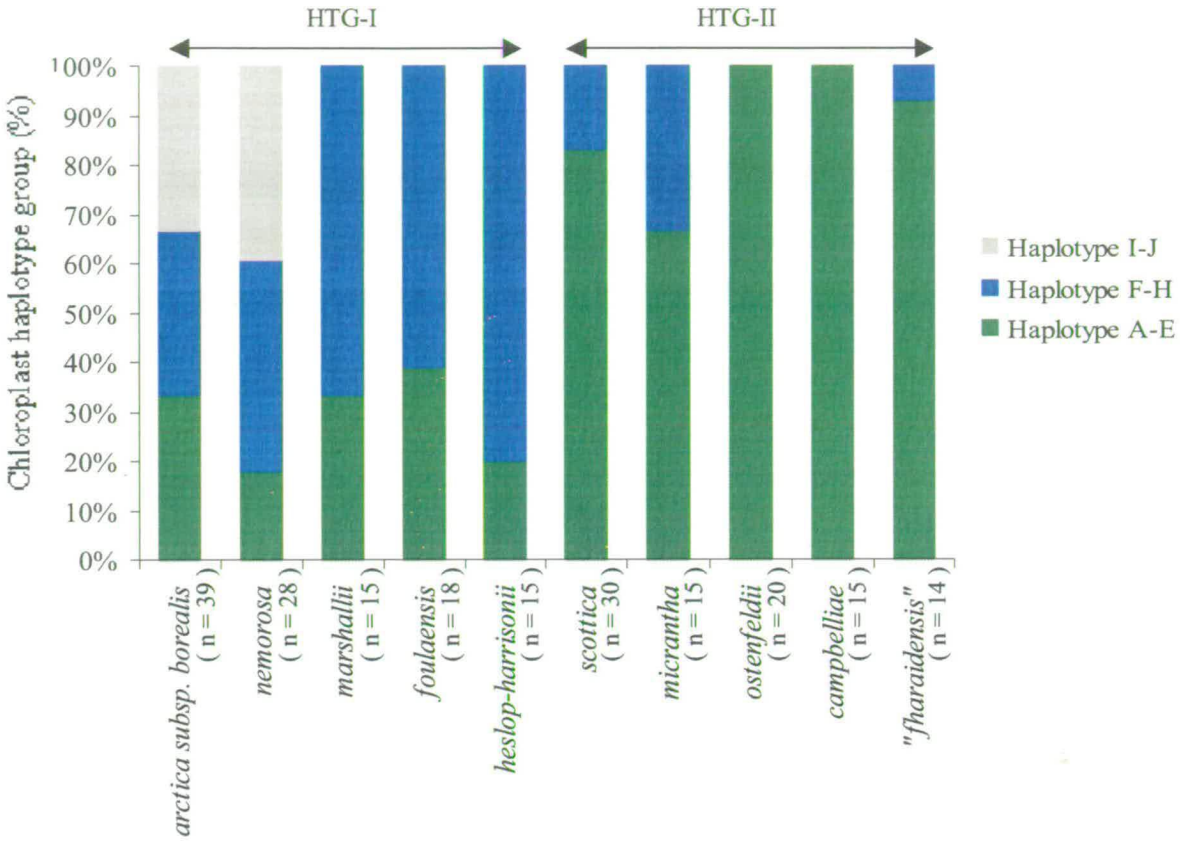


Figure 7:2. Percentage of three chloroplast haplotypes groups within each species (adapted from Chapter 6). n = number of individuals; HTG = haplotype-taxonomic group.

homogeneity of variance were performed on all average environmental variables, using Kolmogorov-Smirnov and Bartlett's statistical analysis. Transformation of non-parametric data was performed by conversion to natural logarithmic values. Environmental differences between the two haplotype-taxonomic groups were tested using nested analysis of variance. All statistical analyses were performed using the statistical package, MINITAB®.

Table 7:2. Associated species recorded within 37 populations of 10 British tetraploid taxa. Taxa have been allocated to either of two haplotype-taxonomic groups (HTG) depending on the predominant presence of chloroplast haplotype groups; HTG-I = F to H and I and J; HTG-II = A to E (Figure 7:1; Figure 7:2).

Number	Taxon	Region	Location	Grid reference	No. of associated species
HTG-I					
T8	*Euphrasia arctica* subsp. *borealis*	north Scotland	Betty Hill	NC/700.621	24
T9	*Euphrasia arctica* subsp. *borealis*	north Scotland	Durness	NC/405.669	21
T10	*Euphrasia arctica* subsp. *borealis*	north Scotland	Reay	NC/958.653	19
T46	*Euphrasia nemorosa*	north-west Scotland	Dail Beag	NB/227.458	13
T47	*Euphrasia nemorosa*	north Scotland	Betty Hill	NC/702.608	23
T48	*Euphrasia nemorosa*	north Scotland	Fhariad Head	NC/392.687	18
T49	*Euphrasia nemorosa*	north Scotland	Dunnett Bay	ND/223.700	25
T37	*Euphrasia marshallii*	north Scotland	Betty Hill	NC/705.628	19
T38	*Euphrasia marshallii*	north Scotland	Melness	NC/577.653	21
T39	*Euphrasia marshallii*	north Scotland	Strathy Point	NC/827.694	14
T23	*Euphrasia foulaensis*	north-west Scotland	Mealasta	NA/993.240	17
T24	*Euphrasia foulaensis*	north-west Scotland	Mangurstadh	NB/005.315	14
T25	*Euphrasia foulaensis*	north Scotland	Scourie Bay	NC/144.449	23
T27	*Euphrasia foulaensis*	north Scotland	Coldbackie	NC/611.598	36
T28	*Euphrasia foulaensis*	north Scotland	Betty Hill	NC/605.627	25
T31	*Euphrasia heslop-harrisonii*	north-west Scotland	Kensaleyre	NM/418.518	10
T32	*Euphrasia heslop-harrisonii*	north-west Scotland	Kentra Bay	NM/648.698	11
T33	*Euphrasia heslop-harrisonii*	north-west Scotland	Ardtoe	NM/627.712	14
T34	*Euphrasia heslop-harrisonii*	north-west Scotland	Newton of Ardtoe	NM/627.712	19
T36	*Euphrasia heslop-harrisonii*	north-west Scotland	Seilebost	NG/083.668	13
HTG-II					
T59	*Euphrasia scottica*	mid Scotland	Dalwhinnie	NN/642.850	19
T60	*Euphrasia scottica*	north Scotland	Newton	NC/153.308	21
T61	*Euphrasia scottica*	north Scotland	Gleann Leireag	NC/153.308	13

Table 7:2. *Continued.*

Number	Taxon	Region	Location	Grid reference	No. of associated species
T62	*Euphrasia scottica*	north-west Scotland	Carlabhagh	NB/190.413	20
T64	*Euphrasia scottica*	north Scotland	Lochan Hackel	NC/572.533	13
T40	*Euphrasia micrantha*	south-west England	Wheal Busy	SW/742.423	23
T41	*Euphrasia micrantha*	south-west England	Chapel Porth	SW/697.494	16
T52	*Euphrasia ostenfeldii*	north Scotland	Inchnadamp	NC/272.210	23
T53	*Euphrasia ostenfeldii*	north Scotland	An-t-sron	NC/443.580	17
T12	*Euphrasia campbelliae*	north-west Scotland	Reinigeadal	NB/218.017	13
T13	*Euphrasia campbelliae*	north-west Scotland	Mealasta	NA/994.235	16
T14	*Euphrasia campbelliae*	north-west Scotland	Islibhig	NA/993.280	9
T18	"*Euphrasia fharaidensis*"	north Scotland	Inchnadamp	NC/272.210	20
T19	"*Euphrasia fharaidensis*"	north Scotland	Rhiconich	NC/253.523	23
T20	"*Euphrasia fharaidensis*"	north Scotland	Lochan Hackel	NC/572.533	31
T21	"*Euphrasia fharaidensis*"	north Scotland	Betty Hill	NC/605.627	22
T22	"*Euphrasia fharaidensis*"	north Scotland	Fharaid Head	NC/387.713	20
Total					174

7.4. Results

The two haplotype-taxonomic groups differed siginificantly in mean Ellenberg values for two environmental variables (Figure 7:3), reaction and nitrogen values (Nitrogen: $F_{1,8} = 20.56$, $p < 0.05$; Reaction: $F_{1,8} = 7.72$, $p < 0.05$; Moisture: $F_{1,8} = 1.90$, $p > 0.05$; Light: $F_{1,8} = 5.16$, $p > 0.05$; Table 7:3).

Table 7:3. Nested analysis of variance of mean Ellenberg values for four environmental variables, among two tetraploid haplotype-taxonomic groups (A to E and F to J; Figure 7:1) and among taxa within these groups. D.f. = degrees of freedom; Adj. MS = adjusted mean square; ‡ = log transformed. (*p < 0.05, NS = not significant).

Source of variation	d.f.	Adj. MS: Nitrogen	Adj. MS: Reaction	Adj. MS: ‡Moisture	Adj. MS: Light
Among groups	1	*15.5812	*20.1381	^{NS}0.18540	^{NS} 0.35133
Among taxa within groups	8	0.7579	2.6092	0.09753	0.06810
Error	27	0.3210	0.3306	0.00690	0.02464
Total	36	16.6601	23.0779	0.28983	0.44407

Taxa within HTG-I (chloroplast haplotypes F to H and I to J; *Euphrasia arctica* subsp. *borealis*, *Euphrasia nemorosa*, *Euphrasia marshallii*, *Euphrasia foulaensis* and *Euphrasia heslop-harrisonii*), tended to occur in more fertile, less acidic, drier grassland habitats than taxa within HTG-II (chloroplast haplotypes A to E). With the exception of *Euphrasia ostenfeldii*, taxa in HTG-II tended to occur in less fertile, more acidic conditions. Habitats of the taxa within this group also showed marked variation in moisture content. The endemic taxa *Euphrasia campbelliae* and "*Euphrasia fharaidensis*" and widespread *Euphrasia scottica* occurred in wetter habitats than *Euphrasia micrantha*.

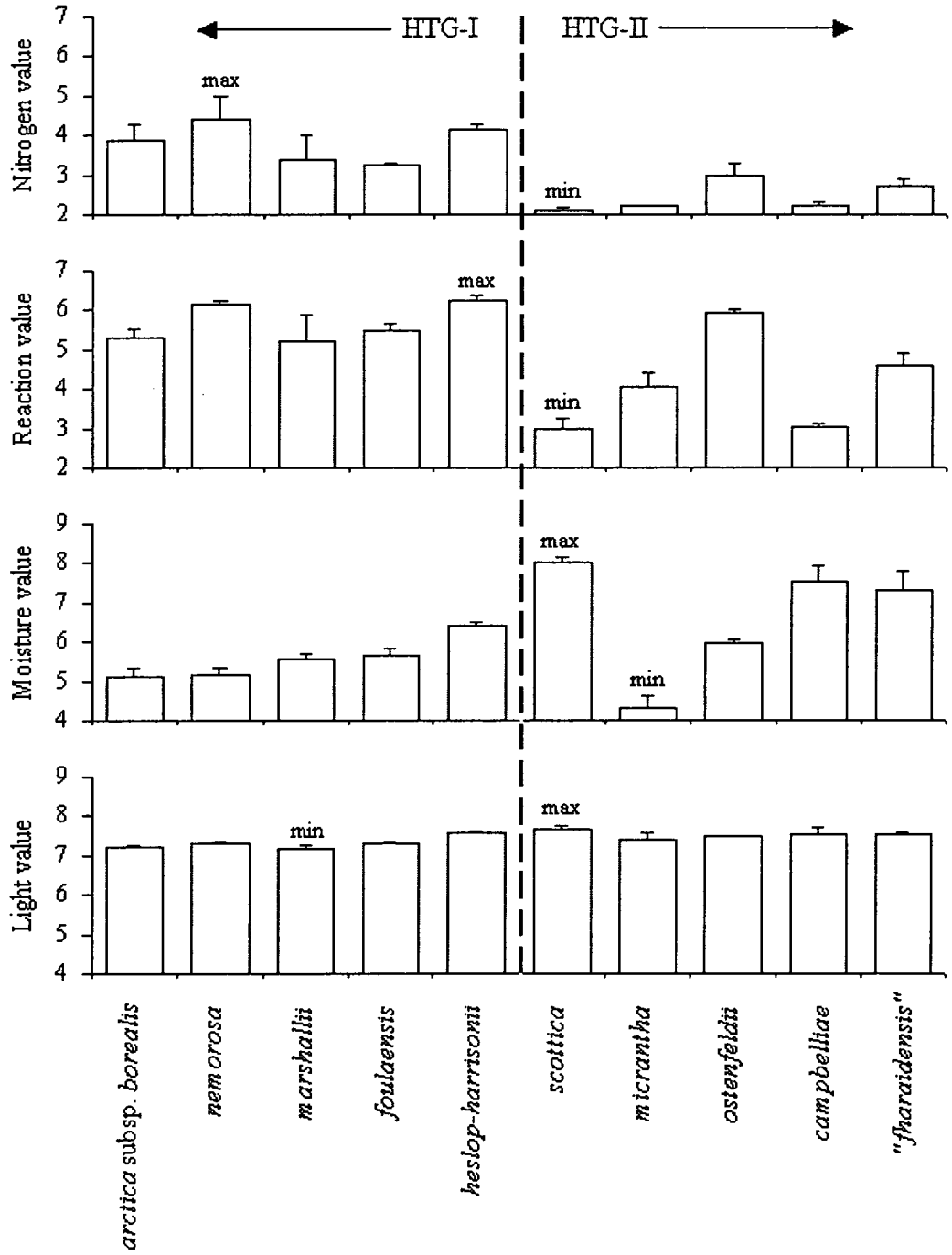


Figure 7:3. Mean Ellenberg values for four environmental variables, calculated from associated species of 10 taxa, grouped within two tetraploid haplotype-taxonomic groups in British *Euphrasia*. HTG = haplotype-taxonomic group; min = lowest Ellenberg value; max = highest Ellenberg value.

7.5. Discussion

With a limited ecological survey, using non standardised sampling techniques, coupled with uncertainty over the accuracy of inferring environmental variables from the uncorroborated use of mean Ellenberg values and its application to the British flora (Wamelink *et al.*, 2002; Schaffers and Sỳkora, 2000; Hill *et al.*, 1999), interpretation from this ecological work on British *Euphrasia* must be regarded as preliminary. Survey of associated species has, however, indicated environmental differences between taxa as well as hypothesised haplotype-taxonomic groups within the tetraploid group in Britain.

Out of all the 10 taxa examined, *Euphrasia scottica* within HTG-II (chloroplast haplotype group A to E), occurred in the most distinct environmental conditions. This taxon tends to occur in the wettest, most nutrient poor and acidic habitats (Figure 7:3). Some of these conditions are shared by the endemic taxa *Euphrasia campbelliae* and *Euphrasia "fharaidensis"*. Also these taxa share the presence of haplotype D with the more widespread *Euphrasia scottica* (Chapter 6). The sharing of this haplotype possibly indicates the important role that *Euphrasia scottica* may play in the formation of a number of endemic taxa in Britain. Haplotype D also occurs within the drier grassland taxa, *Euphrasia confusa.* An explanation for this may be the common occurrence of the hybrid *Euphrasia scottica* x *E.confusa*, found in calcareous hill-flushes (Silverside, 1998).

This preliminary study therefore confirms the importance that ecological specialisation may have on the formation and isolation of restricted taxa, as well as indicating the possible important involvement of *Euphrasia scottica* in the origin of a number of the endemic taxa. Recommendations for future research need to include the importance of gaining greater understanding of habitat diversity, and its interaction with genetic variation, within British *Euphrasia.* The incorporation of genetic and ecological factors, with the variation found in the breeding system of this group, is an important first step to understanding the generation of the high diversity of *Euphrasia* in Britain. These interactive processes are the subject of the next chapter.

CHAPTER 8: Conservation genetics of British *Euphrasia*

Abstract: Species concepts based on the general assumption of monophyly and reproductive isolation have been difficult to apply to the genus *Euphrasia*. In this thesis, molecular analyses, using a combination of chloroplast PCR-RFLP and AFLP techniques, has shown that, despite some degree of genetic congruence with taxonomic divisions within British *Euphrasia*, support for the currently accepted taxonomy is not universal throughout the group. This is especially seen in the more diverse tetraploid group where individuals of the tetraploid taxa did not form mutually exclusive groups in ordination analyses of the molecular data. In this chapter I argue that a species-based approach to conservation in this group is un-workable and an alternative process-orientated conservation strategy may be a better approach to describing the diversity in British *Euphrasia*. This may also allow an understanding of why Britain has so many *Euphrasia* taxa, compared to the rest of Europe. Here I give one hypothesis, relating to the formation of new taxonomic diversity in the context of the interaction of morphological, ecological, breeding system and genetic variation, occurring within the oceanic and hyperoceanic climate experienced in western and northern Britain.

8.1. Introduction

Numerous attempts have been made to classify biological diversity into discrete fundamental evolutionary units, defined at the species level (Mishler and Donoghue, 1982). This sorting of diversity has been the subject of intense debate among biologists and philosophers (Schemske, 2000), resulting in increasing numbers of species concepts (Mayden, 1997). These concepts generally differ in the emphasis they place on the description of patterns or processes that occur in nature. For example, concepts such as the Biological Species Concept (Mayr, 1940) or the Cohesion Species Concept (Templeton, 1989), by stressing the importance of the processes of reproductive isolation or intrinsic cohesion mechanisms, have a different approach to species delimitation, than either the Phenetic Species Concept (Sneath, 1976) or Phylogenetic Species Concepts (Rosen, 1978; de Queiroz and Donoghue, 1988; McKitrick and Zink, 1988). These latter two concepts place more emphasis on patterns resulting from either phenetic clustering or monophyletic grouping.

However, most species are currently defined phenetically, delimitated by both morphological and ecological differences (McDade, 1995). This approach, as applied to the genus *Euphrasia* in Britain (Pugsley, 1930; Yeo, 1978a), remains controversial. As current conservation prioritisation is based on defined taxa at species rank, resolution of this controversy is an important goal of the existing biodiversity action plan for British *Euphrasia* (Anon, 1995b). In an attempt to address this goal, three chapters of this thesis have analysed the distribution of molecular marker variation within the genus *Euphrasia* in Britain. This has been done under the assumption that observable patterns of morphological variation used to define species boundaries have an underlying genetic basis (McDade, 1995). Under this assumption the finding of a high degree of congruence between genetic marker variation and the accepted taxonomic divisions would lend support to current taxonomic treatment and strengthen the current conservation strategy of targeting resources towards preservation of rare endemic species. This chapter initially summarises the degree of congruence that has been found between partitioning of genetic marker variation and current taxonomic divisions, among and between both ploidy groups within British *Euphrasia*.

The direct relationship between species recognition and conservation prioritisation has been described as "rather mindless", being misguided with no scientific justification (Mishler, 1999, pg. 313). Given such strong criticism, I shall also discuss whether or not support for taxonomic treatment of *Euphrasia* can further justify the current conservation strategy as applied to the genus. In this respect I shall show that the use of molecular markers, by providing insight into processes generating diversity within British *Euphrasia*, has been valuable in highlighting shortcomings in the current species orientated conservation policy, and suggest a more appropriate conservation strategy for this particular plant group.

8.2. Genetic congruence with the current taxonomic treatment

Controversy over the placement of species boundaries within *Euphrasia* has existed since modern attempts at taxonomic division within the genus were initiated just over 100 years ago (Townsend, 1884; Wettstein, 1896). At that time, Townsend's opening

Ploidy	Tetraploid			Diploid
Series	*Boreales*	*Nemorosae*	*Parviflorae*	*Euphrasia*
Taxa	*arctica* subsp. *borealis* *arctica* subsp. *arctica*	*tetraquetra* *nemorosa* *pseudokerneri* *confusa*	*frigida* *foulaensis* *cambrica* *ostenfeldii* *marshallii* *rotundifolia* *campbelliae* *micrantha* *scottica* *heslop-harrisonii* *"fharaidensis"*	*officinalis* subsp. *monticola* *officinalis* subsp. *rostkoviana* *rivularis* *officinalis* subsp. *anglica* *vigursii*

Figure 8:1. Modern taxonomic divisions within the genus *Euphrasia* in Britain (Yeo, 1978a; Silverside 1991d). The tetraploid series *Majoriflorae* has not been included as its only representative in Britain, *Euphrasia stricta*, is presently recognised only in Guernsey.

lines in his paper on *Euphrasia officinalis* (1884, pg. 161) typified the differing views that nineteenth century botanists held, stating that

"The question whether *Euphrasia officinalis* L. represents a single polymorphic species, or a collective species, and what rank its numerous forms should take as representatives of the genus, are questions upon which botanists have been much divided."

Only 11 years previously Ball (1873, pg. 271) in his description of a botanical excursion to Morocco with J.D.Hooker and G.Maw, had stated that

"In our islands [Britain] the forms included under this name [*Euphrasia officinalis*] differ so slightly that, as I believe, no botanist has proposed to designate them by distinct specific names."

Wettstein's (1896) monograph of the genus, with Townsend's (1897) translation, as applied to British forms, were influential in changing this prevailing view, dividing the genus up into a number of distinct morphological entities. This has been continued by a number of British botanists, culminating in the British monograph by Pugsley (1930) and the European monograph of Yeo (1978a). These works have led to the modern taxonomic treatment of the genus in Britain (Figure 8:1). Divisions, based on phenetic morphological discontinuities within an ecological context,

represent a balance between the need to guard against the creation of "cumbersome polynomial nomenclature" (Yeo, 1978a) and an attempt to adequately describe "clear natural units with high predictive information content, which will prove useful for evolutionary and ecological studies" (Vitek, 1998). Despite this pragmatic use of "somewhat flexible characters" and the higher number of taxonomic divisions than would be expected under a strict biological species concept (Sell and Yeo, 1970), the use of both chloroplast PCR-RFLP and AFLP techniques have shown partitioning of genetic variation within British *Euphrasia*, that at least at some levels concurs with presently recognised taxonomic divisions.

Genetic support for the current taxonomic divisions can be seen in some cases at both series and species level within British *Euphrasia* (Figure 8:2). The division between the diploid series *Euphrasia*, and the tetraploid series *Boreales*, *Nemorosae* and *Parviflorae* is the most pronounced. Division among these two ploidy groups explains approximately 30% of both the total chloroplast haplotype and AFLP marker variation and produces the highest number of group-limited AFLP bands (Chapter 4). This supports the presence of an inter-ploidy isolating barrier to gene flow, as previously inferred by chromosomal counts (Yeo, 1954; Löve, 1956, as reported by Yeo, 1966), low hybrid fertility following inter-ploidy cross-fertilisation (Yeo, 1966), and separation of discrete indumentum characters (Silverside, 1990b).

At the species level, the degree of genetic support for the current taxonomic divisions depends on the ploidy group to which the taxon belongs. Diploid taxa are clearly discriminated along the first axis of principal coordinate axis which explained 21% of the variation within the analysis. This is associated with significant level of partitioning of genetic variation among taxa, explaining 20% of the AFLP band and 57% of the chloroplast haplotype variation in the diploid group (Chapter 5). Genetic discrimination of the tetraploid taxa is less apparent. Significant partitioning of genetic variation among tetraploid taxa explains an additional 19% of the AFLP band and 11% of the total chloroplast haplotype variation. However, the majority of the taxa occupy overlapping phenetic space along the first axis of principal coordinate analysis which only explains 8% of the variation within this analysis (Chapter 6).

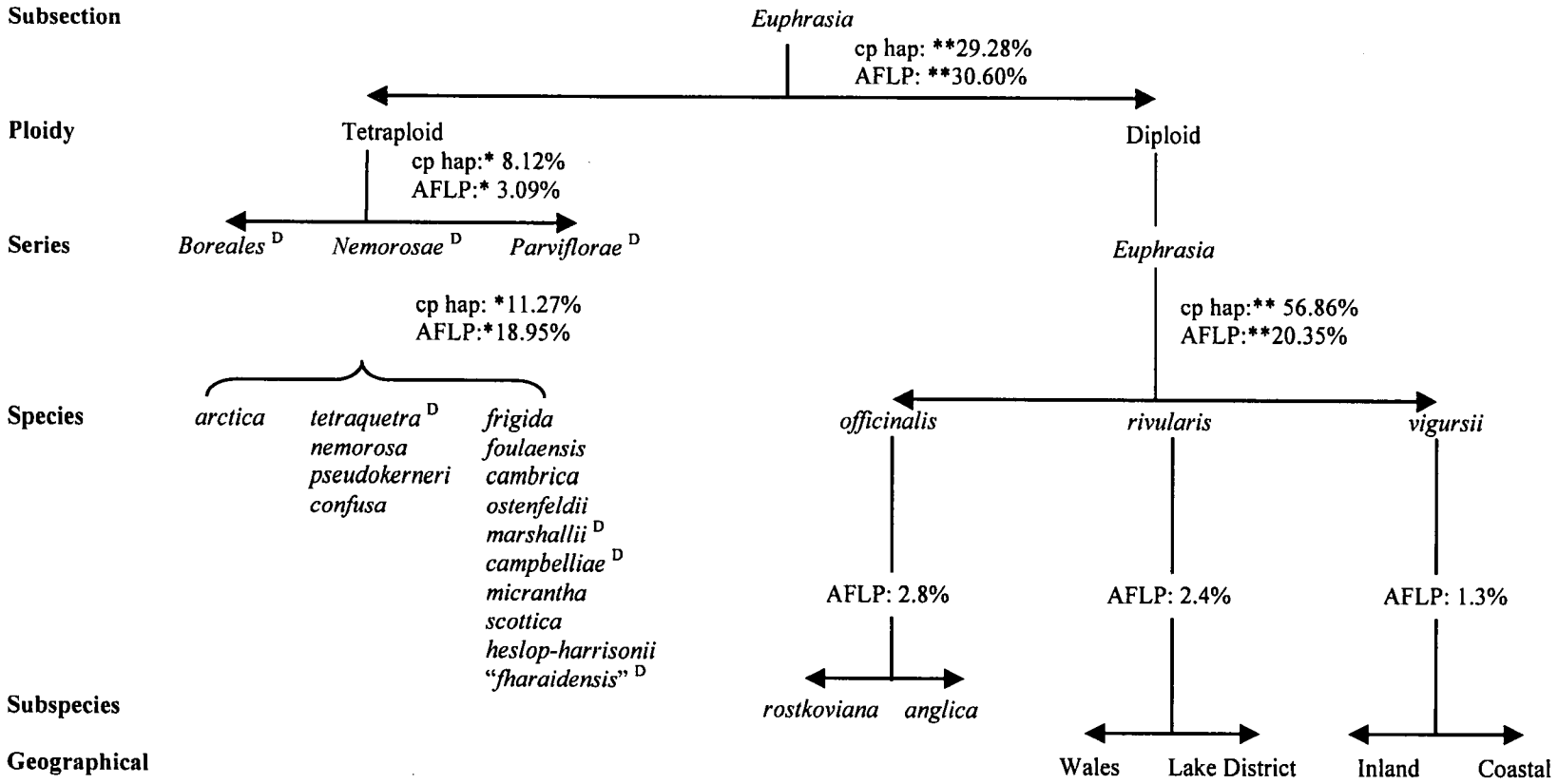


Figure 8:2. Partitioning of chloroplast haplotype and AFLP band variation within the subsection *Euphrasia* in Britain. cp hap. = chloroplast haplotype, ** $p < 0.001$; *$p < 0.05$; D = species or series occupying discontinuous space in principal coordinate analysis.

Populations from a number of tetraploid taxa also occupy a discontinuous phenetic space (with individuals from other taxa nested within their phenetic space). This discontinuity may suggest independent origin for populations within these taxa and so violate the common requirement of monophyly for species rank.

8.3. Species orientated conservation

Assignment of taxa to species rank commonly carries the implication of monophyly and reproductive isolation (Ereshefsky, 2002). These assumptions are based on an over-simplification of the processes that lead to phenetically discrete taxonomic units currently recognised at species rank (Mishler and Donoghue, 1982). They do, however, form the basis of conservation prioritisation, with importance attached to species that are rare, declining or have a long history of phylogenetic distinctiveness (Anon, 1995a). Problems with these assumptions of monophyly and reproductive isolation may arise in genera with marked hybridisation between species. In these genera, extensive reticulation make species definition problematic due to reduced phenetic discontinuity, low reproductive isolation and a tendency towards paraphyly. In a survey of 104 plant monographs, covering 1790 species, however, only 13% were found to include significant hybridisation between species, with only 1% of these marked enough to cause difficulties in species delimitation (McDade, 1995). Therefore, for the majority of plant genera, assumptions regarding monophyly and reproductive isolation are met, leading to the development of biologically sensible conservation strategies.

A survey of the level of hybridisation resulting in serious difficulty in species delimitation within the British flora estimated its occurrence to be approximately 3% of species (Stace, 1975, as reported by McDade, 1995). In addition, this propensity to hybridise was concentrated in a relatively small proportion of genera. One of these genera was *Euphrasia* (Stace, 1975). In these complex genera, assumptions regarding monophyly and reproductive isolation are not met and so conservation prioritisation of species based on these two assumptions are erroneous and indeed are misguided with no scientific justification (Mishler, 1999).

With the assumption that conservation strategies should be backed up by current scientific understanding, conservation prioritisation within taxonomically complex plant genera remains problematic for conservation practitioners. Adherence to strict species orientated conservation where species are "discrete evolutionary units", arising from monophyly and maintained by reproductive isolation, may simplify guidance for conservation management, but do so at the risk of not encompassing the overall diversity found within these complex groups. An alternative more pluralistic approach is to maintain species recognition within these groups, despite extensive reticulation, but remove underlying assumptions that these represent "distinct evolutionary units" (Mishler and Donoghue, 1982).

Out of these two alternative conservation approaches, I wish to argue that the more pluralist approach would be more appropriate for conservation strategy within British *Euphrasia*. Apart from removing the erroneous idea that the current taxonomic treatment represents "discrete evolutionary units", taking a strict species approach to achieve distinct monophyletic units would result in too much lumping of diversity under one or two described taxonomic entities. This view has been previously raised regarding morphological diversity (Sell and Yeo, 1970; Yeo, 1978a), but the molecular work I have done within the genus in Britain demonstrates that this can equally be applied when considering neutral genetic diversity. Under current conservation legislation, description of a few taxa would lead to lower conservation prioritisation given to the genus, as well as lowering the monitoring of the diversity within the genus. Given the unique diversity held within British *Euphrasia*, this would be undesirable.

In addition, setting conservation prioritisation on the basis of a better understanding of the processes that are generating the high diversity of *Euphrasia* in Britain seems more intuitively correct than setting priorities based on assumptions extrapolated from Linnean ranks, formed from pre-evolutionary biological thinking (Ereshefsky, 2002). In this respect, I may be reflecting the feelings of Stuart Thompson (1919, pg. 336), who in response to the botanical discussion over the identity of a tetraploid population of *Euphrasia* in Exmoor said

"the subject has shown me how much is to be learnt from the polymorphic genus *Euphrasia* in regard to plant evolution and distribution .... and if the vexed and unimportant question of specific rank were the only *raison d'être* of the study of these critical plants, I, for one, would regret the time so many have devoted to it."

This more evolutionary way of thinking of complex plant genera may be especially pertinent within the recently colonised flora of northern Europe, where plant groups displaying high degrees of endemism often show active diversification resulting from processes related to deviation from random sexual mating (Squirrell *et al.*, 2002). The complexity and diversity found in *Euphrasia* in Britain makes this country an ideal study area for understanding evolutionary processes in this genus (Yeo, 1968).

8.4. Process orientated conservation

Setting conservation priorities based on a more pluralistic approach to species definition requires changing the current species orientated approach (Anon, 1995a), to a process orientated approach. This would allow better targeting of resources to help preserve and promote the evolutionary potential of the group as a whole and not just the rare defined endemic species. In this respect, the use of molecular markers may be instrumental in guiding the formulation of new and more appropriate conservation strategies for *Euphrasia*, providing insights into factors promoting diversification in this group.

A number of north Atlantic endemic plant taxa occur in areas previously covered by the Weichselian glacial ice sheet and are hence assumed to have arisen since the retreat of this ice sheet (Brochmann *et al.*, 2003). Genera containing these endemic taxa are often polyploid, with fixed heterozygosity and high rates of self fertilisation (Brochmann *et al.*, 2003). These genera therefore share biological similarities with the polyploid complex found within British *Euphrasia*, and so may provide clues as to which processes are important in the generation of diversity.

These polyploid complexes have been increasingly recognised to possess considerably more dynamic genetic systems than previously thought (Brochmann *et al.*, 1992b). A combination of processes including increased heterozygosity, reduced

inbreeding depression, increased selfing rates, increased rate of genomic rearrangements and increased genetic diversity, as a result of multiple origins from a number of divergent diploid genomes, have been cited as contributors to the rapid diversification of polyploid plants (Soltis and Soltis, 2000). The arctic alpine genus *Draba* L., for example, contains many allopolyploid autogamous species, with a high degree of fixed heterozygosity (Brochmann *et al.*, 1992a). It has been proposed that this degree of heterozygosity, by effectively reducing the deleterious consequences of inbreeding may have allowed these selfing taxa to occur in severe ecological conditions found in the Arctic (Brochmann *et al.*, 1992a). In addition, inter-ploidal gene flow and extensive post glacial range expansion have contributed to the observed blurring of species boundaries in this genus (Scheen *et al.*, 2002).

Multiple shifts in breeding system may occur over a relatively short timescale. Following only two generations of directional selection, flowers of the winter annual *Phlox drummondii* Hook. decreased in size from 28.87 mm to 25.28 mm. If this magnitude of response were maintained over subsequent generations, this might result in the evolution of the smaller flowered *Phlox cuspidata* Scheele from a *Phlox drummondii*-like ancestor in only eight generations (Lendvai and Levin, 2003). Changing floral traits may therefore play an important role in the rapid diversification and generation of taxonomic complexity within a plant group (Lendvai and Levin, 2003). For example, within the *Epipactis leptochila* complex this may have led to the repeated formation of selfing taxa from the outcrossing parental species *Epipactis helleborine* (L.) Crantz. Morphological differentiation of these reproductively isolated selfing taxa has been proposed to have occurred by the rapid fixation of new multilocus genotypes and adaptation to differing ecological conditions (Squirrell *et al.*, 2002).

Many of these processes are likely to be also important in generating the complexity and diversity within British *Euphrasia*. In the remaining part of this chapter I will use evidence from molecular markers to construct a hypothesis to explain the generation of diversity in British *Euphrasia*. This hypothesis will then be used to propose a

more appropriate biodiversity action plan for *Euphrasia* in Britain. This proposal is the subject of the final chapter.

8.5. Diversifying processes in British *Euphrasia*

Diversification in British *Euphrasia* is likely to have been a continuous process since its re-colonization after the end of the last glacial period. During this time new discrete entities are likely to have evolved and have since been expanding their range or have declined towards extinction. The present day taxonomy of *Euphrasia* therefore represents a "snapshot" of this process. Important stages within this process may, however, be inferred from the examination of the present diversity found in British *Euphrasia*. Within this continual process, three main steps may be recognised. These are allopolyploid formation of European tetraploid progenitor taxa, migration of diploid and tetraploid progenitor taxa into Britain, and diversification in Britain, resulting from the interaction of genetic variation with ecological diversity found within an oceanic and hyper-oceanic climate.

8.5.1. Diversification Process I: Multiple allopolyploid events

The geographical distribution of British *Euphrasia* taxa is varied, some have a widespread European distribution (Tutin *et al.*, 1972), while others are endemic, with a markedly restricted distribution (Preston *et al.*, 2002). These endemic taxa are likely to have originated within Britain, forming from a subset of diversity found within the more widespread European taxa. Hence, high diversity presently found amongst British endemic taxa may partly be explained by a high degree of diversity within the European taxa, especially the progenitor taxa that migrated into Britain after the end of the last glacial period (Yeo, 1968).

It is likely that both diploid and tetraploid *Euphrasia* taxa acted as progenitor taxa. The finding of abnormal meiosis in a British triploid hybrid between diploid *Euphrasia officinalis* subsp. *anglica* and tetraploid *Euphrasia micrantha*, with 11 bivalents and 11 univalents (Yeo, 1956) has led to the suggestion that the progenitor tetraploid taxa originally arose from progenitor diploid taxa by one or more allopolyploid events (Yeo, 1978b). Genetically different diploid groups have been

demonstrated in Europe (see Box 8:1). Hybridisation experiments between diploid taxa from differing series within subsection *Euphrasia* produced hybrids resembling some of the widespread European tetraploid taxa. Chromosomal pairing within these hybrids was often abnormal, indicating genetically different diploid parents (Yeo, 1976). Also crosses between tetraploid taxa from different series within Britain, though resulting in fertile hybrids, produce some univalents at meiosis. This failure of normal chromosomal pairing occurred in a cross between *Euphrasia pseudokerneri* in the series *Nemorosae* and *Euphrasia scottica* in the series *Parviflorae* (Yeo, 1966).

The formation of the tetraploid group by allopolyploid events, between distinct diploid taxa, is supported by the distribution of AFLP bands among the two ploidy groups in Britain. Out of 139 scorable AFLP bands, 74 were found to be common across ploidy groups, while 10 bands occur only within the diploid group and 55 are bands limited to the tetraploid group (Chapter 4). This suggests that the tetraploid genome consists of at least two genetically distinct diploid genomes, one of which is found within Britain and one of outside Britain.

Tracing phylogenetic relationships from current tetraploid taxa back to the initial allopolyploid events and finding the specific diploid progenitor taxa may, however, be difficult, if not impossible, due to extensive reticulation that has since taken place within this group (Yeo, 1968; Karlsson, 1974). Recent hypothetical schemes for the formation of the tetraploid group have been suggested by Yeo (1978b) and Vitek (1986). Within Europe, the genus *Euphrasia* consists of two subsections, *Euphrasia* and *Angustifoliae* (Yeo, 1978a). Within Britain, only tetraploid taxa from the subsection *Euphrasia* are present. Within this subsection, both the hypothetical schemes of Yeo and Vitek involve at least three distinct diploid genomes in the formation of the tetraploid group. This includes a small flowered diploid species that has since been described from the series *Parviflorae* (Ehrendorfer and Vitek, 1984).

Box 8:1. Diversity within the progenitor group of British *Euphrasia*

The progenitor group of British *Euphrasia* represents taxa that migrated into this country after the end of the last glacial period. It is likely to have consisted of both diploid and tetraploid groups. The diploid group containing ancestral *Euphrasia officinalis* from the series *Euphrasia* and the tetraploid group containing ancestral taxa from the series *Boreales*, *Nemorosae*, *Majoriflorae* and *Parviflorae*. This latter group is the most diverse in Britain, being hypothesised to have formed from the combination of at least three distinct European diploid genomes (Figure 1). The distribution of AFLP bands and chloroplast haplotype groups in the British tetraploid group supports this multiple allotetraploid hypothesis (Chapter 6). The partitioning of diversity within the tetraploid group into two main divisions with distinct genomes corresponds to the species complexes as defined by Karlsson (1976). Combined series *Boreales*, *Nemorosae* and *Majoriflorae* equivalent to *stricta* complex and series *Parviflorae* equivalent to *micrantha* complex.

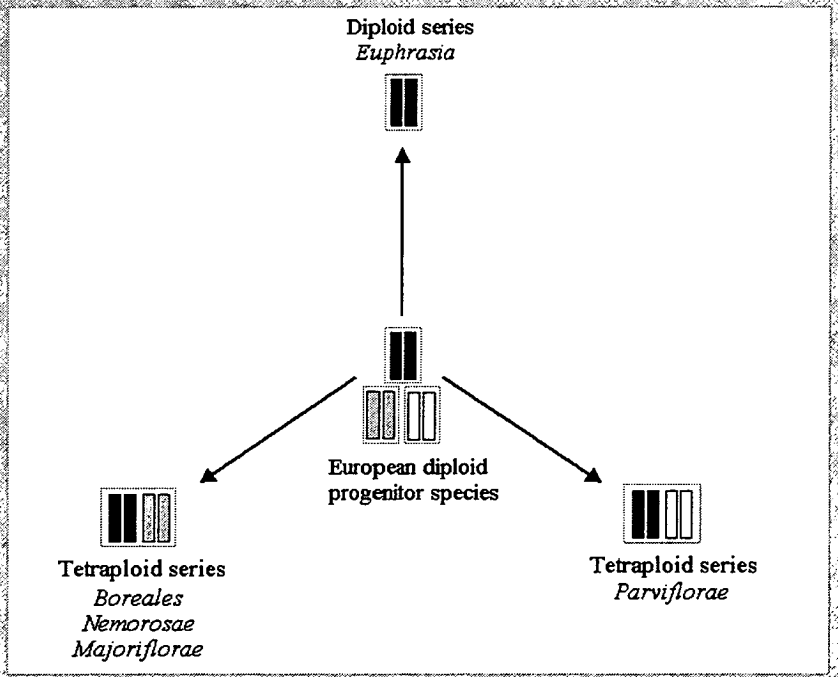


Figure 1. Hypothesised progenitor group of British *Euphrasia* formed from European diploid progenitor species. Coloured boxes represent differing genomes, as described by Yeo (1978b) (see figure 8:3). Black = AA; Grey = BB; White = CC.

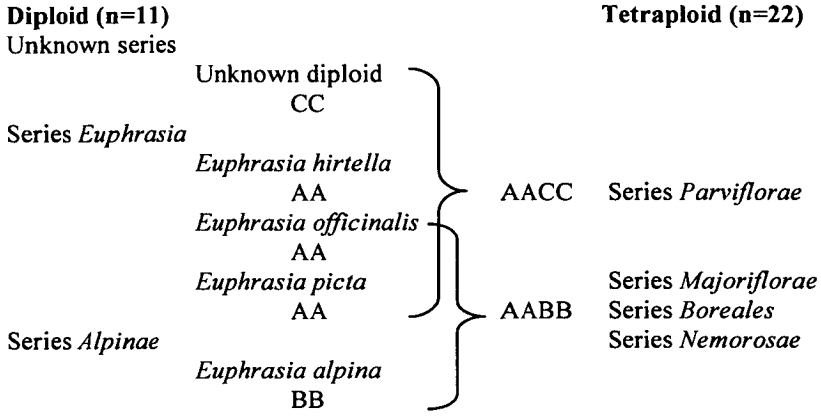


Figure 8:3. Hypothetical scheme of some possible genomic relationships in British *Euphrasia*, as adapted from Yeo (1978b). Letter A, B, C represent different genomes. *Euphrasia hirtella* and *Euphrasia picta* are alpine species in southern Europe and *Euphrasia officinalis* a widespread European species, often occurring at lower altitudes. The unknown diploid taxa, "CC" may represent the European alpine species *Euphrasia inopinata* or *Euphrasia sinuata*, within series *Parviflorae* (Ehrendorfer and Vitek, 1984).

Under the hypothetical scheme of Yeo (1978b), there are two distinct tetraploid genomes, in differing series within the subsection *Euphrasia.* One genome, "AABB", occurring in series *Boreales*, *Nemorosae* and *Majoriflorae* and the other "AACC" occurring in series *Parviflorae* (Figure 8:3; Box 8:1). These two distinct genomes within the tetraploid group may reflect their separate origins from the progenitor diploid taxa. This is reflected by the non-uniform distribution of chloroplast haplotypes found within the tetraploid group in Britain, with significant partitioning of variation (8% of the total chloroplast variation), among tetraploid series (series *Boreales*, *Nemorosae* and *Parviflorae*; $\chi^2 = 86.177$, d.f. = 4, $p < 0.000$; Chapter 6). The separation of chloroplast haplotypes among Yeo's two main tetraploid groups ("AABB" and "AACC") is clearly evident on grouping chloroplast haplotypes according to their distinctiveness from one another, as seen in the minimum spanning network (Table 8:1; Figure 8:4). Here, chloroplast haplotype groups F to H and I to J predominantly occur in the combined series *Boreales* and *Nemorosae* and the chloroplast haplotype group A to E, predominantly in the series *Parviflorae* (Chapter 6). Within the tetraploid "AABB" group, including series *Boreales* and *Nemorosae*, the presence of two distinct chloroplast haplotype groups F to G and I to J, suggests that at least two allopolyploid events were involved in its formation.

Table 8:1. Chloroplast haplotypes detected by RFLP analysis of four PCR products in British *Euphrasia*: AS = *psa*A - *trn*S: CD = *trn*C - *trn*D: HK = *trn*H - *trn*K: KK = *trn*K_1 - *trn*K_2 (Taken from Chapter 4).

Haplotype	AS:*Hinf*I band I	AS:*Hinf*I band II	AS:*Hinf*I band III	CD:*Hinf*I band I	CD:*Hinf*I band II	CD:*Hinf*I band III	HK:*Hae*III band I	KK:*Hinf*I band I
A	1	1	2	1	2	3	2	1
B	1	1	2	1	2	3	2	0
C	1	1	2	2	2	1	2	0
D	1	1	2	1	2	1	2	0
E	3	1	2	1	2	3	2	0
F	3	1	2	1	1	2	2	0
G	3	1	2	?	1	0	2	0
H	3	1	2	?	1	0	1	0
I	2	2	2	2	2	1	2	0
J	2	2	2	2	2	1	3	0
K	1	0	1	1	2	3	2	0
L	1	0	1	1	2	3	1	0

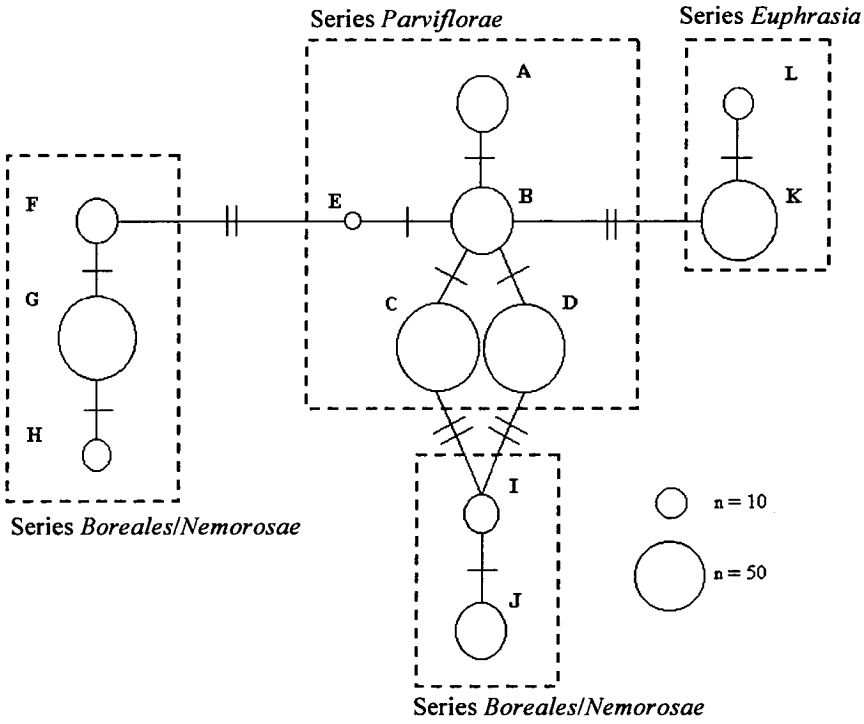


Figure 8:4. Haplotype groups defined according to relationships between chloroplast haplotypes, represented as a minimum spanning network. The two main tetraploid groups are indicated by boxes. Series *Boreales* and *Nemorosae* (genotype "AABB") by haplotype groups F to H and I and J and series *Parviflorae* (genotype "AACC") by haplotype group A to E. Diploid series *Euphrasia* represented by box enclosing haplotype group K to L. Haplotypes are described in Table 8:1. Mutational steps are indicated by single cross line. The areas of the circles represents number of individuals with each haplotype. n = number of individuals (Taken from Chapter 4).

The distribution of chloroplast haplotypes in the British tetraploid group therefore supports the notion that at least three allopolyploid events occurred in the formation of tetraploid taxa within the subsection *Euphrasia*. At least two polyploid events leading to the formation of the combined series *Boreales* and *Nemorosae* (and series *Majoriflorae*) and at least one separate event leading to the formation of series *Parviflorae*. This main division within the tetraploid group generally corresponds to the definition of a number of fundamentally distinct units within Swedish *Euphrasia* (Karlsson, 1976). These are the *micrantha* complex (series *Parviflorae* with mainly chloroplast haplotypes A to E) and the *stricta* complex (combined series *Boreales Nemorosae* (and *Majoriflorae*) with chloroplast haplotypes F to H and I to J). The only exception to this, in the *stricta* complex, is *Euphrasia frigida* (series *Parviflorae*, fixed for chloroplast haplotype C).

8.5.2. Diversification Process II: Post glacial migration of progenitor groups into Britain

During post-glacial migration, many of the progenitor *Euphrasia* taxa would probably have occurred sympatrically in Britain. Reproductive isolation, resulting from ploidal differences, though not absolute, is likely to have been great enough to keep the diploid progenitor taxa distinct from the tetraploid progenitor taxa (Chapter 4; see Box 8:2). This is not the case for the homoploid tetraploid groups. With no significant reproductive barriers (Yeo, 1966), extensive hybridisation might have occurred between sympatric populations of differing tetraploid progenitor taxa. This may reflect the widespread distribution of a number of chloroplast haplotypes throughout the tetraploid group and the limited separation of tetraploid taxa along the first axis of principal coordinate analysis (Chapter 6).

Despite this extensive reticulation, chloroplast haplotype groups are not randomly distributed within the tetraploids. This suggests that hybridisation has not been extensive enough to completely merge the tetraploid progenitor groups together (series *Boreales* and *Nemorosae* with series *Parviflorae*). The presence of barriers to gene flow between these series may be inferred from the present frequency of their hybrids seen in the field. In Sweden, where the two tetraploid series groups are equivalent to species complexes, hybrid populations are common between taxa

within species complexes but rare between taxa among species complexes (Karlsson, 1976; Karlsson, 1986).

Barriers to gene flow among progenitor tetraploid groups are likely to have been the result of differences in ecological habitat (Chapter 7), combined with reproductive isolation from high selfing rates (Chapter 3). The progenitor taxa within series *Parviflorae*, for example, are smaller flowered and occupy different ecological habitats to taxa within the other tetraploid series. The different hypothesised genome within series *Parviflorae* compared to series *Boreales* and *Nemorosae* ("AACC" compared to "AABB", respectively, Figure 8:3) may have contributed to this ecological and reproductive isolation (see Box 8:2).

Cultivation experiments have shown that variation in corolla size is genetically inherited (Yeo, 1959; Yeo, 1962; Bobear, 1969; Karlsson, 1984; Zopfi, 1998a). Thus, genes for small, selfing flowers in series *Parviflorae* may have been inherited from the small flowering progenitor diploid taxa with genome "CC" (Figure 8:3). Whether or not ecological specialisation is inherited has been less well studied but a similar inheritance of genes for survival in often wetter, less fertile and more acidic habitats in tetraploid taxa of the series *Parviflorae* may also have been derived from the same diploid progenitor parents. Alternatively, both sets of genes may have been acquired from segregation of genes within the two larger flowered diploid parental taxa within series *Euphrasia* (genome "AA") and series *Alpinae* (genome "BB"). Further work is required to help determine which of these scenarios is likely to have resulted in the transfer of these quantitative morphological traits, as well as the role that they played in the isolation of tetraploid groups from one another during post-glacial migration.

Box 8:2. Maintaining the distinctiveness of progenitor groups

Despite extensive reticulation within British *Euphrasia*, structuring of chloroplast haplotypes have persisted among the main progenitor groups. A strong ploidal barrier may account for the persisting distinction between the diploid and tetraploid groups. This barrier does not exist between the homoploid tetraploid groups. At this ploidal level, a combination of genome differences, habitat differences and high rates of selfing are likely to have been the isolating factors. The hypothesised diploid genome "CC" within series *Parviflorae*, not shared by the series *Boreales* or *Nemorosae*, may indicate the transfer of distinct genes from a progenitor diploid taxa into this tetraploid group. This hypothesised diploid progentitor group may be the source of genes leading to the predominance of small flowers in tetraploid taxa of the series *Parviflorae* and also the ability of some of their taxa to survive in wet, nutrient poor, acidic habitats.

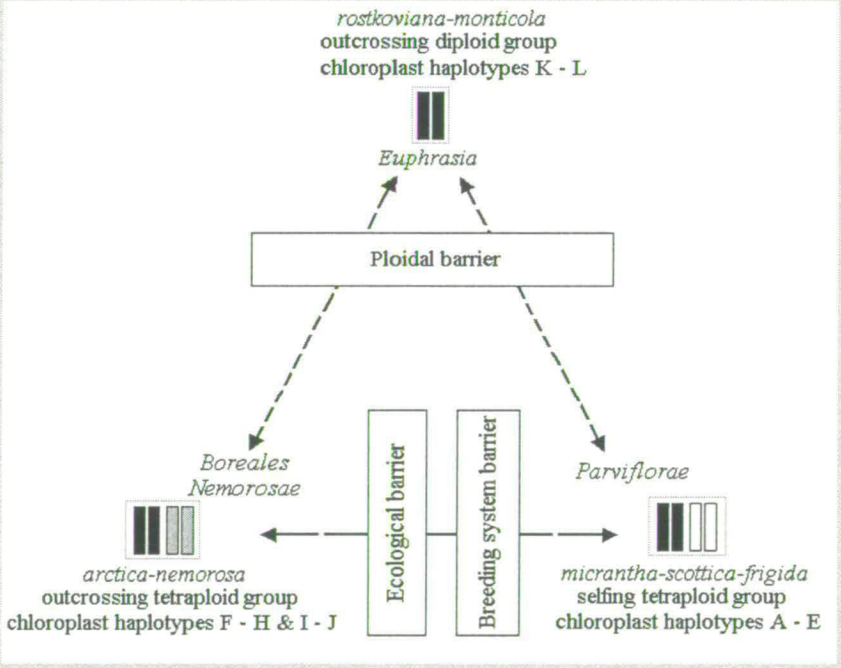


Figure I. Hypothesised barriers to gene flow keeping the three main progenitor groups (haplotype-taxonomic groups) distinct, as inferred by non-uniform distribution of chloroplast haplotype groups among present day taxa in Britain. Coloured boxes represent differing nuclear genomes, as described by Yeo (1978b) (see figure 8:4). Black = AA; Grey = BB; White = CC.

8.5.3. Diversification Process III: Formation of restricted British *Euphrasia* taxa

The three proposed mechanisms limiting gene flow (ploidal, ecological and breeding system barriers) that are important in maintaining a degree of distinctiveness between migrating diploid and tetraploid progenitor groups, may also be crucial to the generation of unique diversity found within British *Euphrasia*. During migration, repeated habitat expansion and founder events associated with colonisation, combined with multiple formation of selfing lines, may have lead to the generation and maintenance of new multilocus genotypes, with the fixation of novel adaptive gene complexes to differing ecological habitats. Genetic diversity within the diploid progenitor group may also have been enhanced by gene flow, across the ploidal barrier, from the genetically diverse tetraploid groups. The introduction of these genes may have included genes that were initially present in other non-British diploid progenitor taxa so that new diploid multilocus genotypes acquired a greater degree of adaptability to a wider range of ecological conditions (see Box 8:3).

The generation and fixation of new genotypes is a continual process, forming novel populations that combine characters from the progenitor parental taxa. Many of these populations are likely to be either relatively indistinct or short lived and so not nameable under current *Euphrasia* taxonomic concepts. Some however may persist over a longer periods of time, forming distinct entities in discrete ecological habitats, over specific geographical areas. It is these that have been recognised as new endemic taxa, often being given species status, under present concepts of *Euphrasia* taxonomy.

European *Euphrasia* have an annual lifestyle with seedbanks lasting up to only five years (see Yeo, 1961; Karlsson, 1984; Vitek, 1998; Lammi *et al.*, 1999). This rapid turnover of generations with limited seedbank reserves increases the potential for adaptive differentiation over short time periods and so allows populations in different habitats to quickly diverge.

Box 8:3. Generating diversity within British *Euphrasia*

Processes important in the diversification of *Euphrasia* occur throughout Europe. Alteration in the breeding system with repeated shifts from allo- to autogamy is an important mechanism of morphological change in this group, with the formation and fixation of novel multilocus genotypes, that may adapt and survive to differing ecological habitats. The higher ecological diversity found within the oceanic and hyper-oceanic climate of Britain may therefore allow a greater expression of this morphological change, as seen by the higher number of recognised endemic taxa. These taxa represent the attainment of differing "adaptive optima" within the possible interactions between ecological, breeding system and genetic variation found in Britain. Important genetic interactions, both within and among the genomes of tetraploid and diploid groups, have been governed by the partitioning of genes from at least three progenitor diploid European taxa, during a number of allotetraploid events.

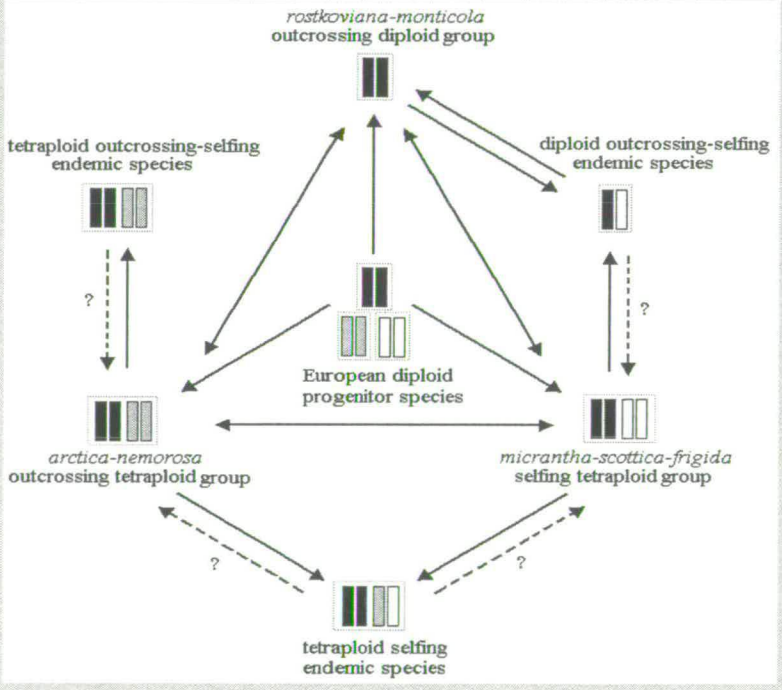


Figure I. Hypothesised processes generating diversity within British *Euphrasia.* Coloured boxes represent differing nuclear genomes, as described by Yeo (1978b) (see figure 8:4). Black = AA; Grey = BB; White = CC. Arrows represent gene flow with dotted lines representing presence of non-absolute barriers. ? = gene flow uncertain in this direction.

In addition, hemi-parasitism (see Yeo, 1959; Yeo, 1961; Yeo, 1964; Wilkins, 1963; Seel and Press, 1993; Seel and Press, 1994; Matthies, 1998 for hemi-parasitism in *Euphrasia*), by allowing successful competit-ion with perennial plants for nutrients and water, increases the range of available habitats (Karlsson, 1974). The ability to survive in these closed plant communities may have been particularly important in respect to diversification of the genus in Europe, with human disturbance, especially, increasing the diversity of these habitat types. Introduction of agricultural practices into the landscape has not only resulted in the expansion and increasing connectiveness of the forestless grassland and heathland communities but also produced a high variety of these communities by variation in practices, such as timing of grazing and mowing (Karlsson, 1974).

Within this high diversification, Britain has been particularly prolific in the generation of new taxa. This raises the question as to why *Euphrasia* should be so particularly diverse as compared to the rest of Europe. This is especially pertinent in the relatively recently colonised and species depauperate flora of the British Isles. It is true that this genus has been particularly well studied in Britain, by workers who have tended to give taxonomic recognition to a large degree of variation found within this country (Pugsley, 1930; Yeo, 1978a) but the group has also been intensively studied in Europe (see Wettstein, 1896; Chabert, 1902; Jørgensen, 1919; Jalas and Kukkonen, 1973; Hartl, 1975 for examples of monographic and floral accounts of European *Euphrasia*) which, though often with a more taxonomic "lumping" approach, does not fully account for this discrepancy in rates of diversification. Indeed the last *Euphrasia* monograph incorporating British taxa (Yeo, 1978a), does so on a European wide scale.

An important ecological difference between Britain and mainland Europe results from a higher level of ecological diversity found within the oceanic and hyper-oceanic climate, and arctic-alpine conditions, experienced in Britain (Preston and Hill, 1997). Evolution of new taxa within *Euphrasia* represents the attainment of "adaptive optima" produced from the possible myriad of morphological, ecological, breeding system and genetic interactions. Within Britain, the high diversity of

ecological niches allows the attainment of a greater number of differing optima. This is especially found within the western and northern coastline of Scotland, where a high number of endemic taxa occur. Diploid taxa do not extend this far north so that all the *Euphrasia* taxa at this latitude are tetraploid (Preston *et al.*, 2002). This may reflect the advantages obtained from doubling of the genome, allowing tetraploid taxa to survive in harsher environmental conditions, as well as exploiting a broader ecological amplitude (Brochmann and Elven, 1992). It is also noteworthy that endemic or restricted taxa in this region are predominantly small flowered (Chapter 3). This may reflect the advantage of autogamy, providing reproductive assurance in the face of pollinator service unpredictability.

In allopolyploid taxa, inbreeding depression may be relatively low as a result of the buffering effect of additional genomes (Richards, 1986) and presence of high levels of fixed heterozygosity (Brochmann *et al.*, 1992a). Therefore, the advantages of increased selfing may not be outweighed by the disadvantages of increased inbreeding depression (Jarne and Charlesworth, 1993; Schoen *et al.*, 1996). Higher rates of selfing may thus be advantageous and smaller flowered taxa may evole. This may be the case in the tetraploid *Euphrasia* group in west and north Scotland. The hypothesised allotetraploid origin of the group, as supported by the presence of fixed heterozygosity in allozyme analysis of the tetraploid taxon, *Euphrasia heslop-harrisonii* in Scotland (Oliver, 1999), has allowed the possibility of more extreme allo- to autogamous shifts in breeding system. This may have occurred in response to the pollinator service unpredictability, which in turn by leading to the fixation of novel genotypes, has allowed exploitation of the great diversity of habitats present in this geographical region.

Evolving taxa within west and north Scotland may therefore result from the attainment of a number of differing "adaptive optima", made possible by the greater range of ecological, breeding system and genetic interactions between the progenitor tetraploid taxa. An important component that has increased the scope of these possible interactions has been the genetic diversity found within this tetraploid progenitor group, generated by the hypothesised multiple allopolyploid events

between at least three distinctly different diploid progenitor groups. The distribution of chloroplast haplotype groups within the north Scottish endemic and restricted taxa may divide the tetraploid taxa into two distinct sets (Chapter 7). *Euphrasia foulaensis* and *Euphrasia heslop-harrisonii*, within one of these sets, share the chloroplast haplotypes group commonly found within the tetraploid progenitor group "AABB" (series *Boreales*, *Nemorosae* and potentially *Majoriflorae*). These taxa also share similar environmental conditions to this progenitor group (Chapter 7). Evolution of these taxa may have resulted from the segregation of both "selfing" and "environmental" genes found within this progenitor group. *Euphrasia campbelliae* and "*Euphrasia fharaidensis*", in contrast, share their chloroplast haplotype groups and common environmental conditions with the progenitor taxa within the tetraploid group "AACC" (series *Parviflorae*). In these small flowered taxa, both selfing and the ability to survive in wetter, nutrient poor, acidic environments may have been acquired from diversity of genes from progenitor tetraploid group "AACC". Both these endemic taxa do though contain morphological characters in common with the progenitor tetraploid group "AABB" (Silverside, 1991c). Hybridisation between taxa within differing progenitor groups may therefore have been important in generating the novel genotypes of these endemic taxa.

Diversity within the progenitor tetraploid group may be higher than that estimated by the use of AFLP and chloroplast PCR-RFLP markers (Chapter 6). Limitations of this study, introduced by excluding plants with morphologies intermediate between named taxa, may have been particularly important within the small flowered tetraploid progenitor group "AACC" (series *Parviflorae*). In this group, presumed hybrid populations between the progenitor taxa, *Euphrasia micrantha*, *Euphrasia scottica* or *Euphrasia frigida* were excluded. Given the small flower size, and so presumably high selfing rates of these taxa, formation of hybrid populations is likely to be rare. Populations that have been proposed on morphological grounds to be hybrid may instead be the result of separate allopolyploid events between the progenitor diploid taxa. Alternatively in this group, selfing rates may not be entirely related to flower size with hybrid populations forming between progenitor taxa with higher outcrossing rates than expected. For example, a higher outcrossing rate for the

small flowered *Euphrasia micrantha* has been hypothesised, resulting from Batesian mimicry of *Euphrasia micrantha* to flowers of *Calluna vulgaris* (L.) Hull (Yeo, 1968). Further work is still needed to test these alternate hypotheses regarding the hybrid populations of this tetraploid progenitor group and their importance in the generation of diversity within British *Euphrasia.*

A high rate of taxon formation has also occurred along the oceanic climate of western England and Wales. Diversification within both the diploid and tetraploid groups of British *Euphrasia* have been important in this geographical area. In the diploid group the evolution of new taxa within *Euphrasia,* representing the attainment of "adaptive optima", would be expected to be restricted by its single segregating genome "AA". Here both the degree of ecological amplitude and extent of allo- to autogamous shifts in breeding system would be expected to be lower than that found in the tetraploid group. Indeed none of the British diploid taxa attain the small flower sizes of many of the tetraploid taxa (Chapter 3). For example, the average middle lower lobe of a small flowered population of the diploid endemic taxon, *Euphrasia officinalis* subsp. *anglica* was 5.0 ± 1.17 mm^2. This was only 3.8 mm^2 smaller than the average middle lobe of its putative progenitor diploid taxon, *Euphrasia officinalis* subsp. *rostkoviana.* This contrasts with a population of the tetraploid endemic taxon, *Euphrasia heslop-harrisonii* where average middle lower lobe sizes of 2.65 ± 0.98 mm^2 was 7.4 mm^2 smaller than its progenitor tetraploid taxon, *Euphrasia arctica* subsp. *borealis* (Chapter 3).

Both endemic diploid taxa, *Euphrasia vigursii* and *Euphrasia rivularis* have similar flower sizes to *Euphrasia officinalis* subsp. *anglica,* though one population of *Euphrasia rivularis* had an average middle lower lobe of 3.95 ± 0.85 mm^2, with an estimated 87% selfing rate (Chapter 3). These taxa, though, share a number of morphological characters and habitat requirements with the tetraploid progenitor group "AACC" (series *Parviflorae*) and not the diploid progenitor group "AA" (Silverside, 1990b). *Euphrasia rivularis* also possesses chloroplast haplotypes within the haplotype group A to E (Chapter 5). In common with the generation of diversity of the tetraploid group, hybridisation with the tetraploid progenitor group "AACC"

(series *Parviflorae*), may therefore also be important in generating novel genotypes and endemic taxa within the diploid group. This inter-ploidal gene flow is facilitated by the non-absolute ploidal barrier between the two ploidal groups (Chapter 4).

8.6. Summary

A hypothesis may therefore be given where diversification in British *Euphrasia* involves complex processes combining ecological, breeding system and genetic variation with an allopolyploid group. Data gathered in this thesis from molecular markers suggests that caution is required when applying an alternative approach involving the simplistic interpretation of species concepts in *Euphrasia*. Certainly for the tetraploid taxa, the named entities do not represent discrete reproductively isolated monophyletic units. However, the amount of taxonomic lumping required to achieve such units would result fusing together a large amount of diversity and would hence lead to too much loss of information. A more pluralistic, processes orientated approach, allows a better description of the diversity found within the genus in Britain and so leads to the development of a more appropriate conservation strategy, aimed at preserving and promoting this diversity. Therefore, switching the focus of using molecular markers away from tools for taxonomic clarification to using them as tools to gain insight into important processes in the generation of the unique diversity within British *Euphrasia* is a fundamental step to understanding and so conserving this genus in Britain. This chapter has been an attempt at this, showing that hypotheses can be suggested into how diversification within British *Euphrasia* may have occurred. The final chapter will examine how increased understanding of these processes can be converted into the development of more practical conservation management strategies.

CHAPTER 9: Conservation strategy for British *Euphrasia*

Abstract: Currently, Britain strives to meet its obligations to the 1992 Rio de Janeiro Convention on Biological Diversity by implementing a number of species-based and habitat-based action plans. In doing this, there has been a failure to explicitly account for diversification patterns in a recently colonised flora. In the overall UK Action Plan, this equal weighting of all endemic species, regardless of their mode of origin has led to the over representation of taxonomically complex groups. In this chapter I suggest one approach to remedy this discrepancy, namely a process orientated approach. Reduction of the processes to five general components allows the development of general guidelines which may prove helpful when forming process-based action plans for taxonomically complex groups in Britain. The production of an individual process-based action plan for the genus *Euphrasia* in Britain, is given as an example.

9.1. Introduction

Article 6a of the Convention on Biological Diversity, at the Earth Summit in Rio de Janeiro, 1992, required each participating country

"to develop national strategies, plans and programmes for the conservation and sustainable use of biological diversity or adapt for this purpose existing strategies, plans or programmes which shall reflect, inter alia, the measures set out in this Convention relevant to the Contacting Party concerned"

In response to this, Britain launched *Biodiversity: The UK Action Plan* in 1994 and established a Biodiversity Steering Group to carry out its obligations as laid down in the objectives of the Convention of Biodiversity Diversity. One of these obligations is "the conservation of biodiversity", the overall goal of this UK Action Plan being

"to conserve and enhance biological diversity within the UK, and to contribute to the conservation of global biodiversity through all appropriate mechanisms" (Anon, 1995a, pg. 15)

This has been implemented by the formation of a number of species and habitat action plans whose inclusion criteria have been laid down in the UK Steering group report (Anon, 1995a). The species for which action plans have been written are those that are endemic, whose numbers have declined substantially in recent years, are

under a high degree of international threat or are also covered by existing relevant Conventions, Directives or legislation.

In Britain, implementation of this species-based conservation approach has led to the over-representation of taxonomically complex plant groups, with approximately half of the "short list" for vascular plants comprising of species that are recognised as taxonomically controversial (Squirrell *et al.*, 2002). This is not surprising given the geographical position and glacial history of the British Isles. The retreat of the last glacial ice sheet from Britain, 12000 years ago (Hewitt, 1996; Brochmann *et al.*, submitted), has resulted in a relatively young flora with endemic plant species that predominantly come from groups currently undergoing active diversification and so not falling into neat, tidy taxonomies (Hollingsworth, 2003). By adhering to prioritised species lists, the national strategy as set out in the Biodiversity Steering Group report (Anon, 1995a), has failed to recognise and so incorporate the unique conservation problems associated with this relatively young flora.

The consequences of this failure can be seen in the practical implementation of these action plans. The assessment of conservation needs often relies on the ability of botanists to identify and accurately record species distributions. In taxonomically complex groups, smaller pools of botanists are available to confidently carry out this initial assessment step. This can often lead to delays in, or even prevention of, successful implementation of action plans within complex plant groups. Widening the identification expertise to a greater number of botanists may go some way to alleviating this problem but this can only occur in groups where defined species represent clear morphological entities. In groups where this is not the case, problems in the accurate identification and recording of species remain intractable. If a population is not easily assignable to any species within a group this often results in them being ignored and so dismissed during the implementation of the action plan for that group.

It could be argued that the difficulties surrounding species definition within these taxonomically complex groups merely indicates that they do not reflect definable

units and are hence not worthy of the conservation importance currently attached to them. Within the relatively recently colonised flora of Britain, endemic taxa will not represent ancient endemic lineages whose loss would involve major tracts of phylogenetic diversity (Moritz, 2002). Therefore, one view may be that conservation resources should not be used to conserve species within taxonomically complex groups but instead may be diverted to conserving species within more "typical" plant groups, with well defined species.

In this chapter I shall disagree with this view, believing that these endemic taxa are the outcome of active evolutionary processes, representing unique assemblages of morphological and genetic variation within a group. Their uniqueness occurs irrespective of whether or not they may be "pigeon holed" as discrete morphological entities. Conservation policies based solely on the attempt to categorize the total range of diversity, generated within a continuum of biological evolution, will, by omitting groups that do not easily fit into this conceptual framework, run the risk of loosing important progenitors for future biodiversity. As endemic taxa within taxonomically complex groups are the product of active evolutionary processes, they also provide ideal opportunities for increasing our understanding of the evolution of, and hence ability to conserve, future biodiversity. In a local flora, endemic taxa may raise the profile of biodiversity, after all people focus on their local specialities. These taxa may thus serve to promote the case that biodiversity, at the local, national and global scales, is of importance and should be afforded protection.

If this view is generally accepted, then it follows that in actively evolving taxonomically complex groups conserving discrete taxonomic units should not be the only goal for setting conservation strategies (Hollingsworth, 2003). Therefore, adherence to typical species- and habitat-based action plans in Britain, may not only fail to fulfil the conservation aims of the UK Action Plan, but also not fully meet Britain's obligations laid down in the 1992 Rio de Janeiro Convention on Biological Diversity. This raises the difficult question of how best to incorporate the conservation of taxonomically complex groups in the overall action plan framework.

In an attempt to answer this question, I shall suggest one possible approach. Conservation strategies in actively evolving groups are best directed towards protecting the process rather than the products themselves (Cowling and Pressey, 2001; Moritz, 2002). I therefore propose the formation of a third group of action plans, directed more to the conservation of biological processes. This third approach, in conjunction with current species and habitats approaches, may allow a better achievement of the overall goal of the UK Action Plan. By way of example I will show that conservation of the biodiversity found within the taxonomically complex genus *Euphrasia* is best implemented by a process rather than a species-based conservation approach.

9.2. Process action plans

Diversification within taxonomically complex plant groups typically occurs as a consequence of processes other than random sexual mating in diploid taxa (Squirrell *et al.*, 2002). Relevant processes, including self-pollination, agamospermy, hybridisation and changes in chromosome numbers, give rise to novel variation within a group. Generation of new taxonomic entities may originate as a result of subjecting this new variation to differing selection pressures within an ecologically diverse habitat (Hollingsworth, 2003). The relative importance of each of these processes may vary from group to group, but common key components across groups may be defined. From these components, a general strategy for the conservation of evolutionary processes can therefore be produced to aid conservation planners when considering conservation of processes within taxonomically complex groups.

One such strategy, suggested by Moritz (2002), divides the overall process of diversification into three main parts. The first relates to the maintenance of progenitor species within a group. These species, though often common and so not usually encompassed by existing species action plans, are the foundation from which future diversity is generated. Loss of these species will result in degradation and eventual loss of the overall process. The second part relates to the observation that generation of diversity may be focused in particular geographical areas. These areas, termed "species factories" by Fjeldså and Rahbek (1998), may represent the

necessary ecological conditions required for the meeting and survival of distinct genomic combinations within a group. Loss of these areas may therefore result in the reduction in the formation and survival of novel variations, upon which selection may act. Finally, to ensure persistence of the overall process, contiguous habitats must connect the main progenitor taxa with these "hotspot" areas of diversification. Fragmentation of these two areas may prevent replenishment of progenitor taxa into these "hotspot" areas. This may lead to loss of genetic variation or, in a worse case scenario, eventual extinction of progenitor populations from these areas.

To the three main components of the processes of diversification, as suggested by Moritz (2002), I wish to add a fourth. Taxonomic diversification from newly formed genetic variation may be greatest in areas of high ecological habitat diversity. Loss of this ecological diversity may therefore reduce the overall diversity generated in a particular group. Questions of whether or not taxa formed within these areas of ecological diversity should also be given conservation importance is controversial. These taxa, often endemic and hence having high conservation status within many current species action plans, represent unique assemblages of morphological and genetic variation within a particular group. Their relevance within an evolutionary process is not governed by their degree of uniqueness or distinctiveness, but by whether or not they contribute to the generation of future diversity within the group. Theoretically, if they represent ultimate end products of diversification then they should be afforded little conservation importance. If, however, they are shown not to be end products but part of the overall continuing process, they are of importance and hence warrant some of the conservation status afforded to that group as a whole. It may also be argued that the taxa produced by the diversification process, often by occurring in "hotspot" areas may indicate the presence of important ecological factors for the continuing diversification of that group. Loss of these taxa may therefore indicate loss of important underlying ecological habitats.

Conservation of endemic taxa will still remain controversial in taxonomically complex groups and the importance of these taxa will need to be determined on a case to case basis. It may be prudent to divert resources to some of these taxa, even if

they have no proven role in the generation of future diversity within their group, for reasons other than scientific. This may be especially the case where rates of diversification are low and so generation of new endemics infrequent.

To recap, production of process action plans should be targeted to the unique processes important in the generation of diversity within each particular group. In doing this, overall general points may be considered. These are

1. Importance of the populations of the progenitor species.
2. Importance of the "hotspot" areas of diversification.
3. Importance in assuring connection between the progenitor populations and "hotspot" areas.
4. Importance of ecological habitat diversity.
5. Consideration of the importance of current end products of diversification.

Many of these general points relate to the importance of conserving habitat diversity. Conservation of evolutionary processes may therefore be incorporated into, and so strengthen current habitat action plans.

An example of an actively evolving flora may be found in the North Atlantic region. Here a high number of endemic species have arisen since the end of the last glacial period (Brochmann *et al.*, submitted). Within this flora *Euphrasia* represents a particularly actively evolving plant group (Yeo, 1978a). Diversification within this genus is especially high in Britain, resulting in a particularly high number of endemic and geographically restricted taxa (Preston *et al.*, 2002). This diversification has been proposed to be due to the combination of changes in chromosome numbers, multiple transitions of the breeding system towards selfing, and adaptation to ecologically diverse habitats (Chapter 8). Conservation of the unique diversity within this genus may therefore be a good example of where process-, and not species-based conservation, is more appropriate.

9.3. Process action plan for British *Euphrasia*

9.3.1. Proposed processes involved in generating diversity

Taxonomic diversity within British *Euphrasia* occurs across two ploidy groups. However it is significantly greater within the tetraploid group than the diploid group. Molecular work, using chloroplast PCR-RFLP, co-dominant microsatellite loci and dominant AFLP band distribution, supports previous hypotheses regarding the diversification of *Euphrasia* in Britain (Yeo, 1968; Yeo, 1976; Yeo, 1978b), as well as highlighting the importance that a dynamic mixed mating system may play in this diversification. Under these hypotheses, initial diversity within Europe, contained within at least three differing diploid groups (Yeo, 1978b; Ehrendorfer and Vitek, 1984), has increased by multiple hybridisation events between these groups, with the formation of a number of distinct allotetraploid haplotype-taxonomic groups. Within these groups, the combination of differing diploid genomes, the doubling of chromosome numbers and the variable extent of chromosomal pairing at meiosis, has led to increasing diversification. This diversification has been further increased by repeated development of increasing autogamy, shifting the breeding system away from random sexual mating. This has allowed the fixation of favourable genotypes within selfing lines, giving rise to a greater ability to exploit a wider range of ecological habitat. In addition, further hybridisation within the tetraploid group and between the diploid and tetraploid haplotype-taxonomic groups has generated additional diversity (Chapter 8).

These processes have occurred on the background of high ecological diversity found within Britain (Chapter 7). This ecological diversity, presumably resulting from the combined effects of oceanic climate and extensive human disturbance and management (Preston and Hill, 1997), has allowed a greater adaptive diversification in Britain, compared to other European countries. This is demonstrated by the high taxonomic diversity seen in Britain today, produced by the repeated formation of endemic *Euphrasia* taxa (Yeo, 1978a).

The current combined species action plan for *Euphrasia* incorporates six endemic species. Unique *Euphrasia* diversity is though not solely confined to these, with equally important taxa not currently having species status or populations not easily assignable to nameable entities. Conservation strategies need to incorporate genetic variation found within these additional unique *Euphrasia* taxa and populations. Given the intractable difficulties involved in applying a species-based approach to this genus, this will be best achieved by using an alternative process-based approach.

In applying a process conservation approach to *Euphrasia*, the general points already considered to be important to the conservation of taxonomically complex groups as a whole should be considered. These are the importance of progenitor taxa, "hotspot" areas of diversification, connection of the progenitor taxa to these "hotspot" areas, ecological habitat diversity and the current end products of diversification. Changing from a species- to process-based approach will result in a shift in the prioritisation and resourcing within the group, increasing the emphasis on the importance of common species and habitat diversity. In doing this, there is a danger that this will lead to increasing resource allocation to the genus, to the detriment of other plant groups. In *Euphrasia*, the common progenitor species tend to occupy habitats that are in decline and are of national importance. These may therefore already be recognised under current habitat action plans and so the conservation of a significant part of *Euphrasia* diversity may fall under a number of habitat action plans already in place. Converting from a species- to process-based conservation approach in this genus may therefore not require the diversion of a large amount of additional resources.

9.3.2. Progenitor populations

The important progenitor groups are;

1. diploid *Euphrasia officinalis* subsp. *rostkoviana*, *Euphrasia officinalis* subsp. *monticola* and hybrids between them.
2. tetraploid *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa* and hybrids between them.
3. tetraploid *Euphrasia scottica*, *Euphrasia micrantha*, *Euphrasia frigida* and hybrids between them.

These groups consist of common species that occur in more than 15 10km squares in Britain and thus are not classified as rare or endangered (Wigginton, 1999). The two diploid subspecies of *Euphrasia officinalis*, subsp. *rostkoviana* and subsp. *monticola* are scarce, occupying less than 100 10km squares (Stewart *et al.*, 1994). However when compared with the average change in the overall British flora in the last 25 years, *Euphrasia* species per se have declined (Preston *et al.*, 2002). Difficulties arising from identification of these common species precludes a detailed survey of their individual distributions (Chris Preston pers. comm.), but this overall decline probably reflects a general decline in the common species of British *Euphrasia* and so represent significant threat to future development of diversity within the group as a whole.

Species within the first two groups, (*Euphrasia officinalis* subsp. *rostkoviana* and *Euphrasia officinalis* subsp. *monticola*, and *Euphrasia arctica* subsp. *borealis* and *Euphrasia nemorosa*), are facing marked decline due to extensive loss of their unimproved grassland habitat. This has especially occurred over the last 30 years as a result of extensive agricultural intensification (Fuller, 1987; Chamberlain *et al.*, 2000). Species within the third group, (*Euphrasia scottica*, *Euphrasia micrantha* and *Euphrasia frigida*), may not be declining as rapidly as the species of unimproved grassland. The lowland heathland habitat of *Euphrasia micrantha*, though, has shown a marked decline over recent years (Haskins, 2000). Increasing urbanisation and changing habitat management, has occurred to such an extent in south-east England that in this geographical region *Euphrasia micrantha* may now be almost extinct (Preston *et al.*, 2002).

With the decline in common *Euphrasia* species predominantly resulting from marked loss of key unimproved grassland and heathland habitats, their conservation is one example where possible incorporation in current habitat action plans may be considered. These include lowland and upland hay meadows, calcareous grassland and lowland and upland heathland (Anon, 1995b).

9.3.3. "Hotspot" areas of diversification

Diversification of British *Euphrasia* occurs predominantly in the west and northern oceanic regions, with all six action plan species restricted to this geographical area (Wigginton, 1999; Preston *et al.*, 2002). *Euphrasia* diversity is not uniformly distributed within this broad geographical area, with "hotspot" areas leading to the formation of a greater number of endemic species than may be found in other areas. These "hotspot" areas include inland and coastal heathland habitats of south-west England, montane habitats of north Wales and north-west England and coastal habitats of western and northern Scotland. Many of the known "hotspot" areas within these geographical regions are covered in existing legislation, but further work is required to identify additional important "hotspots", adding them to the legal protective framework if necessary.

9.3.4. Connecting progenitor populations with "hotspot" areas

In determining the importance of conserving particular populations of common progenitor taxa, the overall metapopulation dynamics of each taxon should be considered with increasing importance being given to populations allowing greater connectiveness with "hotspot" areas of diversity. Within populations, seed dispersal is generally low (Molau, 1993) but may be markedly higher between populations as a result of transport of hay (Karlsson, 1984). This anthropogenic seed movement may be important to ensure increasing connectiveness of some populations within a metapopulation and so form an important contribution to the overall process based action plan for the group.

9.3.5. Ecological habitat diversity

The combined actions of oceanic climate, human disturbance and land management have probably led to a high number of differing grassland and heathland habitats in Britain. Many of these habitats may now be under threat from agricultural intensification, changing grazing pressures and loss of traditional management practices. The continuing generation of diversity within *Euphrasia* requires the maintenance of a mosaic of grassland and heathland habitats for new evolving taxa to adapt and survive in. The conservation of these mosaics of habitats may be particularly important in areas of close proximity to the "hotspot" areas of

diversification. This is another example where conservation of *Euphrasia* diversity may be incorporated into existing habitat action plans. These may include inland and coastal heathland of south-west England, montane habitats of north Wales and north-west England, and maritime cliff and slope, machair and saltmarsh habitats of western and northern Scotland (Anon, 1995b).

9.3.6. Current end products of diversification

The role of currently defined endemic taxa, as well as other rare unique, often unnamed populations, in the overall process generating diversity within *Euphrasia* is currently unknown. Many of these taxa are small flowered species and so presumably highly selfing (Chapter 3). They may therefore represent end products within the overall process, where their current conservation status would be unwarranted. This conclusion though is far from certain, with work still required to be performed before their importance in the overall process can be reliably assessed. Given additional factors, of unknown turnover rate (this may be relatively slow) and their possible role as indicators of ecological habitat diversity, conservation recommendations should presently include incorporating these endemic taxa within the overall *Euphrasia* action plan. This recommendation may change with increasing understanding of their role in generating future diversity in British *Euphrasia*.

9.4. Summary

Given the relatively depauperate flora of the British Isles, biodiversity contained within taxonomically complex groups represents an important overall part of the total biodiversity found within this country. Conservation strategies as laid down in UK action plans should reflect this. In these actively evolving groups this requires a change from a species- to a process-based approach to conservation. The focus of conservation in this genus should incorporate the maintenance of the commoner progenitor species, whilst also preserving the rare endemic species until their role in the overall process is clarified. In British *Euphrasia* many of these common species occur in declining internationally important habitats and so strategies for their conservation may be incorporated in and strengthen existing key habitat action plans.

The use of molecular markers can be instrumental in understanding the processes involved in generating diversity within a complex taxonomic group and in leading to the production of more appropriate action plans. This has been demonstrated in this thesis by the examination of the critical genus *Euphrasia* in Britain. As a practical outcome of this molecular study, an example of an appropriate process-based action plan for British *Euphrasia* is proposed (Appendix III). This conservation strategy is the outcome of an increased understanding of the processes generating diversity within this taxonomically complex group. It has been achieved by synthesising previous knowledge provided by cultivation, fertilisation, ecological and taxonomic work, with that acquired by the use of contemporary molecular techniques.

References

Adams R.P. and Demeke T. (1993). Systematic relationships in *Juniperus* based on random amplified polymorphic DNAs (RAPDs). *Taxon* 42: 553-571.

Allard R.W., Jain S.K. and Workman P.L. (1969). Genetics of inbreeding population. *Advances in Genetics* 14: 55-131.

Amos W. and Balmford A. (2001). When does conservation genetics matter? *Heredity* 87: 257-265.

Anon. (1995a). *Biodiversity: the UK steering group report. Volume 1. Meeting the Rio challenge.* London. HMSO.

Anon. (1995b). *Biodiversity: the UK steering group report. Volume 2. Action Plans.* London. HMSO.

Armbruster S.W., Mulder C.P.H., Baldwin B.G., Kalisz S., Wessa B. and Nute H. (2002). Comparative analysis of late floral development and mating-system evolution in Tribe *Collinsieae* (Scrophulariaceae). *American Journal of Botany* 89: 37-49.

Armstrong J.V. and Sell P.D. (1996). A revision of British elms (*Ulmus* L., Ulmaceae): the historical background. *Botanical Journal of the Linnean Society* 120: 39-50.

Ashton P.A. and Abbott R.J. (1992). Multiple origins and genetic diversity in the newly arisen allopolyploid species, *Senecio cambrensis* Rosser (Compositae). *Heredity* 68: 25-32.

Bachmann K. (1994). Molecular markers in plant ecology. *New Phytologist* 126: 403-418.

Ball J. (1873). Descriptions of some new species, subspecies, and varieties of plants collected in Morocco by J.D.Hooker, G.Maw and J.Ball. *Journal of Botany* 11: 267-273.

Barker W.R. (1982). Taxonomic studies in *Euphrasia* L. A revised infrageneric classification, and a revision of the genus in Australia. *Journal of the Adelaide Botanic Gardens* 5: 1-304.

Barrett S.C.H., Harder L.D. and Worley A.C. (1996). The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society of London Series B.* 351: 1271-1280.

Bateman R.M. and Denholm I. (1983). A reappraisal of the British and Irish dactylorchids, 1. The tetraploid marsh-orchids. *Watsonia* 14: 347-376.

Bateman R.M. and Denholm I. (1985). A reappraisal of the British and Irish dactylorchids, 2. The diploid marsh-orchids. *Watsonia* 15: 321-355.

Bateman R.M. and Denholm I. (1988). A reappraisal of the British and Irish dactylorchids, 3. The spotted orchids. *Watsonia* 17: 319-349.

Beasley E.M., Myers R.M., Cox D.R. and Lazzeroni L.C. (1999). Statistical refinement of primer design parameters. In: *PCR Applications.* pp. 55-71. Ed. by M.A.Innis. Academic Press, San Diego.

Bobear J.B. (1969). An analysis of populations of Irish *Euphrasia* L. *Watsonia* 7: 168-190.

Brochmann C. and Elven R. (1992). Ecological and genetic consequences of polyploidy in arctic *Draba* (Brassicaceae). *Evolutionary Trends in Plants* 6: 111-124.

Brochmann C., Gabrielsen T.M., Nordal I., Landvik J.Y. and Elven R. (2003). Glacial survival or *tabula rasa*? The history of North Atlantic biota revisited. *Taxon* 52: 417-450.

Brochmann C., Soltis P.S. and Soltis D.E. (1992a). Recurrent formation and polyphyly of Nordic polyploids in *Draba* (Brassicaceae). *American Journal of Botany* 79: 673-688.

Brochmann C., Stedje B. and Borgen L. (1992b). Gene flow across ploidal levels in *Draba* (Brassicaceae). *Evolutionary Trends in Plants* 6: 125-134.

Brown A.H.D. (1979). Enzyme polymorphism in plant populations. *Theoretical and Applied Genetics* 15: 1-42.

Brown A.H.D. (1989). Genetic characterization of plant mating systems. In: *Plant population genetics, breeding, and genetic resources.* pp. 145-162. Ed. by A.H.D.Brown, M.T.Clegg, A.L.Kahler and B.S.Weir. Sinauer Associates Inv. Massachusetts.

Brummit R.K. (1992). Proposal concerning the names of certain apomictic species aggregates. *Taxon* 41: 596.

Bucknall C. (1917). British Euphrasiae. *Journal of Botany* 55: 1-29.

Buerkle C.A. and Rieseberg L.H. (2001). Low intraspecific variation for genomic isolation between hybridizing sunflower species. *Evolution* 55: 884-691.

Burke T.E., Rainey W.E. and White T.J. (1992). Molecular variation and ecological problems. In: *Genes in Ecology.* pp. 229-254. Ed. by R.J.Berry, T.J.Crawford and G.M.Hewitt. Blackwell Scientific. Oxford.

Casgrain P. and Legendre P. (2001). The R Package for multivariate and spatial analysis, version 4.0. Université de Montréal. Available from: http://www.fas.umontreal.ca/biol/casgrain/en/labo/R/v4/index.html.

Chabert A. (1902). Les *Euphrasia* de la France. *Bulletin de l'Herbier Boissier.* 2ème série 121-152.

Chamberlain D.E., Fuller R.J., Bunce R.G.H., Duckworth J.C. and Shrubb M. (2000). Changes in the abundance of farmland birds in relation to the timing of agricultural intensification in England and Wales. *Journal of Applied Ecology* 37: 771-788.

Charlesworth D. and Mayer S. (1995). Genetic variability of plant characters in the partial inbreeder *Collinsia heterophylla* (Scrophulariaceae). *American Journal of Botany* 82: 112-120.

Chiang T.-Y., Schaal B.A. and Peng C.-I. (1998). Universal primers for amplification and sequencing a noncoding spacer between the *atpB* and *rbc*L genes of chloroplast DNA. *Botanical Bulletin Academia Sinica* 39: 245-250.

Cole C.T. and Kuchenreuter M.A. (2001). Molecular markers reveal little genetic differentiation among *Aconitum noveboracense* and *A. columianum* (Ranunculaceae) populations. *American Journal of Botany* 88: 337-347.

Coleman M., Hollingsworth M.L. and Hollingsworth P.M. (2000). Application of RAPD to the critical taxonomy of the English endemic elm *Ulmus plotii* Druce. *Botanical Journal of the Linnean Society* 133: 241-262.

Cook L.M., Soltis P.M., Brunsfeld S.J., and Soltis D.E. (1998). Multiple independent formations of *Tragopogon* tetraploids (Asteraceae): evidence from RAPD markers. *Molecular Ecology* 7: 1293-1302.

Cowling R.M. and Pressey R.L. (2001). Rapid plant diversification: planning for an evolutionary future. *Proceedings of the National Academy of Sciences of the United States of America* 98: 5452-5457.

Cruden R.W. (1977). Pollen-ovule ratios. A conservative indicator of breeding systems in flowering plants. *Evolution* 31: 32-46.

Darwin C. (1876). *The effects of cross and self fertilisation in the vegetable kingdom.* Murray. London.

Davey F.H. (1907). *Euphrasia vigursii,* sp.n. *Journal of Botany* 45: 217-220.

de Queiroz K. and Donoghue M.J. (1988). Phylogenetic systematics and the species problem. *Cladistics* 4: 317-338.

Demesure B., Sodzi N. and Petit R.J. (1995). A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology* 4: 129-131.

Dole J.A. (1992). Reproductive assurance methods in three taxa of the *Mimulus guttatus* complex (Scrophulariaceae). *American Journal of Botany* 79: 650-659.

Downie S.R. and McNeill J. (1988). Description and distribution of *Euphrasia stricta* in North-America. *Rhodora* 90: 223-231.

Downie S.R., Quintin A. and McNeill J. (1988). Le statut des *Euphrasia borealis*, *E. nemorosa* et *E. stricta* dans l'est de l'Amérigue du Nord: une analyse numérique. *Canadian Journal of Botany* 66: 2208-2216.

Doyle J.J. and Doyle J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemical Bulletin* 19: 11-15.

Drabble E. (1916). *Euphrasia nemorosa* and *E. curta*. *Journal of Botany* 54: 73-75.

Druce G.C. (1930). Plant notes for 1930. *Report for the Botanical Society & Exchange Club of the British Isles* 9: 258-287.

Druce G.C. and Lumb D. (1921). Plant notes, etc., etc., for 1921. *Report for the Botanical Society & Exchange Club of the British Isles* 6: 272-327.

Druce G.C. and Lumb D. (1923). Plant notes, etc., etc., for 1923. *Report for the Botanical Society & Exchange Club of the British Isles* 7: 24-76.

Dudman A.A. and Richards A.J. (1997). *Dandelions of Great Britain and Ireland.* Botanical Society of the British Isles. London.

Dumolin-Lapegue S., Pemonge M.-H. and Petit R.J. (1997). An enlarged set of consensus primers for the study of organelle DNA in plants. *Molecular Ecology* 6: 393-397.

Dzwonko Z. (2001). Assessment of light and soil conditions in ancient and recent woodlands by Ellenberg indicator values. *Journal of Applied Ecology* 38: 942-951.

Eddees E.S. and Newton A. (1988). *Brambles of the British Isles.* The Ray Society. London.

Edwards K.J., Barker J.H.A., Daly A., Jones C. and Karp A. (1996). Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques* 20: 758-760.

Ehrendorfer F. and Vitek E. (1984). Evolution in alpine populations of *Euphrasia* (Scrophulariaceae) - Discovery of small-flowered diploid taxa. *Plant Systematics and Evolution* 144: 25-44.

Ellenberg H. (1979). Zeigerwerte von Gefässpflanzen Mitteleuropas. *Scripta Geobotanica* 18: 1-248.

Ellstrand N.C. and Elam D.R. (1993). Population genetic consequences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics* 24: 217-242.

Ennos R.A., Sinclair W.T., Hu X.-S. and Langdon A. (1999). Using organelle markers to elucidate the history, ecology and evolution of plant populations. In: *Molecular Systematics and Plant Evolution.* pp. 1-19. Ed. by P.M.Hollingsworth, R.M.Bateman and R.J.Gornall. Taylor & Francis. London.

Ereshefsky M. (2002). Linnean Ranks: Vestiges of a bygone era. *Philosophy of Science* 69: S305-S315.

Excoffier L., Smouse P.E. and Quattro J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.

Fjeldså J. and Rahbek C. (1998). Continent-wide conservation priorities and diversification processes. In: *Conservation in a changing world.* pp. 139-160. Ed. by G.M.Mace, A.Balmford and J.R.Ginsberg. Cambridge University Press. Cambridge.

Fryer J. and Hylmö B. (1994). The native British *Cotoneaster* - Great orme berry - renamed. *Watsonia* 20: 61-63.

Fuller R.M. (1987). The changing extent and conservation interest of lowland grasslands in England and Wales: A review of grassland surveys 1930-1984. *Biological conservation* 40: 218-300.

Gómez J.M. (2002). Self-pollination in *Euphrasia willkommii* Freyn (Scrophulariaceae), an endemic species from the alpine of the Sierra Nevada (Spain). *Plant Systematics and Evolution* 232: 63-71.

Goudet J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices, version 2.9.3. Université de Lausanne. Available from: http:// www.unil.ch/izea/softwares /fstat.html.

Gower J.C. (1966). Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* 53: 325-338.

Green J., Bullen S. and Martins F. (2002). *Excel 2000 VBA Programmer's Reference.* Wrox Press Ltd. USA.

Greilhuber J., Vitek E. and Ehrendorfer F. (1984). Evolution in alpine populations of *Euphrasia* (Scrophulariaceae): Chromosome counts on diploid and polyploid taxa from the E.Alps. *Plant Systematics and Evolution* 144: 45-51.

Greuter W., McNeill J. and Barrie F.R. (1994). Report on botanical nomenclature - Yokahama 1993. *Englera* 14: 198-205.

Greuter W., McNeill J., Hawksworth D.L. and Barrie F.R. (2000). Report on botanical nomenclature – Saint Louis 1999. *Englera* 20: 200-205.

Hadrys H., Balick M. and Schierwater B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology* 1: 55-63.

Hamilton M.B. (1998). Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* 8: 513-525.

Hampton H. and Kay Q.O.N. (1995). *Sorbus domestica* L. new to Wales and the British Isles. *Watsonia* 20: 379-384.

Hamrick J.L. and Godt M.J.W. (1996). Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London Series B* 351: 1291-1298.

Harris S.A. and Abbott R.J. (1997). Isozyme analysis of the reported origin of a new hybrid orchid species, *Epipactis youngiana* (Young's helleborine), in the British Isles. *Heredity* 79: 402-407.

Hartl D. (1975). *Euphrasia.* In: *Illustrierte Flora Von Mitteleuropa* 6. pp. 83-100. Ed. by G.Hegi. Parey. Hamburg.

Haskins L. (2000). Heathlands in an urban setting – effects of urban development on heathlands of south-east Dorset. *British Wildlife* 11: 229-237.

Hedrén M. (1996). Genetic differentiation, polyploidisation and hybridisation in northern European *Dactylorhiza* (Orchidaceae): evidence from allozyme markers. *Plant Systematics and Evolution* 201: 31-55.

Hedrén M. (2002). Patterns of allozyme and morphological differentiation in the *Carex flava* complex (Cyperaceae) in Fennoscandia. *Nordic Journal of Botany* 22: 257-301.

Hedrén M., Fay M.F. and Chase M.W. (2001). Amplified fragment length polymorphisms (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). *American Journal of Botany* 88: 1868-1880.

Hewitt G. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* 58: 247-276.

Hiern W.P. (1909). *Euphrasia minima. Journal of Botany* 47: 165-172.

Hill M.O. and Carey P.D. (1997). Prediction of yield in the Rothamsted Park grass experiment by Ellenberg indicator values. *Journal of Vegetation Science* 8: 579-586.

Hill M.O., Mountford J.O., Roy D.B. and Bunce R.G.H. (1999). *Ellenberg's Indicator Values for British Plants.* Institiute of Terrestrial Ecology. Huntington.

Hill M.O., Roy D.B., Mountford J.O., and Bunce R.G.H. (2000). Extending Ellenberg's indicator values to a new area: an algorithmic approach. *Journal of Applied Ecology* 37: 3-15.

Hollingsworth P.M. (2001). British Orchids: taxonomic complexity, molecular markers and conservation. *NERC News* Spring 2001: 14-15.

Hollingsworth P.M. (2003). Taxonomic complexity, population genetics and plant conservation in Scotland. *Botanical Journal of Scotland* 55: 55-63.

Hollingsworth P.M., Hollingsworth M.L. and Coleman M. (2000). The European Elms: molecular markers, population genetics and biosystematics. In: *The Elms: Breeding, conservation, and disease management.* pp. 1-20. Ed. by C.P.Dunn. Kluwer Academic Publishers. Boston.

Huff D.R., Peakell R. and Smouse P.E. (1993). RAPD variation within and among natural populations of outcrossing buffalograss [*Buchlöe dactyloides* (Nutt.) Engelm]. *Theoretical and Applied Genetics* 86: 927-934.

Hughes M., Hollingsworth P.M. and Squirrell J. (2002). Isolation of polymorphic microsatellite markers for *Begonia sutherlandii* Hook. f. *Molecular Ecology Notes* 2: 185-186.

Hultén E. (1975). The total range of *Euphrasia. Botaniska Notiser* 128: 357-364.

Ingrouille M.J. and Stace C.A. (1986). The *Limonium binervosum* aggregate (Plumbaginaceae) in the British Isles. *Botanical Journal of the Linnean Society* 92: 177-217.

Jaccard P. (1912). The distribution of the flora of the alpine zone. *New Phytologist* 11: 37-50.

Jain S.K. (1976). The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics* 7: 469-495.

Jalas J. and Kukkonen I. (1973). Typification of the taxa of *Euphrasia* (Scrophulariaceae) described by Finnish botanists. *Annales Botanici Fennici* 10: 27-42.

Jarne P. and Charlesworth D. (1993). The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annual Review of Ecology and Systematics* 24: 441-466.

Jarne P. and Lagoda P.J.L. (1996). Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* 11: 424-429.

Jermy A.C., Chater A.O. and David R.W. (1982). *Sedges of the British Isles.* Botanical Society of the British Isles. London.

Jørgensen E. (1919). Die *Euphrasia*-Arten Normegens. *Bergens Museums Aarbok 1916-1917, Naturvidenskabelig Raekke* 5: 1-337.

Karlsson T. (1974). Recurrent ecotypic variation in Rhinantheae and Gentianaceae in relation to hemiparasitism and mycotrophy. *Botaniska Notiser* 127: 527-539.

Karlsson T. (1976). *Euphrasia* in Sweden: hybridization, parallelism and species concept. *Botanisk Notiser* 129: 49-60.

Karlsson T. (1982). *Euphrasia rostkoviana* i Sverige. *Vaxtekologiska Studier* 15: 1-116.

Karlsson T. (1984). Early-flowering taxa of *Euphrasia* (Scrophulariaceae) on Gotland, Sweden. *Nordic Journal of Botany* 4: 303-326.

Karlsson T. (1986). The evolutionary situation of *Euphrasia* in Sweden. *Symbolae Botanicae Upsalienses* 27: 61-71.

Karp A., Seberg O. and Buiatti M. (1996). Molecular techniques in the assessment of botanical diversity. *Annals of Botany* 78: 143-149.

Knuth P. (1909). *Handbook of Flower Pollination 3.* Translated by J.R.Ainsworth Davis. Clarendon Press. Oxford.

Koch M., Hurka H. and Mummenhoff K. (1996). Chloroplast DNA restriction site variation and RAPD-analysis in *Cochlearia* (Brassicaceae): Biosystematics and speciation. *Nordic Journal of Botany* 16: 585-603.

Kraft T., Nybom H. and Werlemark G. (1996). DNA fingerprint variation in some blackberry species (*Rubus* subg. *Rubus*, Rosaceae). *Plant Systematics and Evolution* 199: 93-108.

Lammi A., Siikamäki P. and Salonen V. (1999). The role of local adaptation in the relationship between an endangered root hemiparasite *Euphrasia rostkoviana*, and its host *Agrostis capillaris*. *Ecography* 22: 145-152.

Legg C.J. (unpublished). ComKey V. 0.22 – Community Identification. Institute of Ecology and Resource Management. University of Edinburgh.

Leitch I.J. and Bennett M.D. (1997). Polyploidy in angiosperms. *Trends in Plant Science Reviews* 2: 1360-1385.

Lendvai G. and Levin D.A. (2003). Rapid response to artificial selection on flower size in *Phlox*. *Heredity* 90: 336-342.

Lewis P.O. and Zaykin D. (2001). Genetic Data Analysis: Computer program for the analysis of allelic data, version 1.0 (d16c). University of Connecticut. Available from: http://lewis.eeb.uconn.edu/lewishome/software.html.

Lord R.M. and Richards A.J. (1977). A hybrid swarm between the diploid *Dactylorhiza fuchsii* (Druce) Soó and the tetraploid *D. purpurella* (T. & T.A. Steph.) Soó in Durham. *Watsonia* 11: 205-210.

Lumart R. and Barrientos E. (1990). Phylogenetic relationships and gene flow between sympatric diploid and tetraploid plants of *Dactylis glomerata* (Gramineae). *Plant Systematics and Evolution* 169: 81-96.

Lynch M. and Milligan B.G. (1994). Analysis of population genetic-structure with RAPD markers. *Molecular Ecology* 3 : 91-99.

Machon N., Lefranc M., Bilger I. and Henry J.-P. (1995). Isoenzymes as an aid to clarify taxonomy of French elms. *Heredity* 74: 39-47.

Masterson J. (1994). Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. *Science* 264: 421-424.

Matthies D. (1998). Influence of the host on growth and biomass allocation in the two facultative root hemiparasites *Odontites vulgaris* and *Euphrasia minima*. *Flora* 193: 187-193.

May B. (1998). Starch gel electrophoresis of allozymes. In: *Molecular genetic analysis of populations*: *A practical approach*. pp. 1-27. Ed. by A.R.Hoezel. Oxford University Press. Oxford.

Mayden R.L. (1997). A hierarchy of species concepts: The denouement in the saga of the species problem. In: *Species*: *The units of biodiversity*. pp. 381-424. Ed. by M.F.Claridge, H.A.Dawah and M.R.Wilson. Chapman and Hall. London.

Mayr E. (1940). Speciation phenomena in birds. *The American Naturalist* 74: 249-278.

McDade L.A. (1995). Species concepts and problems in practice: insight from botanical monographs. *Systematic Botany* 20: 606-622.

McKitrick M.C. and Zink R.M. (1988). Species concepts on ornithology. *The Condor* 90: 1-14.

Melville R. (1975). *Ulmus*. In: *Hybridisation and the Flora of the British Isles*. pp 292-299. Ed. by C.A.Stace. Academic Press. London.

Milligan B.G., Leebens-Mack J. and Strand A.E. (1994). Conservation genetics: beyond the maintenance of marker diversity. *Molecular Ecology* 3: 423-435.

Mishler B.D. (1999). Getting rid of species?. In: *Species*: *New interdisciplinary essays*. pp. 307-315. Ed. by R.A.Wilson. MIT Press. Cambridge.

Mishler B.D. and Donoghue M.J. (1982). Species concepts: A case for pluralism. *Systematic Zoology* 31: 491-503.

Molau U. (1993). Phenology and reproductive ecology in six subalpine species of Rhinanthoideae (Scrophulariaceae). *Opera Botanica* 121: 7-17.

Moritz C. (2002). Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic Biology* 51: 238-254.

Muller U.G. and Wolfenbarger L.L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* 14: 389-394.

Oliver C. (1999). Conservation genetics of *Euphrasia*. MSc thesis. University of Edinburgh and Royal Botanic Garden Edinburgh.

Palacios C. and González-Candelas F. (1997a). Analysis of population genetic structure and variability using RAPD markers in the endemic and endangered *Limonium dufourii* (Plumbaginaceae). *Molecular Ecology* 6: 1107-1121.

Palacios C. and González-Candelas F. (1997b). Lack of genetic variablity in the rare and endangered *Limonium cavanillesii* (Plumbaginaceae). *Molecular Ecology* 6: 671-675.

Palacios C. and González-Candelas F. (1999). AFLP analysis of the critically endangered *Limonium cavanillesii* (Plumbaginaceae). *Journal of Heredity* 90: 485-495.

Palacios C., Kresovich S. and González-Candelas F. (1999). A population genetic study of the endangered plant species *Limonium dufourii* (Plumbaginaceae) based on amplified fragment length polymorphism (AFLP). *Molecular Ecology* 8: 645-657.

Palacios C., Rosselló J.A. and González-Candelas F. (2000). Study of the evolutionary relationships among *Limonium* species (Plumbaginaceae) using nuclear and cytoplasmic molecular markers. *Molecular Phylogenetics and Evolution* 14: 232-249.

Palop M., Palacios C. and González-Candelas F. (2000). Development and across-species transferability of microsatellite markers in the genus *Limonium* (Plumbaginaceae). *Conservation Genetics* 1: 177-179.

Palop-Esteban M. and González-Candelas F. (2002). Development of microsatellite markers for the critically endangered *Limonium dufourii* (Girard) Kuntze (Plumbaginaceae). *Molecular Primer Notes* 2: 521-523.

Paran I., Gidioni D. and Jacobsohn R. (1997). Variation between and within broomrape (*Orobanche*) species revealed by RAPD markers. *Heredity* 78: 86-74.

Park S.D.E. (2001). Microsatellite toolkit, version. 3.1. University of Dublin. Available from: http://acer.gen.tcd.ie /~sdepark/ms-toolkit/.

Persson S. (1981). Ecological indicator values as an aid in the interpretation of ordination diagrams. *Journal of Ecology* 69: 71-84.

Pons O. and Petit R.J. (1995). Estimation, variance and optimal sampling of gene diversity I. Haploid locus. *Theoretical and Applied Genetics* 90: 462-470.

Preston C.D. and Hill M.O. (1997). The geographical relationships of British and Irish vascular plants. *Botanical Journal of the Linnean Society* 124: 1-120.

Preston C.D., Pearman D.A. and Dines T.D. (2002). *New Atlas of the British and Irish Flora.* Oxford University Press. Oxford.

Pritchard N.M. (1959). *Gentianella* in Britain I. *G.amarella, G.anglica* and *G.uliginosa. Watsonia* 4: 169-193.

Pritchard N.M. (1960a). *Gentianella* in Britain II. *Gentianella septrentrionalis* (Druce) E.F.Warb. *Watsonia* 4: 218-237.

Pritchard N.M. (1960b). *Gentianella* in Britain III. *Gentianella germanica* (Willd.) Böner. *Watsonia* 4: 290-303.

Proctor M.C.F., Proctor M.E. and Groenhof A.C. (1989). Evidence from peroxidase polymorphism on the taxonomy and reproduction of some *Sorbus* populations in south-west England. *New Phytologist* 112: 569-575.

Pugsley H.W. (1919). Notes on the British Euphrasias – I. *Journal of Botany* 57: 169-175.

Pugsley H.W. (1922). Notes on the British Euphrasias – II. *Journal of Botany* 60: 1-5.

Pugsley H.W. (1929). New British Species of *Euphrasia. Journal of Botany* 67: 224-225.

Pugsley H.W. (1930). A revision of the British Euphrasiae. *Journal of the Linnean Society of London, Botany* 48: 467-544.

Pugsley H.W. (1933). The Euphrasias of Iceland and the Faeroes. *Journal of Botany* 71: 303-309.

Pugsley H.W. (1936). Notes on the British Euphrasias – IV. *Journal of Botany* 74: 71-75.

Pugsley H.W. (1940). Notes on the British Euphrasias – VI. *Journal of Botany* 78: 11-13.

Pugsley H.W. (1945). The Eyebrights of Rhum. *The Naturalist* April/June 41-44.

Raybould A.F., Gray A.J., Lawrence M.J. and Marshall D.F. (1991). The evolution of *Spartina anglica* C.E.Hubbard (Gramineae): origin and genetic variability. *Biological Journal of the Linnean Society* 43: 111-126.

Reiseberg L.H., Van Fossen C. and Desrochers A. (1995). Hybrid speciation accompanied by genomic reorganization in wild sunflowers. *Nature* 375: 313-316.

Rice W.R. (1989). Analyzing tables of statistical tests. *Evolution* 43: 223-225.

Rich T.C.G. (1991). *Crucifers of Great Britain and Ireland.* Botanical Society of the British Isles. London.

Rich T.C.G. and Dalby D.H. (1996). The status and distribution of mountain scurvygrass (*Cochlearia micacea* Marshall) in Scotland, with ecological notes. *Botanical Journal of Scotland* 48: 187-198.

Richards A.J. (1986). *Plant Breeding Systems.* Allen and Unwin. London.

Richards A.J. (1997). *Epipactis.* In: *Plant Crib.* pp. 378-379. Ed. by. T.C.G. Rich and A.C.Jermy. Botanical Society of the British Isles. London.

Richards A.J. and Porter A.F. (1982). On the identity of a Northumberland *Epipactis. Watsonia* 14: 121-128.

Richens R.H. (1968). The correct designation of the European Field Elm. *Feddes Repertorium Speciorum Novarum Regni Vegetablis* 79: 1-2.

Ritland K. (1990). A series of FORTRAN computer programs for estimating plant mating systems. *Journal of Heredity* 81: 235-237.

Robertson A., Newton A.C. and Ennos R.A. (submitted). Multiple hybrid origins, genetic diversity and population genetic structure of two endemic *Sorbus* taxa on Arran. *Molecular Ecology.*

Rodriguez S., Luisa Palop M., Palacios C. and González-Candelas F. (2003). Molecular and morphological differentiation in *Limonium dufourii* (Plumbaginaceae), an endangered Mediterranean plant. *Conservation Genetics* 4: 383-391.

Román B., Alfaro C., Torres A.M., Moreno M.T., Satovic Z., Pujadas A. and Rubiales D. (2003). Genetic relationships among *Orobanche* species as revealed by RAPD analysis. *Annals of Botany* 91: 637-642.

Rosen D.E. (1978). Vicariant patterns and historical explanation in biogeography. *Systematic Zoology* 27: 159-188.

Rozen S. and Skaletsky H.J. (2000). *Primer3* on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* pp. 365-386. Ed. by S.Krawetz and S.Misener. Humana Press. Totowa. (Code available from http://wwwgenome.wi.mit.edu/genome_software/other/ primer3.html).

Rumsey F.J. and Jury S.L. (1991). An account of *Orobanche* L. in Britain and Ireland. *Watsonia* 18: 257-295.

Saghai-Maroof M.A., Soliman K.M., Jorgensen R.A. and Allard R.W. (1984). Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America* 81: 8014-8019.

Sambrook J., Fritsch E.F. and Maniatis T. (1989). *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbour Laboratory Press. New York.

Schaffers A.P. and Sỳkora K.V. (2000). Reliability of Ellenberg indicator values for moisture and soil reaction: comparison with field measurements. *Journal of Vegetation Science* 11: 225-244.

Scheen A., Elve R. and Brochmann C. (2002). A molecular-morphological approach solves taxonomic controversy in arctic *Draba* (Brassicaceae). *Canadian Journal of Botany* 80: 59-71.

Schemske D.W. (2000). Understanding the origin of species. *Evolution* 54: 1069-1073.

Schneider S., Roessli D. and Excoffier L. (2000). *ARLEQUIN, version* 2.000: *A software for population genetics data analysis.* University of Geneva. Available from: http://lgb.unige.ch/arlequin/.

Schoen D.J., Morgan M.T. and Batallion T. (1996). How does self-pollination evolve? Inferences from floral ecology and molecular genetic variation. *Philosophical Transactions of the Royal Society of London Series B* 351: 1281-1290.

Seel W.E. and Press M.C. (1993). Influence of the host on three sub-Arctic annual facualtive root hemiparasites. I. Growth, mineral accumulation and above-ground dry-matter partitioning. *New Phytologist* 125: 131-138.

Seel W.E. and Press M.C. (1994). Influence of the host on three sub-Arctic annual facualtive root hemiparasites. II. Gas exchange characteristics and resource use-efficiency. *New Phytologist* 127: 34-44.

Segraves K.A, Thompson J.N., Soltis P.S. and Soltis D.E. (1999). Multiple origins of polyploidy and the geographic structure of *Heuchera grossulariifolia. Molecular Ecology* 8: 253-262.

Sell P.D. and Yeo P.F. (1970). A revision of the North American species of *Euphrasia* L. (Scrophulariaceae). *Botanical Journal of the Linnean Society* 63: 189-234.

Sepp S., Bobrova V.K., Troitsky A.K. and Glazunova K.P. (2000). Genetic polymorphism detected with RAPD analysis and morphological variability in

some microspecies of apomictic *Alchemilla*. *Annales Botanici Fennica* 37: 105-123.

Shi Y., Gornall R.J., Draper J. and Stace C.A. (1996). Intraspecific molecular variation in *Hieracium* sect. *Alpina* (Asteraceae), an apomictic group. *Folia Geobotanica et Phytotaxonomica Bohemoslavaca* 31: 305-313.

Silverside A.J. (1990a). A guide to eyebrights (*Euphrasia*) - I. Introduction: *E. nemorosa*, *E. confusa* and *E. arctica*. *Wild Flower Magazine* 417: 30-34.

Silverside A.J. (1990b). A guide to eyebrights (*Euphrasia*) - II. The *E. rostkoviana* group. *Wild Flower Magazine* 418: 31-34.

Silverside A.J. (1991a). A guide to eyebrights (*Euphrasia*) - III. Species of restricted habitats. *Wild Flower Magazine* 419: 31-35.

Silverside A.J. (1991b). A guide to eyebrights (*Euphrasia*) - IV. On northern coasts and western hills. *Wild Flower Magazine* 420: 29-33.

Silverside A.J. (1991c). A guide to eyebrights (*Euphrasia*) - V. Local endemics and concluding remarks. *Wild Flower Magazine* 421: 32-36.

Silverside A.J. (1991d). The identity of *Euphrasia officinalis* L. and its nomenclature implications. *Watsonia* 18: 343-350.

Silverside A.J. (1998). *Euphrasia*. In: *Plant Crib*. pp. 269-272. Ed. by T.C.G.Rich and A.C.Jermy. Botanical Society of the British Isles. London.

Sledge W.A. (1975). Further comments on the supposed occurrence of *Euphrasia salisbergensis* in Yorkshire. *The Naturalist, Leeds* 934: 87-89.

Sneath T.F. (1976). Phenetic taxonomy at the species level and above. *Taxon* 25: 437-450.

Soltis D.E. and Soltis P.S. (1993). Molecular data and the dynamic nature of polyploidy. *Critical Reviews of Plant Sciences* 12: 243-273.

Soltis D.E. and Soltis P.S. (1995). The dynamic nature of polyploid genomes. *Proceedings of the National Academy of Sciences of the United States of America* 92: 8089-8091.

Soltis D.E. and Soltis P.S. (1999). Polyploidy: recurrent formation and genome evolution. *Trends in Ecology and Evolution* 14: 348-352.

Soltis P.S. and Soltis D.E. (2000). The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America* 97: 7051-7057.

Squirrell J. and Wolff K. (2001). Isolation of polymorphic microsatellite loci in *Plantago major* and *P. intermedia*. *Molecular Ecology Notes* 1: 179-181.

Squirrell J., Hollingsworth P.M., Bateman R.M., Tebbit M.C. and Hollingsworth M.L. (2002). Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex. *Molecular Ecology* 11: 1957-1964.

Squirrell J., Hollingsworth P.M., Woodhead M., Russell J., Lowe A., Gibby M. and Powell W. (2003). How much effort is required to isolate nuclear microsatellites from plants? *Molecular Ecology* 12: 1339-1348.

Stace C.A. (1975). *Hybridisation and the Flora of the British Isles.* Academic Press. London.

Stace C.A. (1978). Breeding systems, variation patterns and species delimitation. In: *Essays in Plant Taxonomy.* pp. 57-78. Ed. by H.E.Street. Academic Press. London.

Stace C.A. (1997). *New Flora of the British Isles.* Cambridge University Press. Cambridge.

Stace C.A., Gornall R.J. and Ying S. (1997). Cytological and molecular variation in apomictic *Hieracium* sect. *Alpina. Opera Botanica* 132: 39-51.

Stammers M., Harris J., Evans G.M., Hayward M.D. and Forster J.W. (1995). Use of random PCR (RAPD) technology to analyse phylogenetic relationships in the *Lolium/Festuca* complex. *Heredity* 74: 19-27.

Stebbins G.L. (1970). Adaptive radiation of reproductive characteristics in Angiosperms I: Pollination mechanisms. *Annual Review of Ecology and Systematics* 1: 307-326.

Stewart A., Pearman D.A. and Preston C.D. (1994). *Scarce Plants in Britain.* Joint Nature Conservation Committee. Peterborough.

Stewart O.M. (1997). *Calamagrostis.* In: *Plant Crib.* pp. 363-364. Ed by T.C.G.Rich and A.C.Jermy. Botanical Society of the British Isles. London.

Stuart Thompson H. (1919). The genus *Euphrasia* and *E. minima. Journal of Botany* 57: 335-337.

Taberlet P., Geilly L., Pautou G. and Bouvet J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105-1109.

Templeton A.T. (1989). The meaning of species and speciation: a genetic perspective. In: *Speciation and Its Consequences.* pp. 3-27. Ed. by D.Otte and J.A.Endler. Sinauer Associates. Massachusetts.

Thompson D.J. and Lumaret R. (1992). The evolutionary dynamics of polyploid plants: Origins, establishment and persistence. *Trends in Ecology and Evolution* 7: 302-306.

Townsend F. (1884). On *Euphrasia officinalis* L. *Journal of Botany* 22: 161-168.

Townsend F. (1897). Monograph of the genus *Euphrasia*. *Journal of Botany* 35: 321-336; 395-406; 417-426; 465-476.

Travis S.E., Maschinski J. and Keim P. (1996). An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. *Molecular Ecology* 5: 735-745.

Tutin T.G., Heywood V.H., Burges N.A., Moore D.M., Valentine D.H., Walter S.M. and Webb D.A. (1972). *Flora Europaea* 3. Cambridge University Press. Cambridge.

van Oostrum H., Sterk A.A. and Wijsman J.W. (1985). Genetic variation in agamospermous microspecies of *Taraxacum* sect. *Erythrosperma* and sect. *Obliqua*. *Heredity* 55: 223-228.

Vitek E. (1985). Evolution in alpine populations of *Euphrasia* (Scrophulariaceae): *E. alpina* and *E. christii*. *Plant Systematics and Evolution* 149: 1-18.

Vitek E. (1986). Evolution in Alpine Populations of *Euphrasia* (Scrophulariaceae): The tetraploid *E. minima*. *Plant Systematics and Evolution* 151: 241-269.

Vitek E. (1998). Are the taxonomic concepts of agamospermous genera useful for autogamous groups - a critical discussion using the example of *Euphrasia* (Scrophulariaceae). *Folia Geobotanica* 33: 349-352.

Vogler D.M. and Kalisz S. (2001). Sex among the flowers: the distribution of plant mating systems. *Evolution* 55: 202-204.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M. and Zabeau M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407-4414.

Walters S.M. (1949). *Alchemilla vulgaris* L. agg. *Watsonia* 1: 6-18.

Wamelink G.W.W., Joosten V., van Dobben H.F. and Berendse F. (2002). Validity of Ellenberg indicator values judged from physio-chemical field measurements. *Journal of Vegetation Science* 13: 269-278.

Warburg E.F. (1962). *Sorbus* L. In: *Flora of the British Isles*. pp. 423-437. Ed by A.R.Clapham, T.G.Tutin and E.F.Warburg. Cambridge University Press. Cambridge.

Waugh R., van de Ven W.T.G., Phillips M.S. and Powell W. (1990). Chloroplast DNA diversity in the genus *Rubus* (Roseaceae) revealed by southern hybridisation. *Plant Systematics and Evolution* 172: 65-75.

Weber H.E. (1998). Proposal concerning the names of certain apomictic species aggregates. *Taxon* 41: 495.

Weising K., Nybom H., Wolff K. and Meyer W. (1995). *DNA Fingerprinting in Plants and Fungi.* CRC Press. Florida.

Wettstein R. von. (1896). *Monographie der Gattung Euphrasia.* Wilhelm Engelmann. Leipzig.

White G. and Powell W. (1997). Isolation and characterization of microsatellite loci in *Swietenia humilis* (Meliaceae): an endangered tropical hardwood species. *Molecular Ecology* 6: 851-860.

Wigginton M.J. (1999). *British Red Data Books 1: Vascular Plants.* Joint Nature Conservation Committee. Peterborough.

Wilkins D.A. (1963). Plasticity and establishment in *Euphrasia. Annuals of Botany* 27: 533-552.

Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A. and Tingey S.V. (1990). DNA polymorphisms amplified by arbitary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531-6535.

Wolfe A.D. and dePamphilis C.W. (1997). Alternate paths of evolution for the photosynthetic gene rbcL in four nonphotosynthetic species of *Orobanche. Plant Molecular Biology* 33: 965-977.

Wright S. (1965). The interpretation of population structure by *F*-statistics with special regards to systems of mating. *Evolution* 38: 1358-1370.

Yeo P.F. (1954). The cytology of British species of *Euphrasia. Watsonia* 3: 101-108.

Yeo P.F. (1956). Hybridisation between diploid and tetraploid species of *Euphrasia. Watsonia* 3: 253-269.

Yeo P.F. (1959). *Experimental taxonomy on the genus* Euphrasia *L. (Scrophulariaceae – Rhinanthoideae).* PhD thesis. University of Leicester.

Yeo P.F. (1961). Germination, seedlings and the formation of haustoria in *Euphrasia. Watsonia* 5:11-22.

Yeo P.F. (1962). A study of variation *Euphrasia* by means of outdoor cultivation. *Watsonia* 5: 224-235.

Yeo P.F. (1964). The growth of *Euphrasia* in cultivation. *Watsonia* 6: 1-24.

Yeo P.F. (1966). The breeding relationships of some European *Euphrasiae. Watsonia* 6: 216-245.

Yeo P.F. (1968). *Euphrasia stricta* Lehm. in Guernsey. *Proceedings of the Botanical Society of the British Isles* 7: 383-385.

Yeo P.F. (1970a). *Euphrasia brevipilia* and *E. borealis* in the British Isles. *Watsonia* 8: 41-44.

Yeo P.F. (1970b). New chromosome counts in *Euphrasia*. *Candollea* 25: 21-24.

Yeo P.F. (1971). Revisional notes on *Euphrasia*: In: Flora Europaea. Notulae Systematicae ad Floram Europaeam spectantes No.11. Ed. by V.H.Heywood. *Botanical Journal of the Linnean Society* 64: 353-381.

Yeo P.F. (1975). The Yorkshire records of *Euphrasia salisbergensis*. *The Naturalist, Leeds* 934: 83-87.

Yeo P.F. (1976). Artificial hybrids between some European diploid species of *Euphrasia*. *Watsonia* 11: 131-135.

Yeo P.F. (1978a). A taxonomic revision of *Euphrasia* in Europe. *Botanical Journal of the Linnean Society* 77: 223-334.

Yeo P.F. (1978b). *Euphrasia*: a taxonomically critical group with normal sexual reproduction. In: *Essays in Plant Taxonomy*. pp. 143-162. Ed. by H.E.Street. Academic Press. London.

Zane L., Bargelloni L. and Patarnello T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology* 11: 1-16.

Zopfi H.J. (1997). Ecotypic variation of *Euphrasia rostkoviana* Hayne (Scrophulariaceae) in relation to grassland management. *Flora* 192: 279-295.

Zopfi H.J. (1998a). The genetic basis of ecotypic variants of *Euphrasia rostkoviana* Hayne (Scrophulariaceae) in relation to grassland management. *Flora* 193: 41-58.

Zopfi H.J. (1998b). Life-history variation among populations of *Euphrasia rostkoviana* Hayne (Scrophulariaceae) in relation to grassland management. *Biological Journal of the Linnean Society* 64: 179-205.

Zouchair R., Corradini P., Defontaine A. and Hallet J. (2000). RAPD markers for genetic differentiation of species within *Polytrichum* (Polytrichaceae, Musci): a preliminary survey. *Taxon* 49: 217-229.

Appendix I

Nomenclature for generic subdivsions and taxa mentioned in this thesis (= represents an old name not now formally recognised; + represents taxa not currently recognised in Britain, with records currently incorporated into recognised British taxa or generic divisions).

Genus *Euphrasia* L.

Section *Euphrasia* (= *Semicalcaratae* Bentham)

Subsection *Euphrasia* (= *Ciliatae* Jøerg., + *Euphrasia septentrionalis* Druce & Lumb, + *Euphrasia lumbii* Druce (= *Euphrasia variabilis* Druce & Lumb)

Series *Euphrasia* (= *Grandiflorae* Wettst., = *Hirtellae* Pugsley)

***Euphrasia officinalis* L.** (+ *Euphrasia hirtella* Jordan ex Reuter, + *Euphrasia rostkoviana* Hayne subsp. *campestris* (Jordan) P.Fourn., *Euphrasia officinalis* L. subsp. *officinalis* (= *Euphrasia rostkoviana* Hayne subsp. *fennica* (Kihlman) Karlsson))

***Euphrasia officinalis* L. subsp. *rostkoviana* (Hayne) F.Towns.** (= *Euphrasia rostkoviana* Hayne subsp. *rostkoviana*)

***Euphrasia officinalis* L. subsp. *monticola* Silverside** (= *Euphrasia rostkoviana* Hayne subsp. *montana* (Jord.) Wettst.)

***Euphrasia officinalis* L. subsp. *anglica* (Pugsley) Silverside** (= *Euphrasia anglica* Pugsley)

***Euphrasia vigursii* Davey**

***Euphrasia rivularis* Pugsley**

Series *Boreales* Sell & Yeo (= *brevipilae* Pugsley, + *Euphrasia suecica* Murb. & Wettst.)

***Euphrasia arctica* Lange ex Rostrup**

***Euphrasia arctica* Lange ex Rostrup subsp. *borealis* (F.Towns.) Yeo** (+ *Euphrasia breviplia* Wettst., *Euphrasia borealis* (F.Towns.) Wettst., + *Euphrasia arctica* subsp. *borealis* var. *notata*, + *Euphrasia arctica* subsp. *borealis* var. *reayensis*)

Euphrasia arctica* Lange ex Rostrup subsp. *arctica (+ *Euphrasia borealis* var. *speciosa* Pugsley)

Series *Nemorosae* Pugsley (+ *Euphrasia coerulea* Hoppe & Fürnrohr, + *Euphrasia kerneri* Wettst.)

***Euphrasia tetraquetra* (Bréb.) Arrond.** (= *Euphrasia occidentalis* Wettst.)

***Euphrasia nemorosa* (Pers.) Wallr.** (+ *Euphrasia curta* (Fries) Wettst.)

***Euphrasia pseudokerneri* Pugsley**

***Euphrasia confusa* Pugsley** (+ *Euphrasia minima* Jacq. ex DC., + *Euphrasia atroviolacea* Druce & Lumb)

Series *Majoriflorae* Jøerg.

***Euphrasia stricta* D.Wolff ex J.F.Lehm.**

Series *Alpinae* Rothmaler

***Euphrasia alpina* Lam.**

Series *Parviflorae* Wettst. (= *Latifoliae* Pugsley, + *Euphrasia rhumica* Pugsley, + *Euphrasia eurocarpa* Pugsley)

***Euphrasia minima* Jacq. ex DC.**

***Euphrasia frigida* Pugsley** (= *Euphrasia latifolia* Wettst.)

***Euphrasia foulaensis* F.Towns.**

Euphrasia cambrica **Pugsley**

Euphrasia ostenfeldii **(Pugsley) Yeo** (= *Euphrasia curta* var. *ostenfeldii* Pugsley)

Euphrasia marshallii **Pugsley**

Euphrasia rotundifolia **Pugsley**

Euphrasia campbelliae **Pugsley**

Euphrasia micrantha **Rchb.** (= *Euphrasia gracilis* Fries)

Euphrasia scottica **Wettst.** (= *Euphrasia paludosa* F.Towns.; = *Euphrasia scotica*)

Euphrasia heslop-harrisonii **Pugsley**

Euphrasia inopinata **F.Ehrendorfer & E.Vitek**

Euphrasia sinuata **E.Vitek & F.Ehrendorfer**

"*Euphrasia fharaidensis*" Silverside (awaiting formal description)

Series *Petiolares* Pugsley

Euphrasia willkommii **Freyn**

Subsection *Angustifoliae* (Wettst.) Jøerg.

Euphrasia salisbergensis **Funck var. *hibernica* Pugsley**

Appendix II

Visual Basic for Applications: Chloroplast macros

Add chloroplast menubar

```
Option Explicit                          'Requires declaration of variables

Private Sub Workbook_Open()

'Defines private variables
Dim wsMenuBar As CommandBar              'Worksheet Menu Bar
Dim iHelpIndex As Integer                'Help menu index number
Dim menucp As CommandBarPopup            'Chloroplast popup menu
Dim btdefhap As CommandBarButton         'Button to launch def_haplotype
Dim btredefhap As CommandBarButton       'Button to launch redef_haplotype
Dim btarlformat As CommandBarButton      'Button to launch alq_data_format
Dim bthaplodiv As CommandBarButton       'Button to launch haplodiv_data_format
Dim ctlControl As CommandBarControl      'Used to search menu bar controls
Dim i As Long                            'Loop counter

'Removes Chloroplast menu if still present
Set ctlControl = Application.CommandBars("Worksheet Menu Bar").FindControl_
        (Tag:="Chloroplast")
On Error Resume Next
ctlControl.Delete

'Adds Chloroplast popup menu
Set wsMenuBar = Application.CommandBars("Worksheet Menu Bar")
iHelpIndex = wsMenuBar.Controls("Help").Index
Set menucp = wsMenuBar.Controls.Add(Type:=msoControlPopup, Before:=_
iHelpIndex, Temporary:=True)
With menucp
        .Caption = "Chloroplast-RFLP"
        .Tag = "Chloroplast"
End With
Set btdefhap = menucp.Controls.Add(Type:=msoControlButton)
With btdefhap
        .Style = msoButtonIconAndCaption
        .Caption = "Define Haplotypes"
        .OnAction = "def_haplotype"
End With
Set btredefhap = menucp.Controls.Add(Type:=msoControlButton)
With btredefhap
        .Style = msoButtonIconAndCaption
```

```
        .Caption = "Redefine Haplotypes"
        .OnAction = "redef_haplotype"
End With
Set btarlformat = menucp.Controls.Add(Type:=msoControlButton)
With btarlformat
        .Style = msoButtonIconAndCaption
        .Caption = "Arlequin Data Format"
        .BeginGroup = True
        .OnAction = "alq_data_format"
End With
Set bthaplodiv = menucp.Controls.Add(Type:=msoControlButton)
With bthaplodiv
        .Style = msoButtonIconAndCaption
        .Caption = "Haplodiv Data Format"
        .OnAction = "haplodiv_data_format"
End With

End Sub
```

Remove chloroplast menubar

```
Private Sub Workbook_AddinUninstall()

'Defines private variables
Dim ctlControl As CommandBarControl     'Used to search menu bar controls

'Removes Chloroplast popup menu
On Error Resume Next
For Each ctlControl In Application.CommandBars("Worksheet Menu Bar").Controls
        If ctlControl.Tag = "Chloroplast" Then ctlControl.Delete
Next ctlControl

End Sub
```

Define chloroplast macros variables

```
Option Explicit                         'Requires declaration of variables
Option Base 1                           'Sets array indicies to start at 1

'Defines public variables
Public rgcptemprng As range             'Total range
Public rgcptemprow As range             'First row
Public rgcptempcolumn As range          'First column
Public icptempcolumn As Integer         'Number of columns in total range
Public icptemprow As Integer            'Number of rows in total range
Public vcptemparray As Variant          'Total array
Public vcptemprow As Variant            'First row array
Public vcptempcolumn As Variant         'First column array
Public vcppophaplotype As Variant       'Array to store population table
```

```
Public vcphapdescrip As Variant          'Array to store haplotype table
Public wksdata As Worksheet              'Data worksheet
Public m As Long, n As Long, p As Long   'Loop counters

'Defines chloroplast population  and haplotypes as two class objects
Public CpPops As New Collection          'Chloroplast population class collection
Public CpPop As clsCpPop                 'Chloroplast population class object
Public haplotypes As New Collection      'Haplotype class collection
Public Haplotype As clsCpHaplotype       'Haplotype class object
Define chloroplast haplotypes

Private Sub def_haplotype()

'Indicates macro running, stops screen updating and cancels displayalert messages
With Application
        .StatusBar = "Defining Haplotypes"
        .DisplayAlerts = False
        .ScreenUpdating = False
End with

'Delete haplotypes and populations worksheets if present
On Error Resume Next
Worksheets("Haplotypes").Delete
Worksheets("Populations").Delete
Worksheets("Arlequin").Delete
Worksheets("Haplodiv").Delete

'Remembers active worksheet name
Set wksdata = ActiveSheet

'Sets temporary range and array variables
range("A1").Name = "topleftcell"
Call cptemp_range

'Defines haplotype and population class variables
Call cls_pop_haplotype

'Defines array storing haplotype data
Call cphaplotypes

'Returns to first worksheet
wksdata.Select

'Clears macro running message, re-updates screen and turns on displayalert messages
With Application
        .StatusBar = False
        .DisplayAlerts = True
        .ScreenUpdating = True
```

```
End with

End Sub
```

Redefine chloroplast haplotypes

```
Private Sub redef_haplotype()

'Indicates macro running, stops screen updating and cancels displayalert messages
With Application
        .StatusBar = "Redefining Haplotypes"
        .ScreenUpdating = False
        .DisplayAlerts = False
End with

'Cancels program stop if error occurs
On Error Resume Next

'Remembers active worksheet name
Set wksdata = ActiveSheet

'Sets temporary range and array variables
range("A1").Name = "topleftcell"
Call cptemp_range

'Deletes haplotype column
Cells(1, icptempcolumn).EntireColumn.Delete

'Resets temporary range and array variables
range("A1").Name = "topleftcell"
Call cptemp_range

'Deletes haplotypes worksheet
Worksheets("Haplotypes").Delete

'Defines haplotype and population class variables
Call cls_pop_haplotype

'Defines array storing haplotype data
Call cphaplotypes
wksdata.Select

'Defines array storing population haplotypes
Call cp_pop_haplotype

'Returns to first worksheet
wksdata.Select
```

```
'Clears macro running message, re-updates screen and turns on displayalert messages
With Application
        .StatusBar = False
        .DisplayAlerts = True
        .ScreenUpdating = True
End with

End Sub
```

Arlequin data format

```
Private Sub alq_data_format()

'Defines private variables
Dim rng As range                        'Stores activecell position
Dim strhaplotype As String              'Stores haplotype as string

'Indicates macro running and stops screen updating
With Application
        .StatusBar = "Arlequin Data Format"
        .ScreenUpdating = False
End with

'Adds and names new worksheet
Worksheets.Add.Name = "Arlequin"

'Inserts Profile and Data Headings
Cells(1, 1).Value = "[Profile]"
Cells(2, 1).Value = "Title= " & Chr(34) & Chr(34)
Cells(3, 1).Value = "NbSamples= " & CpPops.Count
Cells(4, 1).Value = "DataType= STANDARD"
Cells(5, 1).Value = "GenotypicData= 0"
Cells(6, 1).Value = "CompDistMatrix= 1"
Cells(7, 1).Value = "LocusSeparator= WHITESPACE"
Cells(8, 1).Value = "MissingData= ""?"""
Cells(10, 1).Value = "[Data]"
Cells(12, 1).Value = "[[HaplotypeDefinition]]"
Cells(13, 1).Value = "HaplListName= " & Chr(34) & Chr(34)
Cells(14, 1).Value = "HaplList = {"

'Inserts haplotypes
Set rng = range(Cells(14, 1), Cells(14, 1))
For m = 1 To haplotypes.Count
        Set rng = rng.Offset(1, 0)
        rng.Value = haplotypes(m).Number
        Set rng = rng.Offset(0, 1)
        rng.Value = "*"                 'Inserts * (replaced by comma later on)
        strhaplotype = haplotypes(m).Name  'Haplotype name as string
```

```
'Splits haplotype name and adds spaces in between each character
        For n = 1 To Len(strhaplotype)
                Set rng = rng.Offset(0, 1)
                rng.Value = Mid(strhaplotype, n, 1)
                Set rng = rng.Offset(0, 1)
                rng.Value = Chr(32)                         'Inserts Space
        Next n
        rng.Clear
        Set rng = rng.Offset(0, -2 * Len(strhaplotype) - 1)
Next m
Set rng = rng.Offset(1, 0)
rng.Value = "}"

'Inserts samples
Set rng = rng.Offset(2, 0)
rng.Value = "[[Samples]]"
For m = 1 To CpPops.Count
        Set rng = rng.Offset(1, 0)
        rng.Value = "SampleName= " & Chr(34) & "Population " &_
        CpPops(m).Number & Chr(34)
        Set rng = rng.Offset(0, 1)
        rng.Value = Chr(32)
        Set rng = rng.Offset(0, 1)
        rng.Value = "#" & CpPops(m).Name
        Set rng = rng.Offset(1, -2)
        rng.Value = "SampleSize= " & CpPops(m).Size
        Set rng = rng.Offset(1, 0)
        rng.Value = "SampleData={"
'Inserts haplotype numbers and totals for each population
        For n = 3 To (haplotypes.Count + 2)
                If vcppophaplotype(m + 1, n) > 0 Then
                        Set rng = rng.Offset(1, 0)
                        rng.Value = n - 2
                        Set rng = rng.Offset(0, 1)
                        rng.Value = Chr(32)
                        Set rng = rng.Offset(0, 1)
                        rng.Value = vcppophaplotype(m + 1, n)
                        Set rng = rng.Offset(0, -2)
                End If
        Next n
        Set rng = rng.Offset(1, 0)
        rng.Value = "}"
Next m

'Inserts structure section
Set rng = rng.Offset(2, 0)
rng.Value = "[[Structure]]"
Set rng = rng.Offset(1, 0)
```

```
rng.Value = "StructureName= " & Chr(34) & Chr(34)
Set rng = rng.Offset(1, 0)
rng.Value = "NbGroups= 1"
Set rng = rng.Offset(1, 0)
rng.Value = "IndividualLevel= 0"
Set rng = rng.Offset(1, 0)
rng.Value = "Group= {"
Set rng = rng.Offset(1, 0)
For m = 1 To CpPops.Count
        rng.Value = Chr(34) & "Population " & CpPops(m).Number & Chr(34)
        Set rng = rng.Offset(1, 0)
Next m
rng.Value = "}"

'Formats cells
For n = 1 To 2 * Len(strhaplotype) + 1
        With Cells(1, n).EntireColumn
                .ColumnWidth = 2
                .HorizontalAlignment = xlCenter
        End With
        Cells(1, 1).EntireColumn.HorizontalAlignment = xlLeft
Next n

'Clears macro running message and re-updates screen
With Application
        .StatusBar = False
        .ScreenUpdating = True
End with

End Sub
```

Haplodiv data format

```
Private Sub haplodiv_data_format()

'Defines private variables
Dim rng As range                        'Stores activecell position

'Indicates macro running and stops screen updating
With Application
        .StatusBar = "Haplodiv Data Format"
        .ScreenUpdating = False
End with

'Adds and names new worksheet
Worksheets.Add.Name = "Haplodiv"

'Adds number of haplotypes and populations to first row
```

```
Cells(1, 1).Value = haplotypes.Count
Cells(1, 2).Value = Chr(32) & Chr(32)
Cells(1, 3).Value = CpPops.Count

'Adds population haplotype numbers
Set rng = range(Cells(1, 1), Cells(1, 1))
For n = 1 To CpPops.Count
        Set rng = rng.Offset(1, 0)
        For m = 1 To haplotypes.Count
                rng.Value = vcppophaplotype(n + 1, m + 2)
                Set rng = rng.Offset(0, 1)
                rng.Value = Chr(32) & Chr(32) & Chr(32)
                Set rng = rng.Offset(0, 1)
        Next m
        Set rng = rng.Offset(0, -1)
        rng.Clear
        Set rng = rng.Offset(0, -2 * haplotypes.Count + 1)
Next n

'Formats cells
For n = 1 To 2 * haplotypes.Count - 1
        With Cells(1, n).EntireColumn
                .ColumnWidth = 2
                .HorizontalAlignment = xlCenter
        End With
Next n

'Clears macro running message and re-updates screen
With Application
        .StatusBar = False
        .ScreenUpdating = True
End with

End Sub
```

Set temporary table and array variables

```
Private Sub cptemp_range()

'Determines and sizes required range
Set rgcptemprng = range("topleftcell").CurrentRegion
With rgcptemprng
        .icptempcolumn = .Columns.Count
        .icptemprow = .Rows.Count
End With
ReDim vcptemparray(icptemprow, icptempcolumn) As Variant
vcptemparray = rgcptemprng.Value
```

```
'Determines 1st column and row as ranges
range("topleftcell").Offset(0, 1).Select
Set rgcptempcolumn = range(ActiveCell, ActiveCell.End(xlToRight))
ReDim vcptempcolumn(1, (icptempcolumn - 1)) As Variant
vcptempcolumn = rgcptempcolumn.Value
range("topleftcell").Offset(1, 0).Select
Set rgcptemprow = range(ActiveCell, ActiveCell.End(xlDown))
ReDim vcptemprow((icptemprow - 1), 1) As Variant
vcptemprow = rgcptemprow.Value

End Sub
```

Define chloroplast population and haplotype class variables

```
Private Sub cls_pop_haplotype()

'Defines private variables
Dim vcppopname As Variant              'Stores population name
Dim icppopcount As Integer             'Count individuals in each population
Dim vcphaplotype As Variant            'Stores restriction fragment sequence
Dim Bnewhaplotype As Boolean           'Stores whether new haplotype exists

'Removes previous chloroplast populations
For m = 1 To CpPops.Count
        CpPops.Remove 1
Next m

'Removes previous haplotype populations
For m = 1 To haplotypes.Count
        haplotypes.Remove 1
Next m

'Removes haplotype column if present
If Cells(1, icptempcolumn).Value = "Haplotype" Then
        Cells(1, icptempcolumn).EntireColumn.Delete
        Call cptemp_range
End If

'Adds haplotype to end column
Cells(1, icptempcolumn + 1).Value = "Haplotype"

'Counts individuals in each population and places them in population collection
vcppopname = Cells(1, 1).Value
icppopcount = 0
For p = 1 To icptemprow
        If p = icptemprow Then Exit For
        vcppopname = vcptemprow(p, 1)
        Do
```

```
            Select Case vcppopname
                Case Is = vcptemprow(p, 1)
                    icppopcount = icppopcount + 1
                    p = p + 1
                    If p = icptemprow Then Exit Do
                Case Else
                    Exit Do
            End Select
        Loop

'Places each population name and number of individuals in collection
        Set CpPop = New clsCpPop
        With CpPop
            .Number = CpPops.Count + 1
            .Name = vcppopname
            .Size = icppopcount
        End With
        CpPops.Add CpPop, CpPop.Name
        icppopcount = 0
        p = p - 1
Next p

'Combines all restriction fragment alleles into one haplotype
For p = 1 To icptemprow
        For m = 3 To icptempcolumn
            Select Case IsEmpty(Cells(p, m))
                Case Is = True
                    vcphaplotype = "Undefined"
                    Exit For
                Case Is = False
                    vcphaplotype = vcphaplotype & vcptemparray(p, m)
            End Select
        Next m

'Names different haplotypes, places them in haplotype collection and places the
haplotypes on last column
        For n = 1 To haplotypes.Count
            Select Case vcphaplotype
                Case Is = haplotypes(n).Name
                    Bnewhaplotype = False
                    Cells(p, icptempcolumn + 1).Value = _
                        haplotypes(n).Number
                    Exit For
                Case Is = "Undefined"
                    Bnewhaplotype = False
                    Cells(p, icptempcolumn + 1).Value = vcphaplotype
                    Exit For
            End Select
```

```
        Next n
        If Bnewhaplotype = True Then
                Set Haplotype = New clsCpHaplotype
                With Haplotype
                        .Number = haplotypes.Count + 1
                        .Name = vcphaplotype
                End With
                haplotypes.Add Haplotype, Haplotype.Name
                Cells(p, icptempcolumn + 1).Value = haplotypes(n).Number
        End If
        vcphaplotype = ""
        Bnewhaplotype = True
Next p

End Sub
```

Produce table of haplotype descriptions

```
Private Sub cphaplotypes()

'Defines private variables
Dim strhapname As String                    'Stores restriction fragment sequence
strhapname = haplotypes(1).Name
ReDim vcphapdescrip((haplotypes.Count) + 1, Len(strhapname) + 1)

'Adds haplotype numbers to first column
For m = 1 To haplotypes.Count
        vcphapdescrip(m + 1, 1) = haplotypes(m).Number
Next m

'Adds haplotype title
vcphapdescrip(1, 1) = "Haplotype"

'Adds restriction fragment labels to first row
For m = 2 To icptempcolumn - 1
        vcphapdescrip(1, m) = rgcptempcolumn(1, m).Value
Next m

'Adds haplotype description for each haplotype
For m = 1 To haplotypes.Count
        strhapname = haplotypes(m).Name
        For n = 1 To Len(strhapname)
                vcphapdescrip(m + 1, n + 1) = Mid(strhapname, n, 1)
        Next n
Next m

'Adds and names new worksheet and determines new range
Worksheets.Add.Name = "Haplotypes"
```

```
range("A1").Name = "HaplotypeData"
With range("HaplotypeData")
        .Resize((haplotypes.Count) + 1, Len(strhapname) + 1).Name =_
                "HaplotypeData"
End With

'Transfers data from array to range
With range("HaplotypeData")
        .Value = vcphapdescrip
End With

'Format cells
With range("HaplotypeData")
        .ColumnWidth = 5
        .Font.Name = "Times New Roman"
        .Font.Size = 12
        .HorizontalAlignment = xlCenter
End With
With Cells(1, 1)
        .EntireColumn.ColumnWidth = 10
        .EntireColumn.Font.Bold = True
        .EntireRow.Font.Bold = True
End With

End Sub
```

Produce table of population haplotype number

```
Private Sub cp_pop_haplotype()

'Defines private variables
Dim ipoprow As Integer                  'Stores row number for population
Dim vhapcolumn As Variant               'Stores no. of haplotypes in population
Dim badddata As Boolean                 'Haplotype been defined or not
Dim vcppopname As Variant               'Stores name of population
ReDim vcppophaplotype((CpPops.Count + 1), (haplotypes.Count + 3))

'Adds labels to top row
vcppophaplotype(1, 1) = "Pop.No"
vcppophaplotype(1, 2) = "Pop.Name"
For p = 1 To haplotypes.Count
        vcppophaplotype(1, p + 2) = haplotypes(p).Number
Next p
vcppophaplotype(1, (haplotypes.Count + 3)) = "Total"

'Adds population number, name and total number of individuals
For p = 1 To CpPops.Count
        vcppophaplotype(p + 1, 1) = CpPops(p).Number
```

```
        vcppophaplotype(p + 1, 2) = CpPops(p).Name
        vcppophaplotype(p + 1, haplotypes.Count + 3) = CpPops(p).Size
Next p

'Counts up haplotypes in each population and inputs into array
For p = 1 To icptemprow
        If p = icptemprow Then Exit For
        vcppopname = vcptemprow(p, 1)
        For m = 1 To CpPops.Count
                If vcppopname = CpPops(m).Name Then
                        ipoprow = CpPops(m).Number
                End If
        Next m
        vhapcolumn = Cells(p + 1, icptempcolumn + 1).Value

'Excludes undefined haplotypes
        Select Case vhapcolumn
                Case Is = "Undefined"
                        badddata = False
                Case Is = "Haplotypes"
                        badddata = False
                Case Else
                        badddata = True
        End Select

'Adds haplotypes to array
        If badddata = True Then
                vcppophaplotype((ipoprow + 1), (vhapcolumn + 2)) = _
                vcppophaplotype((ipoprow + 1), (vhapcolumn + 2)) + 1
        End If
Next p

'In temporary array replaces all empty cells with zero
For p = 1 To CpPops.Count + 1
        For m = 1 To haplotypes.Count + 3
                If vcppophaplotype(p, m) = "" Then vcppophaplotype(p, m) = 0
        Next m
Next p

'Adds and names new worksheet and determines new range
Worksheets.Add.Name = "Populations"
range("A1").Name = "PopulationData"
With range("PopulationData")
        .Resize((CpPops.Count + 1), (haplotypes.Count + 3)).Name =_
                "PopulationData"
End With

'Transfers data from array to range
```

```
With range("PopulationData")
        .Value = vcppophaplotype
End With

'Formats cells
With range("PopulationData")
        .ColumnWidth = 3
        .Font.Name = "Times New Roman"
        .Font.Size = 12
        .HorizontalAlignment = xlCenter
End With
With Cells(1, 1)
        .EntireColumn.ColumnWidth = 8
        .EntireRow.Font.Bold = True
End With
With Cells(1, 2).EntireColumn
        .ColumnWidth = 28
        .HorizontalAlignment = xlLeft
End With
With Cells(1, haplotypes.Count + 3).EntireColumn
        .ColumnWidth = 5
End With

End Sub
```

Class module for chloroplast haplotype object definition

```
Option Explicit                         'Requires declaration of variables

'Defines properties of chloroplast haplotypes object
Public Number As Integer                'Haplotype number
Public Name As String                   'Haplotype name
```

Class module for chloroplast population definition

```
Option Explicit                         'Requires declaration of variables

'Defines properties of chloroplast populations object
Public Number As Integer                'Population number
Public Name As String                   'Population name
Public Size As Integer                  'Number of individuals in population
```

Visual Basic for Applications: AFLP macros

Add AFLP menubar

```
Option Explicit                          'Requires declaration of variables

Private Sub Workbook_Open()
'Defines private variables
Dim wsMenuBar As CommandBar              'Worksheet Menu Bar
Dim iHelpIndex As Integer                'Help menu index number
Dim menuAFLP As CommandBarPopup          'AFLP popup menu
Dim btr As CommandBarButton              'Button to launch r_data_format
Dim btPCOplot As CommandBarButton        'Button to launch PCO_plot
Dim btarlequin As CommandBarButton       'Button to launch arlequin_data_format
Dim PSbands As CommandBarButton          'Button to launch Population_Bands()
Dim ctlControl As CommandBarControl      'Used to search menu bar controls
Dim i As Long                            'Loop counter

'Removes AFLP menu if still present
Set ctlControl = Application.CommandBars("Worksheet Menu Bar").FindControl_
        (Tag:="AFLP")
On Error Resume Next
ctlControl.Delete

'Adds AFLP popup menu
Set wsMenuBar = Application.CommandBars("Worksheet Menu Bar")
iHelpIndex = wsMenuBar.Controls("Help").Index
Set menuAFLP = wsMenuBar.Controls.Add(Type:=msoControlPopup, before:=_
iHelpIndex, Temporary:=True)
With menuAFLP
        .Caption = "AFLP"
        .Tag = "AFLP"
End With
Set btarlequin = menuAFLP.Controls.Add(Type:=msoControlButton)
With btarlequin
        .Style = msoButtonIconAndCaption
        .Caption = "Arlequin Data Format"
        .OnAction = "arlequin_data_format"
End With
Set btr = menuAFLP.Controls.Add(Type:=msoControlButton)
With btr
        .Style = msoButtonIconAndCaption
        .Caption = "R Data Format"
        .OnAction = "r_data_format"
End With
Set btPCOplot = menuAFLP.Controls.Add(Type:=msoControlButton)
With btPCOplot
        .Style = msoButtonIconAndCaption
```

```
        .Caption = "PCO Plot"
        .OnAction = "PCO_plot"
End With
Set PSbands = menuAFLP.Controls.Add(Type:=msoControlButton)
With Psbands
        .Style = msoButtonIconAndCaption
        .Caption = "Group Bands"
        .OnAction = "Population_Bands"
End With

End Sub
```

Remove AFLP menubar

```
Private Sub Workbook_AddinUninstall()

'Defines private variables
Dim ctlControl As CommandBarControl     'Used to search menu bar controls
On Error Resume Next
For Each ctlControl In Application.CommandBars("Worksheet Menu Bar").Controls
        If ctlControl.Tag = "AFLP" Then ctlControl.Delete
Next ctlControl

End Sub
```

Define AFLP macro variables

```
'Option Explicit                        'Requires declaration of variables
Option Base 1                           'Sets array indicies to start at 1

'Defines public variables
Public rgtemprng As Range               'Total range
Public rgtemprow As Range               'First row
Public rgtempcolumn As Range            'First column
Public itempcolumn As Integer           'Number of columns in total range
Public itemprow As Integer              'Number of rows in total range
Public vtemparray As Variant            'Total array
Public vtemprow As Variant              'First row array
Public vtempcolumn As Variant           'First column array
Public wksAFLP As Worksheet             'Stores active worksheet
Public vPSdata As Variant               'Array for storing population data
Public iPSpopcount As Integer           'Stores no. of individuals in population
Public strPSpopname As String           'Stores population name
Public i As Long, j As Long, k As Long  'Loop counters

'Defines AFLP population as class object
Public AFLPpops As New Collection       'Population class collection
Public AFLPpop As clsAFLPpop            'Population class object
```

Arlequin data format

```
Private Sub arlequin_data_format()

'Defines private variables
Dim vaflppopname As Variant                 'Stores population name
Dim iaflppopcount As Integer                'Counts no. of individuals in each pop.
Dim rng As Range                            'Stores activecell position
Dim iarraypos                               'Stores position in vtemparray
Dim isamplenumber As Integer                'Stores sample number

'Indicates macro running, stops screen updating and cancels displayalert messages
With Application
        .StatusBar = "Arlequin Data Format "
        .DisplayAlerts = False
        .ScreenUpdating = False
End with

'Remembers activeworksheet
Set wksAFLP = ActiveSheet

'Removes previous class population data
For i = 1 To AFLPpops.Count
        AFLPpops.Remove 1
Next I

'Sets temporary range and array variables
Range("A1").Clear
Range("A1").Name = "topleftcell"
Call temp_range

'Counts individuals in each population and places them in population collection
vaflppopname = Cells(1, 1).Value
iaflppopcount = 0
For i = 1 To itemprow
        If i = itemprow Then Exit For
        vaflppopname = vtemprow(i, 1)

'Counts individuals in each population
        Do
                Select Case vaflppopname
                        Case Is = vtemprow(i, 1)
                                iaflppopcount = iaflppopcount + 1
                                i = i + 1
                                If i = itemprow Then Exit Do
                        Case Else
                                Exit Do
                End Select
```

```
        Loop

'Places each population name and number of individuals in collection
        Set AFLPpop = New clsAFLPpop
        With AFLPpop
                .Number = AFLPpops.Count + 1
                .Taxon = vaflppopname
                .Size = iaflppopcount
        End With
        AFLPpops.Add AFLPpop, AFLPpop.Taxon
        iaflppopcount = 0
        i = i - 1
Next i

'Removes any existing arlequin sheet
On Error Resume Next
Worksheets("Arlequin").Delete

'Adds and names new worksheet
Worksheets.Add.Name = "Arlequin"

'Inserts profile and data headings
Cells(1, 1).Value = "[Profile]"
Cells(2, 1).Value = "Title= " & Chr(34) & Chr(34)
Cells(3, 1).Value = "NbSamples= " & AFLPpops.Count
Cells(4, 1).Value = "DataType= STANDARD"
Cells(5, 1).Value = "GenotypicData= 0"
Cells(6, 1).Value = "CompDistMatrix= 1"
Cells(7, 1).Value = "LocusSeparator= "","""
Cells(8, 1).Value = "MissingData= ""?"""
Cells(10, 1).Value = "[Data]"
Cells(12, 1).Value = "[[Samples]]"
Set rng = Range(Cells(13, 1), Cells(13, 1))

'Inserts population data
iarraypos = 1
isamplenumber = 0
For i = 1 To AFLPpops.Count
        Set rng = rng.Offset(1, 0)
        rng.Value = "SampleName= " & Chr(34) & "Population " & AFLPpops(i)_
                .Number & Chr(34)
        Set rng = rng.Offset(0, 1)
        rng.Value = Chr(32)
        Set rng = rng.Offset(0, 1)
        rng.Value = "#" & AFLPpops(i).Taxon
        Set rng = rng.Offset(1, -2)
        rng.Value = "SampleSize= " & AFLPpops(i).Size
        Set rng = rng.Offset(1, 0)
```

```
        rng.Value = "SampleData={"
        For j = 1 To AFLPpops(i).Size
                isamplenumber = isamplenumber + 1
                Set rng = rng.Offset(1, 0)
                rng.Value = isamplenumber & "*"
                Set rng = rng.Offset(0, 1)
                rng.Value = 1 & "*"
                Set rng = rng.Offset(0, 1)
                iarraypos = iarraypos + 1
                For k = 2 To itempcolumn
                        Select Case k
                                Case Is = itempcolumn
                                        rng.Value = vtemparray(iarraypos, k)
                                Case Else
                                        rng.Value = vtemparray(iarraypos, k)& ","
                        End Select
                        Set rng = rng.Offset(0, 1)
                Next k
                Set rng = rng.Offset(0, -(itempcolumn + 1))
        Next j
        Set rng = rng.Offset(1, 0)
        rng.Value = "}"
Next i

'Inserts structure section
Set rng = rng.Offset(2, 0)
rng.Value = "[[Structure]]"
Set rng = rng.Offset(1, 0)
rng.Value = "StructureName= " & Chr(34) & Chr(34)
Set rng = rng.Offset(1, 0)
rng.Value = "NbGroups= 1"
Set rng = rng.Offset(1, 0)
rng.Value = "IndividualLevel= 0"
Set rng = rng.Offset(1, 0)
rng.Value = "Group= {"
Set rng = rng.Offset(1, 0)
For i = 1 To AFLPpops.Count
        rng.Value = Chr(34) & "Population " & AFLPpops(i).Number & Chr(34)
        Set rng = rng.Offset(1, 0)
Next i
rng.Value = "}"

'Formats data sheet
Cells.Select
With Selection
        .Columns.AutoFit
End With
Cells(1, 3).EntireColumn.HorizontalAlignment = xlRight
```

```
Range("A1").Select

'Return to data sheet
wksAFLP.Select
Range("A1").Select

'Clears macro running message, re-updates screen and turns on displayalert messages
With Application
        .StatusBar = False
        .DisplayAlerts = True
        .ScreenUpdating = True
End with

End Sub
```

R data format

```
Private Sub r_data_format()

'Indicates macro running, stops screen updating and cancels displayalert messages
With Application
        .StatusBar = "Converting to R "
        .DisplayAlerts = False
        .ScreenUpdating = False
End with

'Remembers activeworksheet
Set wksAFLP = ActiveSheet

'Sets temporary range and array variables
Range("A1").Name = "topleftcell"
Call temp_range

'Deletes previous worksheet
On Error Resume Next
Worksheets("R format").Delete

'Transfers data to new worksheet
Worksheets.Add.Name = "R format"
Range("A1").Name = "Rtable"
With Range("Rtable")
        .Resize(itemprow, itempcolumn).Name = "Rtable"
End With
Range("Rtable").Value = vtemparray
'Resets temporary range and array variables
Range("A1").Name = "topleftcell"
Call temp_range
```

```
'Adds comma to cells except last column
For i = 2 To itemprow
        For j = 1 To itempcolumn - 1
                Select Case j
                        Case Is = 1
                                vtemparray(i, j) = vtemparray(i, j) & ", "
                        Case Else
                                vtemparray(i, j) = vtemparray(i, j) & ","
                End Select
        Next j
Next i

'Transfers new data back to temporary range
rgtemprng.Value = vtemparray

'Deletes band numbers
Cells(1, 1).EntireRow.Delete

'Formats cells
Call format_cells

'Deletes population column
Cells(1, 1).EntireColumn.Delete

'Returns to data worksheet
wksAFLP.Select
Range("A1").Select

'Clears macro running message, re-updates screen and turns on displayalert messages
With Application
        .StatusBar = False
        .DisplayAlerts = True
        .ScreenUpdating = True
End with

End Sub
```

Scatter graph for Principal coordinate analysis plots

```
Private Sub PCO_plot()

'Defines private variables
Dim wkschtdata As Worksheet          'Stores active worksheet name
Dim xcolumn As Integer               'Stores x column number
Dim ycolumn As Integer               'Stores y column number
Dim k As Integer                     'Stores initial column for each plot
Dim rngdatalabels As Range           'Stores which cell for datalabel
Dim rngxvalues As Range              'Stores which cell for x axis
```

```
Dim rngyvalues As Range                 'Stores which cell for y axis
Dim cht As Chart                        'Chart object
Dim wksname As String                   'Stores worksheet name
Dim chtname As String                   'Stores chart name
Dim senewseries As Series               'Chart series object
Dim vseriesxdata As Variant             'Array for x value
Dim vseriesydata As Variant             'Array for y value
Dim Pts As Points                       'Points collection for chart
Dim Pt As Point                         'Points object for chart

'Indicates macro running, stops screen updating and cancels displayalert messages
With Application
        .StatusBar = " Creating PCO Plot "
        .DisplayAlerts = False
        .ScreenUpdating = False
End with

'Sets temporary range and array variables
Range("A1").Name = "topleftcell"
Call temp_range

'Remembers and renames active worksheet name
Set wkschtdata = ActiveSheet
wksname = wkschtdata.Name

'Deletes any previous charts (first three axis only)
On Error Resume Next
Charts("PCO Plot 1 - 2").Delete
Charts("PCO Plot 1 - 3").Delete
Charts("PCO Plot 2 - 3").Delete

'Plots each column against each other
k = 2
For xcolumn = 2 To itempcolumn
        k = k + 1
        For ycolumn = k To itempcolumn
                Application.StatusBar = "PCO Plot " & xcolumn - 1 & " v "_
                        & ycolumn – 1

'Adds chart to active worksheet
                Set cht = Charts.Add
                Set cht = cht.Location(Where:=xlLocationAsObject,_
                        Name:=wksname)

'Delete any present series
                For j = cht.SeriesCollection.Count To 1 Step -1
                        cht.SeriesCollection(j).Delete
                Next j
```

```
'Gets data point one at a time
                With cht
                        .ChartType = xlXYScatter
                        .HasLegend = False
                End With
                For i = 2 To itemprow
                        Application.StatusBar = "PCO Plot " & xcolumn - 1 & " v " &
                                _ycolumn - 1 & " : " & "Data Point " & i - 1
                        With wkschtdata
                                .Range("A1").Select
                                .Range(Cells(i, xcolumn), Cells(i, xcolumn)).Name =_
                                        "xvalues"
                                .Range(Cells(i, ycolumn), Cells(i, ycolumn)).Name =_
                                        "yvalues"
                                .Range(Cells(i, 1), Cells(i, 1)).Name = "datalabel"
                                Set rngxvalues = wkschtdata.Range("xvalues")
                                Set rngyvalues = wkschtdata.Range("yvalues")
                                Set rngdatalabels = wkschtdata.Range("datalabel")
                                vseriesydata = rngyvalues
                                vseriesxdata = rngxvalues
                        End With

'Places each data point on chart as new series
                        With cht
                                Set senewseries = .SeriesCollection.NewSeries
                                senewseries.Values = vseriesydata
                                senewseries.XValues = vseriesxdata
                                senewseries.Name = Cells(i, 1).Value
                                senewseries.HasDataLabels = True

'Makes points invisible
                                Set Pts = senewseries.Points
                                For Each Pt In Pts
                                        With Pt
                                                .MarkerBackgroundColorIndex =_
                                                        xlColorIndexNone
                                                .MarkerForegroundColorIndex =_
                                                        xlColorIndexNone
                                        End With

'Adds data label to centre of each point
                                        With Pt.DataLabel
                                                .Text = "=" & rngdatalabels.Cells(1, 1)_
                                                .Address(Rowabsolute:=True, _
                                                columnabsolute:=True, ReferenceStyle_
                                                        :=xlR1C1, External:=True)
                                                .Font.Size = 10
```

```
                                        .Font.Name = "Times New Roman"
                                        .Font.Bold = False
                                        .Position = xlLabelPositionCenter
                                        .AutoScaleFont = False
                                    End With
                                Next Pt
                            End With
'Selects active worksheet to get next data point
                            wkschtdata.Select
                        Next i
                        Application.StatusBar = "PCO Plot " & xcolumn - 1 & " v " & _
                            ycolumn - 1 & " : " & "Formatting Plot"

'Formats chart
                        With cht
                            .PlotArea.Interior.ColorIndex = xlNone
                            .PlotArea.Border.LineStyle = xlNone
                            .Axes(xlValue).MajorGridlines.Delete
                            With .Axes(xlValue).TickLabels.Font
                                .Size = 12
                                .Name = "Times New Roman"
                                .AutoScaleFont = False
                            End With
                            With .Axes(xlCategory).TickLabels.Font
                                .Size = 12
                                .Name = "Times New Roman"
                                .AutoScaleFont = False
                            End With
                            With .Axes(xlValue, xlPrimary)
                                .HasTitle = True
                                .AxisTitle.Characters.Text = vtemparray(1, ycolumn)
                                .AxisTitle.Font.Name = "Times New Roman"
                                .AxisTitle.Font.FontStyle = "Regular"
                                .AxisTitle.Font.Size = 12
                                .AutoScaleFont = False
                            End With
                            With .Axes(xlCategory, xlPrimary)
                                .HasTitle = True
                                .AxisTitle.Characters.Text = vtemparray(1, xcolumn)
                                .AxisTitle.Font.Name = "Times New Roman"
                                .AxisTitle.Font.FontStyle = "Regular"
                                .AxisTitle.Font.Size = 12
                                .AutoScaleFont = False
                            End With
                        End With

'Places chart on new sheet
                        chtname = "PCO Plot " & xcolumn - 1 & " - " & ycolumn - 1
```

```
                Set cht = cht.Location(Where:=xlLocationAsNewSheet, Name:=_
                    chtname)
        Next ycolumn
        If xcolumn = itempcolumn - 1 Then Exit For
Next xcolumn

'Returns to data worksheet
wkschtdata.Select
Range("A1").Select

'Clears macro running message, re-updates screen and turns on displayalert messages
With Application
        .StatusBar = False
        .DisplayAlerts = True
        .ScreenUpdating = True
End with

End Sub
```

Group individual bands together

```
Private Sub Population_Bands()

'Defines private variables
Dim ibandtotal As Integer                   'Counts band no. in each population
Dim ibandnumber As Integer                  'Removes ? bands from number counted
Dim ibandvalue As Variant                   'Stores band value

'Indicates macro running, stops screen updating and cancels displayalert messages
With Application
        .StatusBar = "Grouping individual bands "
        .DisplayAlerts = False
        .ScreenUpdating = False
End with

'Remembers active worksheet name
Set wksAFLP = ActiveSheet

'Removes any previous grouped bands worksheets
On Error Resume Next
Worksheets("Grouped Bands").Delete

'Set temporary range and array variables
Range("A1").Name = "topleftcell"
Call temp_range

'Removes previous class population data
For i = 1 To AFLPpops.Count
        AFLPpops.Remove 1
```

```
Next I

'Counts individuals in each population and places them in population data collection
strPSpopname = Cells(2, 1).Value
iPSpopcount = 0
For i = 2 To itemprow + 1
        If i = itemprow + 1 Then Exit For
        strPSpopname = vtemparray(i, 1)

'Counts individuals in each population
        Do
                Select Case strPSpopname
                        Case Is = vtemparray(i, 1)
                                iPSpopcount = iPSpopcount + 1
                                i = i + 1
                                If i = itemprow + 1 Then Exit Do
                        Case Else
                                Exit Do
                End Select
        Loop

'Places each population name and number of individuals in collection
        Set AFLPpop = New clsAFLPpop
        With AFLPpop
                .Taxon = vtemparray(i - 1, 1)
                .Size = iPSpopcount
                .Start = i - iPSpopcount
        End With
        AFLPpops.Add AFLPpop
        iPSpopcount = 0
        i = i - 1
Next i

'Places population summary data into array
ReDim vPSdata((AFLPpops.Count + 1), itempcolumn)

'Add labels to top row
vPSdata(1, 1) = "Pop."

'Adds population bands to table
For i = 2 To AFLPpops.Count + 1
        vPSdata(i, 1) = AFLPpops(i - 1).Taxon
        For j = 2 To itempcolumn
                ibandtotal = 0
                ibandnumber = AFLPpops(i - 1).Size
                vPSdata(1, j) = vtemparray(1, j)
                For k = 1 To AFLPpops(i - 1).Size
                        ibandvalue = vtemparray(AFLPpops(i - 1).Start - 1 + k, j)
```

```
                    Select Case ibandvalue
                        Case Is = "?"
                            ibandnumber = ibandnumber - 1
                        Case Is = 1
                            ibandtotal = ibandtotal + 1
                    End Select
                Next k
                On Error Resume Next

'Calcuates frequency of bands in population
                vPSdata(i, j) = ibandtotal / ibandnumber

'Looks for present of absent bands in population only
                If vPSdata(i, j) > 0 Then vPSdata(i, j) = 1
        Next j
Next i

'Adds new population band frequency worksheet
Worksheets.Add.Name = "Grouped Bands"

'Defines new range
Range("A1").Name = "Bandfq"
With Range("Bandfq")
        .Resize((AFLPpops.Count) + 1, itempcolumn).Name = "Bandfq"
End With

'Transfers data from array to range
With Range("bandfq")
        .Value = vPSdata
End With

'Formats cells
With Range("bandfq")
        .NumberFormat = "0"
        .ColumnWidth = 5
        .HorizontalAlignment = xlCenter
End With
With Cells(1, 1)
        .EntireRow.Font.Bold = True
        .EntireRow.NumberFormat = "0"
        .EntireColumn.Font.Bold = True
        .EntireColumn.HorizontalAlignment = xlLeft
        .EntireColumn.AutoFit = True
End With

'Tranfers back to data worksheet
wksAFLP.Select
```

```
'Clears macro running message, re-updates screen and turns on displayalert messages
With Application
        .StatusBar = False
        .DisplayAlerts = True
        .ScreenUpdating = True
End with

End Sub
```

Set temporary table and array variables

```
Private Sub temp_range()

'Determines and sizes required range
Set rgtemprng = Range("topleftcell").CurrentRegion
With rgtemprng
        itempcolumn = .Columns.Count
        itemprow = .Rows.Count
End With
ReDim vtemparray(itemprow, itempcolumn) As Variant
vtemparray = rgtemprng.Value

'Determines 1st column and row as ranges
Range("topleftcell").Offset(0, 1).Select
Set rgtempcolumn = Range(ActiveCell, ActiveCell.End(xlToRight))
ReDim vtempcolumn(1, (itempcolumn - 1)) As Variant
vtempcolumn = rgtempcolumn.Value
Range("topleftcell").Offset(1, 0).Select
Set rgtemprow = Range(ActiveCell, ActiveCell.End(xlDown))
ReDim vtemprow((itemprow - 1), 1) As Variant
vtemprow = rgtemprow.Value

End Sub
```

Format table

```
Private Sub format_cells()

'Sets temporary range and array variables
Range("A1").Name = "topleftcell"
Call temp_range

'Formats cells
With rgtemprng
        .RowHeight = 17
        .Font.Name = "Times New Roman"
        .Font.Size = 12
        .HorizontalAlignment = xlCenter
        .Columns.AutoFit
```

```
End With
With Cells(1, 1)
        .EntireColumn.Font.Bold = True
        .EntireRow.Font.Bold = False
End With

End Sub
```

Class module for AFLP object definition

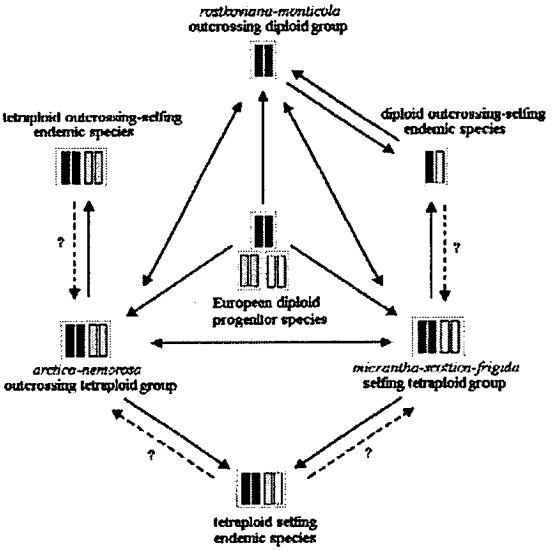
```
Option Explicit                         'Requires declaration of variables

'Defines properties of AFLP object
Public Number As Integer                'Population number
Public Taxon As String                  'Population taxon name
Public Size As Integer                  'Number of individuals in population
Public Start As Integer                 'Number of first individual
```

Appendix III

EYEBRIGHTS (Diploid and allotetraploid groups, allogamous-autogomous shifts in breeding system, ecological diversification and hybridisation)

1. PROCESS SCHEMATIC



* endemic species from previous *Euphrasia* action plan are diploid species *E.vigursii* and *E.rivularis*, and tetraploid species *E.cambrica*, *E.campbelliae*, *E.heslop-harrisonii* and *E.rotundifolia*.

2. CURRENT STATUS

2.1 This action plan covers the above process which are important in continuing the generation of diversity within British *Euphrasia*. Important constituents include taxa within the diploid group (*E.officinalis* subsp. *rostkoviana* and subsp. *monticola*) and within two main allo -tetraploid groups (*E.arctica* subsp. *boreales*, *E.nemorosa* and hybrids between them and *E.micrantha*, *E.scottica*, *E.frigida* and hybrids between them). The diversity is especially generated in "hotspot" areas along a western and northern oceanic distribution.

2.2 Habitats include hay-meadows, pastures and grasslands (*E.officinalis*, *E.arctica* and *E.nemorosa*), heathland and heather moorland (*E.micrantha* and *E.scottica*), non calcareous hill flushes (*E.scottica*) and wet basic montane ledges (*E.frigida*). All of these above species have shown a marked decline in the last 25 years, especially those in hay-meadows and pastures. Sites of "hotspots" of diversity are especially found in heathland habitats of Cornwall and Devon, montane habitats of north Wales and Lake District and coastal habitats of western and northern Scotland. Most of these sites are under no particular threat but maybe affected by changing grazing density. Heathland sites in Devon and Cornwall are declining due to habitat destruction. All of these above species are found in Northern Ireland, these have not however resulted in the generation of diversity seen in the British group.

3. CURRENT FACTORS CAUSING LOSS OR DECLINE

3.1 Agricultural intensification, changing land use and urbanisation.

4. CURRENT ACTION

4.1 SNH & CCW are supporting re-evalution of the distribution of the endemic species in Scotland and Wales.

5. ACTION PLAN OBJECTIVES AND TARGETS

5.1 Clarify the distribution of diploid and allo-tetraploid groups.

5.2 Reduce and reverse decline in common species within these groups.

5.3 Survey areas of "hotspots" of diversity and clarify their protection status.

5.4 Clarify the importance of endemic species and their habitats in overall process.

5.5 Protect known populations of endemic species until their importance in the overall diversification process is clarified.

6. PROPOSED ACTIONS WITH LEAD AGENCIES

6.1 Policy and legislation

6.1.1 Develop policy framework for implementing process-based action plans for *Euphrasia* and other taxonomically complex groups.

6.2 Site safeguard and land acquisition

6.2.1 Promote conservation directives for the maintenance and improvement

of existing neutral unimproved grass-land and lowland heathland sites and the re-establishment of such sites if opportunities arise.

6.2.2 Consider notifying key sites of diversity as SSSIs where this is necessary for securing appropriate management.

6.3 Species management and protection

6.3.1 No action proposed.

6.4 Advisory

6.4.1 Ensure land owners and local voluntary conservation groups are aware of the presence and importance of conserving these common species (named in section 2.2) and advise on appropriate methods of habitat management when required.

6.5 Future research and monitoring

6.5.1 Undertake survey of species within diploid and the allo-tetraploid groups to ascertain their distribution.

6.5.2 Promote genetic and ecological investigations to clarify processes in "hotspot" areas of diversity.

6.5.3 Encourage research to increase understanding of formation of allo-tetraploid groups outside UK.

6.5.4 Subject to confidentiality and data ownership, pass any information gathered during surveys of common species to JNCC or BRC to allow incorporation into national databases.

6.6 Communications and publicity

6.6.1 Use process to highlight threat to UK biodiversity from the destruction of grassland and heathland habitats.