Investigating the variability in *Phragmites australis* (Cav.) Trin. ex Steudel in wetlands for habitat enhancement and water treatment

Margaret Anne Keegan

Degree of Doctor of Philosophy

The University of Edinburgh

2006

I declare that the work presented in the thesis is my own unless stated otherwise and that no part of this thesis has been submitted for any other degree or professional qualification.

Abstract

Phragmites australis (Cav.) Trin. ex Steudel is a cosmopolitan, clonal, highly polymorphic, macrophyte. Wetlands dominated by *P. australis* (reedbeds) have great socio-economic and conservation value. In Britain, reedbeds are a rare and declining habitat and their creation and restoration has become a priority. To devise successful strategies for reedbed conservation, it is essential to have a thorough understanding of the ecology and genetic diversity of the species. The aims of this research were to investigate the ploidy levels, the extent and arrangement of genetic variation (detected as both neutral and adaptive traits), breeding system and seed set among British populations of *P. australis.*

Microsatellite analysis was used to estimate clone structure, genetic variability within and among populations and the outcrossing rate. Prior to analysis, all populations were karyotyped by root squashing and were found to be tetraploid ($2n$ = 48). Five microsatellite loci that displayed a diploid inheritance pattern were employed in screening 20 samples from each population. Whilst high clonal diversity was found in nine out of 11 populations ($P_d > 0.95$), Inner Tay had low diversity ($P_d = 0.5$) and Loch Leven was monoclonal and contained one genet approximately 1.9 km in length. There was no evidence of inbreeding within populations (f = - 0.098) and high levels of genetic diversity were found over all populations (He = 0.547). There was moderate genetic structure among populations (θ = 0.177), which was only partly explained by the isolation by distance model ($R^2 = 0.157$, $P < 0.01$), indicating that long distance gene flow (e.g. by seed) still occurs, most probably through anthropogenic activities. Using up to ten offspring of 20 families from the Inner Tay population, the out crossing rate was found to be > 95 %.

Seedset and germination rate were assessed from sampling 11 reedbeds throughout Britain in both 2002 and 2003. Seedset was determined by examining 20 panicles from each population. These seeds were used for the germination trials and the seedlings provided the material for a fully replicated nutrient-stressed and full-nutrient common garden experiment (CGE). In addition, seedlings were grown in synthetic sewage to simulate growth conditions in constructed wetlands.

Significant differences in seedset and germination rates were found amongst populations. Seedset was reduced by the presence of *Claviceps purpurea* infection and was positively associated with mean maximum October temperature (when seed maturation occurs) and site size (possibly a proxy measure of genotypic diversity). All populations, apart from one northerly population, had > 50 % germination rate and germination rate was found to increase with increasing seedset.

From the CGE, substantial genetic differences in morphology were found among but not within natural British populations of *P. australis*. Shoot height and number of shoots showed consistent variation among populations. Under nutrient-stressed conditions, northern populations produced longer shoots than southern ones whilst the opposite was true in full-nutrient conditions. Application of synthetic sewage resulted in a marginal significant decrease in the number of shoots produced per population and an increase in below ground biomass compared to populations not receiving treatment.

A further aim of this study was to assess the intraspecific variation in the effectiveness of *P. australis* in nutrient removal from constructed wetland systems (CWSs). Mini-CWS (~ 70 l volume) were created using five British *P. australis* populations and their efficacy in removing NH_4-N, NO_3-N, P and BOD over a one-year period was compared. There were significant and substantial differences in NH_4-N removal between populations in the CWSs: a difference of 40 % was found between the highest and lowest removal rates. This was most likely attributed to genetic differences among populations.

From the results of this research, the main recommendations for reedbed establishment and conservation were that seeds should be collected from large reedbeds with high clonal diversity. To prevent the loss of genetic diversity over time in established reedbeds, anthropogenic disturbance should be encouraged to promote sexual recruitment. Where populations are monoclonal, and seedset is depauperate, seedlings would need to be planted from an external source. The source of *P. australis* could be important for CWS performance, particularly in the removal of NH_4-N, suggesting that source of *P. australis* should be taken account of in CWS design and construction.

Acknowledgments

I would like to thank the following people who have helped me complete my Ph.D. thesis:

My biggest thanks must go to my partner Curt Finnemore because without his unfailing support, since my career change seven years ago, none of this would have been be possible. He also provided field assistance in sometimes difficult conditions e.g. Insh Marsh (sampling in waist-high water) and, most memorably, sampling in the Suffolk reedbeds, where a combination of metre high nettles, large mosquitoes (he now knows he's allergic to mosquito bites) and bulls did not thwart him.

My main supervisors Dr. Kate Heal and Dr. Richard Ennos for their lasting support and the generous amount of time they have devoted to discussing my research with me. Richard also provided a few comedy moments whilst sampling in the Scottish reedbeds which still make me laugh, whilst Kate gave very generously of her time in accompanying me to the East Anglian reedbeds on numerous occasions. I will never forget those early morning breakfasts *chez* Mrs Maguilp. Also, my third supervisor Dr. Neil Cowie, for his helpful advice, support and enthusiasm for the project.

My neighbour in room 306a, as well as laughing at all of my jokes, always had time for my queries and gave valuable advice and help when needed. Thank you Catherine.

I received help and advice for microsatellite analysis from Dr. Bengt Hansson and Dr. Kate Byrne. I am especially grateful to Bengt for all the help and encouragement he provided when I initially started the analysis and Dr. Josephine Pemberton for allowing me to use the GeneMapper software.

Dr. Michael Möller for his help in cytological analysis, Dr. Karen Dobbie for her advice on 'root washing' and words of encouragement throughout my Ph.D. and Zisis Gagkas for helping me with GIS.

Whilst carrying out the research I have had many field and laboratory assistants: Dr. Catherine Dickey, John Mormon (who lived up to his accident prone reputation and injured both his back and knee whilst assisting me), Julia Drewer, who looked after my CWS experiment whilst I was on holiday, and Patrick White. In addition the

wardens at each reserve were always very helpful, and in particular Norman Sills and his assistant, who both helped in panicle and leaf collection.

Andy Gray and John Mormon for analysing all of my CWS samples - the double act they have developed is second to none.

The Natural Environment Research Council and RSPB for funding the research.

Contents

Abstract i

Acknowledgements iii

Contents v

Tables x

Figures xiii

Chapter 1 – Introduction 1

1.1 Background 1
1.2 Reedbeds 4
1.2.1 Definition 4
1.2.2 Occurrence 4
1.2.3 Reedbed importance 4
1.2.3.1 Plant material 4
1.2.3.2 Ecological value 5
1.2.3.3 Wastewater treatment 5
1.3 Reedbed resource in Britain 5
1.3.1 Distribution 5
1.3.2 Reedbed decline 8
1.3.3 Reedbed importance 8
1.3.3.1 Conservation value 8
1.3.3.2 Economic value 9
1.3.3.3 Constructed wetland systems 9
1.3.4 Reedbed restoration in Britain 10
1.4 Review of the biology and ecology of *P. australis* 12
1.4.1 Taxonomy 12
1.4.2 Distribution 12
1.4.3 Description 12
1.4.4 Habitat 15
1.4.5 Flood tolerance 16
1.4.6 Colonisation 16
1.4.7 Seed recruitment 17
1.4.8 Reproduction 17
1.4.8.1 Asexual reproduction 17
1.4.8.2 Sexual reproduction 18
1.5 Essential features of *P. australis* 18
1.5.1 Chromosomal races 18
1.5.2 Clonal structure 20
1.5.3 Genetic diversity 21
1.5.4 Breeding system 21
1.5.5 Seed production 22
1.5.6 Adaptive genetic variation 23
1.5.7 Genotypes for CWSs 26
1.6 Summary of objectives 26
1.7 Thesis structure and outline 27

Chapter 2 - Materials and methods 28

2.1 Materials 28
2.1.1 Reedbed populations 28

2.1.2 Site description 31
2.1.2.1 Insh Marsh 31
2.1.2.2 Inner Tay 32
2.1.2.3 Loch Leven 33
2.1.2.4 St. Margaret's Marsh 34
2.1.2.5 Mersehead 35
2.1.2.6 Leighton Moss 36
2.1.2.7 Strumpshaw Fen 37
2.1.2.8 Lakenheath 38
2.1.2.9 Walberswick 39
2.1.2.10 Minsmere 40
2.1.2.11 North Warren 41
2.2 Methods 42
2.2.1 Molecular markers 42
2.2.1.1 Allozymes 43
2.2.1.2 RAPDs 43
2.2.1.3 RFLPs 43
2.2.1.4 AFLPs 44
2.2.1.5 Microsatellite markers 44
2.2.2 The choice of appropriate marker for this study 45
2.2.3 Common Garden Experiments (CGEs) 46
2.2.3.1 Detecting quantitative genetic differences 46
2.2.3.2 Genotype x environment interaction 47
2.2.3.3 CGE design for this study 48
2.2.4 Constructed wetland system experiments 48

Chapter 3 – Ploidy levels and genetic marker variation of 11 British populations of *Phragmites australis* 50

3.1 Introduction 50
3.2 Materials and methods 51
3.2.1 Populations 51
3.2.2 Sampling within populations 52
3.2.3 Plant material for cytological investigations 52
3.2.3.1 Cytological protocol 54
3.2.4 DNA extraction 54
3.2.5 Microsatellite analysis 55
3.2.5.1 PCR amplification 55
3.2.6 Statistical methods 56
3.2.6.1 Clonal structure and diversity within populations 56
3.2.6.2 Gene diversity 57
3.2.6.2.1 Differentiation statistics: F_{IS}, F_{ST} and F_{IT} 57
3.3 Results 58
3.3.1 Ploidy 58
3.3.2 Microsatellite analysis 58
3.3.2.1 Amplification of alleles 58
3.3.2.2 Clonal structure and diversity within populations 61
3.3.2.3 Genetic diversity 64
3.3.2.4 Isolation by distance model 67
3.4 Discussion 69
3.4.1 Ploidy Levels 69
3.4.2 Genetic analysis using microsatellite markers 70
3.4.2.1 Genotypic diversity 70
3.4.2.2 Genetic diversity within populations 75
3.4.2.3 Genetic diversity among populations 75
3.4.2.4 Pairwise estimates of F_{ST} 76
3.4.2.5 Isolation by distance model 77
3.5 Summary 78

Chapter 4 – The breeding system of *Phragmites australis* revealed by microsatellite markers 79

4.1 Introduction 79
4.2 Materials and methods 81
4.2.1 Seed collection 81
4.2.2 DNA extraction 83
4.2.3 Microsatellite analysis 83
4.2.4 Statistical methods 83
4.2.4.1 Genetic structure within populations 83
4.2.4.2 Outcrossing rate 84
4.3 Results 85
4.3.1 Amplification of alleles 85
4.3.2 Maternal inbreeding and outcrossing rate 86
4.4 Discussion 86
4.4.1 Breeding system 86
4.4.2 Genetic diversity of the progeny 88
4.4.3 Implications for reedbed management and conservation 89

Chapter 5 – Variability in seedset and germination of British populations of *Phragmites australis* 91

5.1 Introduction 91
5.2 Materials and methods 94
5.2.1 Site details 94
5.2.2 Sampling 94
5.2.3 Seedset and *Claviceps purpurea* infection 94
5.2.4 Germination trials 96
5.2.5 Reproductive potential 97
5.2.6 Weather data 97
5.2.7 Statistical analysis 97
5.3 Results 99
5.3.1 Seedset 99
5.3.2 Panicle length 102
5.3.3 *Claviceps purpurea* infection 103
5.3.4 Germination trials 104
5.3.5 Reproductive potential per spikelet 106
5.3.6 Factors affecting seedset 107
5.3.6.1 Biotic factors 107
5.3.6.2 Abiotic factors 108
5.3.7 Factors affecting *C. purpurea* infection 110
5.4 Discussion 111
5.4.1 Seedset 111
5.4.2 *C. purpurea* infection 114
5.4.3 Seedset *vs C. purpurea* infection 116
5.4.4 Germination 117
5.4.5 Reproductive potential 118
5.5 Summary 119

Chapter 6 – Quantitative genetic differences in British populations of *Phragmites australis* 120

6.1 Introduction 120
6.2 Materials and methods 121
6.2.1 Plant material for experiments 121
6.2.2 Experimental Design 122

6.2.2.1 Experiment 1: nutrient-stressed conditions 122
6.2.2.2 Experiment 2: full-nutrient conditions 123
6.2.2.3 Experiment 3: synthetic sewage 125
6.2.3 Statistical analysis 128
6.2.3.1 Experiments 1 and 2 128
6.2.3.2 Experiment 3 129
6.3 Results 129
6.3.1 Experiment 1: *P. australis* grown in a nutrient-stressed environment 129
6.3.2 Experiment 2: *P. australis* grown in a full-nutrient environment 133
6.3.2.1 Above ground measurements 133
6.3.2.2 Below ground measurements 136
6.3.2.3 Above and below ground biomass 136
6.3.3 Experiment 3. The effects of synthetic sewage on nine *P. australis* populations 139
6.4 Discussion 144
6.5 Implications for reedbed restoration for conservation and use in constructed wetland systems 149

Chapter 7 – Investigation of intraspecific differences in sewage treatment performance of *Phragmites australis* in CWSs 152

7.1 Introduction 152
7.1.1 NH_4-N and NO_3-N removal 152
7.1.2 P removal 153
7.1.3 BOD removal 154
7.1.4 CWS design 154
7.1.5 Role of *Phragmites australis* in CWSs 155
7.1.6 Intraspecific variability in *P. australis* 156
7.1.7 Aims 156
7.2 Materials and methods 156
7.2.1 Plant material 156
7.2.2 Experimental design and procedure 157
7.2.2.1 Mini-constructed wetland system (mini-CWS) 157
7.2.2.2 Rhizome nutrient analysis 160
7.2.2.3 Synthetic sewage 161
7.2.2.4 Application of synthetic sewage 161
7.2.2.5 Influent and effluent nutrient analysis 162
7.2.2.6 Mass balance calculations 163
7.2.2.7 Evapotranspiration (E_T) 164
7.2.2.8 Above and below ground biomass 164
7.2.3 Statistical analysis 165
7.3 Results 166
7.3.1 The system 166
7.3.2 NH_4-N removal 170
7.3.3 NO_3-N removal 173
7.3.4 P removal 175
7.3.5 BOD removal 177
7.3.6 Correlation among nutrient removal, within populations 179
7.3.7 Evapotranspiration (E_T) from mini-CWSs 179
7.3.8 *P. australis* biomass differences amongst populations 181
7.4 Discussion 182
7.4.1 NH_4-N removal 182
7.4.2 NO_3-N removal 186
7.4.3 P removal 188
7.4.4 BOD removal 190
7.5 Summary 191

Chapter 8 – General Discussion 192

8.1 Introduction 192
8.2 Key research questions 192
8.2.1 What are the chromosomal races of British populations of *P. australis*? 192
8.2.2 What is the clonal structure of *P. australis* populations in Britain? 193
8.2.3 What is the population genetic structure of British populations? 194
8.2.4 What is the breeding system *of P. australis*? 195
8.2.5 Are there differences in seedset and germination rates amongst British populations of *P. australis*? 196
8.2.6 Are there quantitative genetic differences in morphology within and among populations of *P. australis* in Britain? 197
8.2.7 Are there genetic differences in ability to remove nutrients among populations? 198
8.3 Management recommendations 199
8.3.1 Reedbed Establishment 199
8.3.1.1 Choice of plant material 199
8.3.1.2 Source of seed 199
8.3.1.2.1 Creating a habitat for wildlife 200
8.3.1.2.2 Creating a habitat for a specific faunal species 200
8.3.1.2.3 Creating a habitat for thatching 200
8.3.1.3 Selection of the source population (s) 201
8.3.1.4 Collection of seed from the source population 202
8.3.1.5 Examining panicles for seed 202
8.3.2 Conservation of an existing reedbed 203
8.3.2.1 Assessing the genetic diversity of a reedbed 203
8.3.2.2 Recommended management practices to increase the genetic diversity of an old reedbed 205
8.3.3 Use of *P. australis* in CWSs 205
8.4 Future research 206
8.4.1 Reedbed conservation 207
8.4.1.1 Does the genetic diversity of a reedbed decline over time? 207
8.4.1.2 Do sexually recruited seedlings survive in an established *P. australis* population? 207
8.4.1.3 Do reedbeds with a high incidence of *C. purpurea* infection contain less invertebrate species? 208
8.4.2 Constructed wetland systems 208
8.4.2.1 Does the genetic diversity of a reedbed in a CWS decrease over time? 208
8.4.2.2 Do CWSs containing southern populations of *P. australis* remove more NH_4-N than northern populations when planted in northern locations? 208
8.4.2.3 What are the genetic differences responsible for variability in nutrient removal among populations? 209
8.4.2.4 Does ploidy level of *P. australis* affect nutrient removal? 209
8.4.2.5 Do other polymorphic species used in CWS show intraspecific genetic differences in nutrient removal? 209

Tables

Table 2.1 Details of 11 populations chosen to represent the natural resource of *P. australis* in Britain. 29

Table 3.1 Summary of strategies employed for sampling panicles and leaf collection in each population of *Phragmites australis*. 53

Table 3.2 Primer sequences, number of repeat units, overall population allele size range and maximum number of alleles per sample of five microsatellite loci of 11 British populations of *P. australis*. 60

Table 3.3 Total number of alleles, *Ho*, *He* and *f* of 11 British populations of *P. australis*. 61

Table 3.4 Genotype and number of individuals sampled of putative clones from 11 populations of *P. australis* and the estimated probability the estimated probability P_{gen} and P_{se}. 62

Table 3.5 Genotypic diversity, measured as the proportion of distinguishable genotypes in 11 *P. australis* populations. 65

Table 3.6 Within population genetic diversity in 11 British *P. australis* populations characterised by number of alleles detected, *Ho*, *He* and *f* for each loci and each population (mean values). 66

Table 3.7 Pairwise F_{ST} values, mean F_{ST} and geographical distances between 10 British populations of *P. australis* populations, excluding Loch Leven. 68

Table 4.1 Details of the twenty families of *P. australis* sampled from the Inner Tay reedbed for estimation of the mating system. 82

Table 4.2 Details of four microsatellite loci of *P. australis*. Allele size range, total number of alleles scored, observed and expected heterozygosity for each locus and over all loci in the eighteen families studied. 85

Table 4.3 Mating system parameters estimated using multilocus estimation of mating systems model (Ritland and Jain, 1981; Ritland, 2002). 86

Table 5.1 Weather stations recording temperature and rainfall data and approximate distance from each population. 98

Table 5.2 A Two-way ANOVA of seedset per spikelet, seedset per panicle and *C. purpurea* infection per spikelet amongst populations in 2002 and 2003. 100

Table 5.3 A One-Way ANOVA comparing mean percentage germination amongst populations. 105

Table 5.4 A One-Way ANOVA comparing mean reproductive potential amongst populations. 107

Table 5.5 Within population Pearson's correlation coefficient of mean seed set per spikelet versus mean *C. purpurea* infection per spikelet in 2002 and 2003 for 10 populations of *P. australis*. 108

Table 5.6 Regression analysis of mean seed set per spikelet in 2002 and 2003 *vs* latitude, site size, and mean maximum temperature and total monthly rainfall in August, September and October. 109

Table 5.7 Multiple regression analysis of mean seedset per spikelet in 2002 and 2003 *vs* mean maximum October temperature and site size. 109

Table 5.8 Regression analysis of mean *C. purpurea* infection per spikelet in 2002 and 2003 *vs* mean maximum temperature and total monthly rainfall in August, September and October. 110

Table 5.9 Multiple regression analysis of mean *C. Purpurea* infection per spikelet in 2002 and 2003 *vs* mean maximum temperature in August and October. 111

Table 6.1 Experiment 1. Results of a nested ANOVA of shoot length, shoot diameter and number of leaves of longest shoot and number of shoots,

amongst and within ten *P. australis* populations grown in nutrient-stressed conditions. 130
Table 6.2 Experiment 1. Mean values of longest shoot length, shoot diameter, number of leaves of longest shoot and number of shoots of ten populations of *P. australis* grown in nutrient-stressed conditions. 130
Table 6.3 Experiment 2. Results of a nested ANOVA of mean shoot length, mean shoot diameter and mean number of leaves of five longest shoot, total number of shoots and width/length ratio of longest leaf on longest shoot, amongst and within nine *P. australis* populations grown in a full-nutrient environment. 133
Table 6.4 Experiment 2. Mean values of shoot length, shoot diameter, number of leaves, number of shoots, width/length ratio of leaf, total rhizome length, number of rhizome internodes, mean diameter of internodes, length between internodes, number of rhizome buds of nine populations of *P. australis* grown in full-nutrient conditions. 134
Table 6.5 Experiment 2. Results of a nested ANOVA of total rhizome length, total number of internodes, mean diameter of two internodes and length between them and total number of buds, amongst and within populations. 137
Table 6.6 Experiment 2. Results of a nested ANOVA of total biomass of root, rhizome, below ground, above ground, and above/below ground biomass, amongst and within populations, October 2004. 137
Table 6.7 Experiment 2. Mean values of root, rhizome, total below ground, and above ground biomass and above/below ground biomass ratio of nine populations of *P. australis*. 138
Table 6.8 Experiment 3. Results of a Two-Way ANOVA testing the main effects of population and synthetic sewage treatment and a population x treatment interaction amongst nine populations of *P. australis* on mean shoot length, shoot diameter, total number of shoots, above and below ground biomass and above/below ground biomass ratio. 139
Table 6.9 Experiment 3. Population mean values of shoot length, shoot diameter, and number of shoots receiving no treatment or treatment with synthetic sewage. 141
Table 6.10 Experiment 3. Population mean values of above and below ground biomass, and above/below ground biomass ratio receiving no treatment or treatment with synthetic sewage. 142
Table 7.1 Layout of 15 mini-CWSs (5 populations x 3 replicates) in randomised block design. 159
Table 7.2 Total number of applications of synthetic sewage to mini-CWSs and length of cycles from 11 May 2004 to 14 April 2005. 161
Table 7.3 Three-way ANOVA of % and mass removal of NH_4-N and % NH_4-N removal over 3 and 9 day cycles and growing and dormant period, amongst populations. 170
Table 7.4 Mean % NH_4-N removal for 9 and 3 day cycles and growing and dormant periods. 173
Table 7.5 Three-way ANOVA of % and mass removal of NO_3-N over 3 and 9 day cycles and growing and dormant period, amongst populations. 174
Table 7.6 Mean % NO_3-N removal for 9 and 3 day cycles and growing and dormant periods. 175
Table 7.7 Three-way ANOVA of % and mass removal of P and % P removal over 3 and 9 day cycles and growing and dormant period, amongst populations. 176
Table 7.8 Mean % P removal for 9 and 3 day cycles and growing and dormant period. 177

Table 7.9 Three-way ANOVA of % and mass removal of BOD_5 and % BOD_5 removal over growing and dormant period, amongst populations. 177
Table 7.10 Mean % BOD_5 removal during growing and dormant period. 178
Table 7.11 Correlation coefficient of % nutrient removal (NH_4-N *vs* NO_3-N; NH_4-N *vs* P; NH_4-N *vs* BOD_5; NO_3-N *vs* P; NO_3 *vs* BOD_5; P *vs* BOD_5) within populations during whole experimental period. 179
Table 7.12 Three-way ANOVA of % evapotranspiration during total experiment and over growing and dormant periods amongst populations. 180
Table 7.13 One way ANOVA of above ground biomass, below ground biomass, total biomass and above/below ground biomass ratio amongst populations. 181
Table 7.14 Mean population above and below ground biomass, total biomass and above/below ground biomass ratio. 182
Table 7.15 Mean % NH_4-N and NO_3-N removal reported in this study and other studies, in horizontal surface, horizontal subsurface, or vertical flow, wetlands planted with *P. australis* or other species. 183
Table 7.16 Mean % P and BOD_5 removal reported in this study and other studies, in horizontal surface, horizontal subsurface or vertical flow wetlands planted with *P. australis* or other species. 189

Figures

Figure 1.1 Distribution of *Phragmites australis* in Britain. 6
Figure 1.2 Distribution of reedbeds in Britain. Map (a), reedbeds > 2 ha in area; map (b), reedbeds > than 20 ha in area. 7
Figure 1.3 Cross-section of horizontal flow constructed wetland system. 10
Figure 1.4 Diagram of *Phragmites australis* illustrating: floret, bearing stamen and stigma; culm; panicle; spikelet; lemmas enclosing floret; seed; ligule; rhizome. 13
Figure 1.5 *Phragmites australis* showing culms bearing purple panicles. 14
Figure 2.1 Location in Britain of the 11 reedbeds studied. 30
Figure 2.2 Insh Marsh. 31
Figure 2.3 Inner Tay. 32
Figure 2.4 Loch Leven. 33
Figure 2.5 St. Margaret's Marsh. 34
Figure 2.6 Mersehead. 35
Figure 2.7 Leighton Moss. 36
Figure 2.8 Strumpshaw Fen. 37
Figure 2.9 Lakenheath. 38
Figure 2.10 Walberswick. 39
Figure 2.11 Minsmere. 40
Figure 2.12 North Warren. 41
Figure 3.1 Root tip mitotic chromosomes (metaphase) of *P. australis*, $2n = 48$. 59
Figure 3.2 Banding pattern of three samples from the Inner Tay population for loci *Pa*GT16. 60
Figure 3.3 Map of spatial distribution of multilocus genotypes of the Inner Tay population. 63
Figure 3.4 Map of spatial distribution of multilocus genotypes comprising one genet (clone) of the Loch Leven population. 64
Figure 3.5 Regression analysis of pairwise F_{ST} and geographical distance among 10 British populations of *P. australis*. Loch Leven was excluded from the analysis. 68
Figure 3.6 Regression analysis of pairwise F_{ST} and geographical distance among 9 British populations of *P. australis*. Inner Tay and Loch Leven were excluded from the analysis. 69
Figure 5.1 Cut panicle from Walberswick population. 95
Figure 5.2 *a* spikelet containing 4 florets, *b* spikelet containing an ergot of *Claviceps purpurea, c* one seed removed from floret. 95
Figure 5.3 Mean seedset per spikelet for 11 populations. 100
Figure 5.4 Interaction plot of mean seedset per spikelet per population in year 2002 and 2003. 101
Figure 5.5 Mean seedset per panicle in 11 populations of *P. australis*. 102
Figure 5.6 Mean panicle length of 11 populations of *P. australis* in 2002 103
Figure 5.7 Mean *C. purpurea* infection per spikelet in 11 populations of *P. australis*. 104
Figure 5.8 Mean percentage germination rate of 10 populations. 105
Figure 5.9 Mean reproductive potential per spikelet of 10 populations. 106
Figure 6.1 Washed rhizomes of *P. australis*, separated from roots. 125
Figure 6.2 Experiment 3. *P. australis* populations arranged in randomised block design, August 2004. 126
Figure 6.3 Experiment 1. Mean shoot length *vs* latitude for ten populations of *P. australis*. 131

Figure 6.4 An ordination plot of values of principal components PC 1 and 2 for each population of *P. australis* for Experiment 1 and Experiment 2. 132
Figure 6.5 Experiment 2. Regression analysis of mean shoot length, total above ground biomass, total root biomass, total below ground biomass *vs* latitude. 135
Figure 6.6 Mean shoot length of each population in each environment against mean shoot length of all populations in stressed and full-nutrient environment. 136
Figure 6.7 Experiment 3. Interaction plots showing the effect of synthetic sewage application on: mean number of shoots; mean below ground biomass; mean above/below ground biomass ratio, for each population. 143
Figure 7.1 Cross section of a mini-CWS, composed of a 72 l plastic container fitted with a tap at the base and filled with a substrate of graded material. 158
Figure 7.2 Experimental mini-CWSs after one year's growth of *P. australis*. 159
Figure 7.3 Mean nutrient concentrations in effluent from each population, sampled after every cycle from 11 May 2004 - 14 April 2005. 167
Figure 7.4 % NH_4-N removal for each replicate per population (11 May 2004 - 14 April 2005). 168
Figure 7.5 Redox potential measured every hour in each of the five populations in the mini-CWSs, 16 - 23 August 2004. 169
Figure 7.6 Mean % nutrient removal for each population over time 11 May 2004 - 14 April 2005. 171
Figure 7.7 Mean total % nutrient removal by population over the experimental period 11 May 2004 - 14 April 2005. 172
Figure 7.8 Mean % evapotranspiration for each population, from 11 May 2004 - 14 April 2005. 180

References 211

Chapter 1 - Introduction

1.1 BACKGROUND

Until relatively recently, active plant population management has been based largely on intuition rather than on empirical or experimental scientific data. However it is becoming increasingly apparent that to domesticate and make use of new plant species to achieve specific management objectives, such as conservation, habitat restoration, environmental amelioration or production, an understanding is required of all aspects of the species' biology and ecology. To utilise and domesticate a wild plant the following biological and ecological features of a species are of particular importance.

1. *The identity of biological species within the taxon under consideration*

Knowledge of biological species boundaries is fundamental to the process of domestication because it is only within biological species that interbreeding is possible. The biological species concept has been defined by Mayr (1942) as:

"Groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups"

Reproductive isolation (the complete absence of interbreeding between individuals from different populations, should they encounter one another) (Schluter, 1998) is determined fundamentally by the extent of karyotypic differences (chromosomal variation) between individuals. For sexual reproduction to occur, homologous chromosomes (derived from one male and one female gamete) become paired and exchange genetic information in meiosis. A mismatching of chromosomes results in irregular disjunction in meiosis and infertility. Since up to 50 % of all angiosperms are polyploids (containing more than two genomes per cell) (Soltis and Soltis, 2000), chromosome mispairing and associated sterility may occur in a species with odd numbered polyploids (e.g. triploids and pentaploids) as well as when different ploidy levels occur together and interbreed. Therefore it is essential to know the extent of chromosomal races within the targeted species, otherwise unforeseen infertility may arise when chromosomal races are inadvertently mixed. This may frustrate attempts

to effectively use the species to form self-sustaining populations for restoration, conservation or production purposes.

2. *The nature of genetic individuals within a population*

Many plant species, in addition to producing seed, reproduce asexually by vegetative reproduction. Therefore numerous individuals within a population (i.e. ramets) may have the same genetic composition (e.g. belong to one clone or constitute one genet). This has profound effects on the genetic diversity of a population. The size and distribution of clones must also be known if effective sampling strategies are to be designed for obtaining germplasm (the genetic material of a species) within populations.

3. *The breeding system of the species*

There are three basic breeding mechanisms employed by plants, which are not mutually exclusive: outcrossing (the fusion of male and female gametes from different genetic individuals), selfing (fusion of gametes from a single genetic entity) and apomixis (reproduction without fertilization, which can be by production of vegetative parts or seeds without sexual fusion) (Jarne and Charlesworth, 1993; Briggs and Walters, 1997). Knowledge of the breeding system is essential for collection of the appropriate material for plant propagation. For example in a highly outcrossing, clonal species it would be undesirable to establish a population by vegetative propagation, as this would decrease the potential genetic diversity of the population and increase the probability of compatible pollen limitation and infertility.

4. *Factors determining seed production of the species*

An intimate knowledge of the abiotic and biotic factors influencing seed production is necessary to ensure reliability in collecting material for propagation, particularly when human resources are limited.

5. *The range of genetic variability found within and between populations*

Information is needed both in terms of adaptive (quantitative traits found by common garden trials) and selectively neutral traits (i.e. those with little or no effect on the phenotype and commonly determined by molecular marker techniques). Adaptive variation manifests itself as variation in phenology, morphology, and growth and

resistance to pathogens (Ennos *et al.*, 1998), and is determined by selective forces acting on the fitness of individuals. Molecular markers reveal the extent and patterns of gene flow and hence genetic diversity within and between natural populations. Knowledge of the genetic resources of the targeted species, for both neutral and adaptive traits, can ensure that the correct choice of plant material is made for both restoration work and commercial harvesting, and that genetic processes occurring in natural populations (e.g. genetic recruitment, gene flow) are replicated in managed populations.

6. *The range of variability found between and within populations for characters that are important for specific types of management*

It is often the case that characters not specifically selected for in natural populations are required in managed populations. It is important to study the range of genetic variation for these utilisation characters in natural populations. Once intra and inter specific variability in such traits are quantified, appropriate individuals can be selected for specific management objectives. Examples include selection of tree genotypes that maximise commercial timber production, or selection of plant genotypes for constructed wetland systems that are most efficient in promoting nutrient removal during water treatment.

Much of the required knowledge, essential to the successful domestication of wild species, has been learned through crop cultivation (e.g. wheat, rice) and from the cultivation of forest tree species for forest habitat restoration, conservation of endangered species, and use in timber production. Another important plant species to humans, that is dominant in reedbed habitats, is the common reed *Phragmites australis*. The effective management of this plant is necessary for the restoration and creation of reedbeds for conservation, as well as for specific management uses in constructed wetland systems and commercial production in the thatching and paper industry.

1.2 REEDBEDS

1.2.1 Definition

Reedbeds are wetlands dominated by stands of *Phragmites australis* in which the water table is at or above ground level for most of the year. They tend to incorporate areas of open water and ditches, and small areas of wet grassland and carr woodland may also be associated with them (EN, 2003).

In hydroseral development (i.e. the sequence of plant communities occurring during the change from shallow open water to bog or forest) (Martin and Hine, 2000), reed domination is a short-lived phase (unless there is management intervention to maintain the sere) and, over time the plant communities change from open water to marshlands, carr and woodland (Bibby and Lunn, 1982).

1.2.2 Occurrence

Reedbeds are commonly found in low-lying areas intermittently or permanently flooded with shallow still water, such as the littoral zones of lakes, along rivers and irrigation/drainage ditches and shallow freshwater swamps. In addition, *P. australis*, being tolerant of brackish waters, is often found in coastal plains and alongside estuaries (Haslam, 1972; Brix, 1999). Some of the largest reedbeds in the world are found in Europe (e.g. the Danube Delta, Romania and Lake Fertó, Hungary) (Brix, 1999).

1.2.3 Reedbed importance

Reedbeds serve a number of socio-economic and conservation functions (Hawke and José, 1996).

1.2.3.1 Plant material

Reed stems are commercially harvested in some parts of the world and used for roofing, fencing and in cardboard and paper manufacture (Haslam, 1972; Brix, 1999). Reeds have been planted to stabilise river and canal banks (Haslam, 1972; Coops *et al.*, 1996). Wave energy is dissipated through the above ground vegetation,

and the bank slope is stabilised by the enormous network of roots and rhizomes reinforcing the soil. An early example of this use dates back to the 1800s, when it is believed that prisoners from the Napoleonic wars planted reed (from the Netherlands) along the banks of the Inner Tay estuary (located in Scotland), to protect the mudflats from erosion (Hawke and José, 1996).

1.2.3.2 Ecological value

P. australis dominated wetlands have great ecological value because they support a number of specialised groups of fauna (Bibby and Lunn, 1982; Hawke and José, 1996; Poulin *et al.*, 2002). For example, rare avian species such as bittern (*Botaurus stellaris*) and moustached warbler (*Acrocephalus melanopogon*), both listed in Annex I of the EC Birds Directive (Europa, 2003), as well as great reed warbler (*Acrocephalus arundinaceus*) which is considered a vulnerable species in Europe, are dependent on reedbeds for their breeding habitat (Bibby and Lunn, 1982; Poulin *et al.*, 2002).

1.2.3.3 Wastewater treatment

Natural wetlands, with the prevalence of macrophytes, have the ability to remove pollutants from water (Brix, 1994; Ansola *et al.*, 1995). Constructed wetland systems (CWSs) are man-made systems that aim to simulate the processes that occur in natural wetlands (Cooper *et al.*, 1996). They came to prominence in the early 1980s and have become popular as they provide a simple and economical way to remove pollutants from wastewater. In Europe, where *P. australis* is the plant of choice, reedbeds are an integral part of CWSs and have been used to treat urban run off (Shutes, 2001) landfill leachate (Peverley *et al.*, 1995), mine water from abandoned coal/metal mines (Ye *et al.*, 2001), agricultural run-off (Kern and Idler, 1999) and secondary and tertiary treated municipal sewage effluent (Greenway and Woolley, 1999).

1.3 REEDBED RESOURCE IN BRITAIN

1.3.1 Distribution

Within Britain *P. australis* is native and widespread, being most abundant in the south and east and more scattered in the north and west (Haslam, 1972) (Figure 1.1).

Figure 1.1 Distribution of *Phragmites australis* in Britain. Each dot represents at least one record of *P. australis* in a 10 km square of the National Grid (Preston *et al.*, 2002).

Most reedbeds occur in river floodplains, low-lying coastal plains and estuaries (Self *et al.*, 1996). Two types of reedbed are generally recognised in Britain: reed swamp, (National Vegetation Classification (NVC) S4) and reed fen (NVC S26) (Rodwell, 1995). In the former the soil is permanently waterlogged and the summer water level is *c.* 20 cm above ground and, in these conditions, the wetlands are often composed of dense pure stands of reed. In reed fen, the water level is at or below the surface in the summer and the reedbeds are botanically more diverse (Rodwell, 1995).

An inventory of reedbeds in Britain 1993 recorded 922 sites > 2 ha covering a total area of *c.* 6,524 ha (Figure 1.2) (Gilbert *et al.*, 1996). For the purposes of this inventory, a reedbed was defined as: 'a site containing areas dominated by, but not necessarily purely composed of the reed species *P. australis*'. The greatest density of reedbeds is located in the low-lying coastal plains of East Anglia, with Norfolk and Suffolk containing 36 % of the total recorded in Britain. However, the largest contiguous reedbed, the Inner Tay, is located alongside the Tay Estuary in Scotland (410 ha) (Gilbert *et al.*, 1996), whilst the largest in England is Walberswick (210 ha) on the Suffolk coast (Adam Burrows, English Nature, pers. comm. 2003).

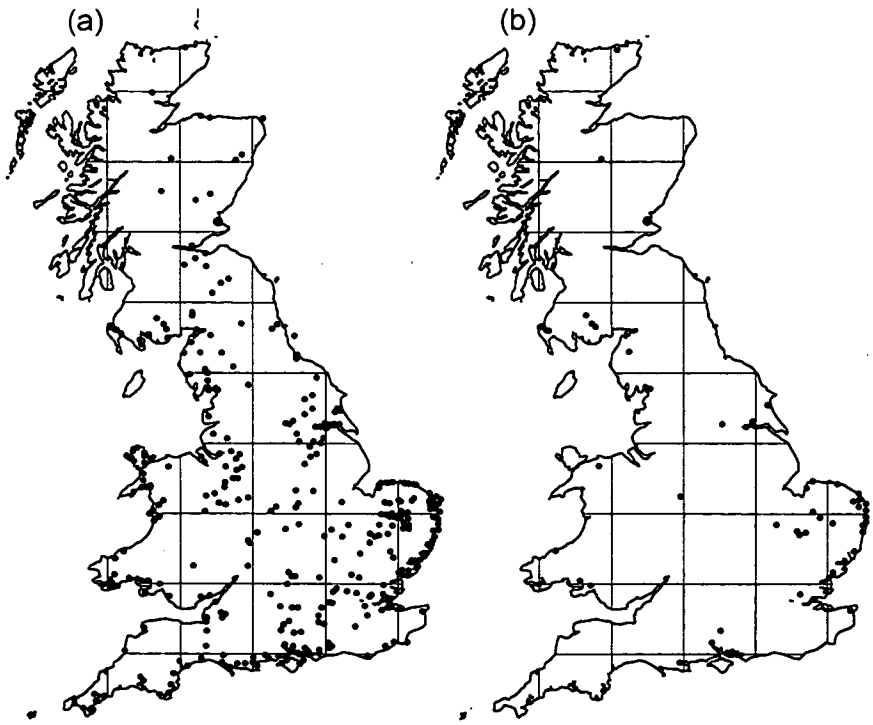


Figure 1.2 Distribution of reedbeds in Britain. Map (a), reedbeds > 2 ha in area; map (b), reedbeds > than 20 ha in area (Gilbert *et al.*, 1996).

1.3.2 Reedbed decline

Overall, there has been a steady decline in reedbeds in Britain, with losses of 10 - 40 % by area recorded between 1945 - 1989 (Gilbert *et al.*, 1996). This figure disguises the fact that some of the remaining reedbeds have experienced serious neglect due to a lack of management which has resulted in habitat deterioration, as well as a decline in reed quality (Hawke and José, 1996). Reasons for reedbed losses include land use change (usually drainage for agriculture), insufficient management (such as rotational cutting and/or burning of old standing vegetation) to arrest natural succession, pollution and water abstraction for domestic, industrial and agricultural purposes (resulting in the reedbed drying out) (Wynne *et al.*, 1995; Self *et al.*, 1996). Reedbeds are far less extensive in Britain than other habitats of conservation concern such as Caledonian pine forest (16,000 ha) (Tuley, 1995), lowland heath (60,000 ha) (Farrell, 1989; cited in Gilbert *et al.*, 1996) and lowland raised bogs (69,400 ha) (Lindsay *et al.*, 1996; cited in Gilbert *et al.*,. 1996).

1.3.3 Reedbed importance

The reedbeds of Britain are geographically important in a global context as they are some of the farthest west in Europe and, although they are considerably smaller than those found elsewhere (e.g. in the Netherlands and Czech Republic), they have high conservation value as they contain a great range of habitats and high numbers of species per hectare (Hawke and José, 1996).

1.3.3.1 Conservation value

In Britain, reedbeds have high conservation value. At least 700 species of invertebrates are associated with reedbeds and 40 species of insect feed solely on reed (Hawke and José, 1996). These include seven Red Data *Lepidoptera* (recorded in 15 or fewer 10 x 10 km squares in Britain), e.g. the Reed leopard moth (*Phragmataecia castanea*), three Red Data *Hymenoptera,* e.g. a solitary wasp (*Rhopalum gracile*) and two Red Data *Diptera* e.g. a hoverfly (*Sphaerophoria loewi*) (Gilbert *et al.,* 1996). However, it is the dependence of rare bird species which is most documented. For instance bittern, requires extensive reedbeds for breeding (> 20 ha) incorporating large areas of thick cover and open pools and ditches (Gibbons

et al., 1996). Marsh harrier (*Circus aeruginosus*), another Red Data species, is dependent on large reedbeds (> 40 ha) for breeding and prefers inland floodplain mires. Migrating birds, such as the sedge warbler (*Acrocephalus schoenobaenus*), although not rare, rely solely on reedbeds for autumn-fattening on the aphid *Hyalopterus pruni* before migration to sub-Saharan Africa (Bibby and Lunn, 1982). Mammals of conservation concern such as water vole (*Arvicola terrestris*), which has experienced a major decline, and otter (*Lutra lutra*), an endangered species in England and Wales, are also associated with reedbeds, as well as other aquatic habitats (Gilbert *et al.*, 1996; Hawke and José, 1996).

1.3.3.2 Economic value

Reed has been used as a roofing material in Britain for many centuries (Haslam, 1972), for example over 90 % of Norfolk's medieval churches were once thatched (Cooper, 1972; cited in Hawke and José, 1996) and the commercial management of reedbeds for thatching material has been a major factor in their maintenance in Britain (Bibby and Lunn, 1982). In 1989, the total UK thatching reed production was estimated at 330,000 bundles (*c* 200 reed stems per bundle), with *c.* 64 % from managed sites with high conservation value, while approximately 1.5 million bundles were imported from Europe (Bateman, 1990; cited in Hawke and José, 1996).

The use of reed for thatching declined in the last century, although the industry still employs some 1,000 people (Hawke and José, 1996). For thatching purposes, reed is cut in winter either annually (single wale) or biennially (double wale). The advantages of the latter are that the reedbed has a period of 'rest' and higher yields are obtained than for single wale (Hawke and José, 1996). The two largest reedbeds in Britain (Inner Tay and Walberswick) have a history of commercial cutting, although commercial production has recently ceased at the former, due to competition from cheaper reed imports from abroad.

1.3.3.3 Constructed wetland systems

Whilst natural wetlands have been declining, there has been a growth in constructed wetlands, and there are now over 400 in Britain (Cooper *et al.*, 1996) and virtually all the water utilities use CWSs for the treatment of sewage. Most systems are small and

deal with polluted water from point sources such as domestic sewage, and agricultural and industrial effluent (Hawke and José, 1996). CWSs are a complex assemblage of water/wastewater, macrophytes (aquatic plant spp, mainly *P. australis*) embedded in a substrate, invertebrates and micro-organisms (Figure 1.3).

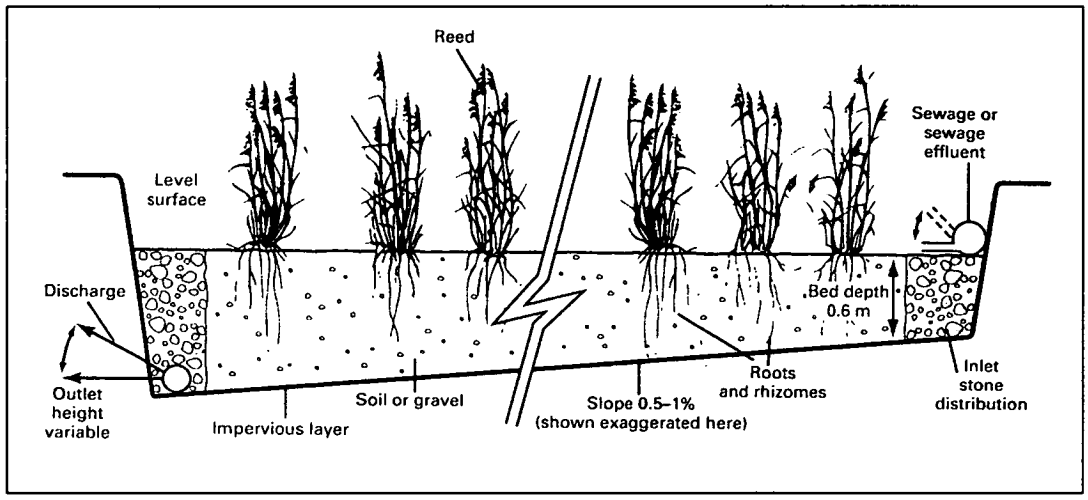


Figure 1.3 Cross-section of horizontal flow constructed wetland system (Cooper *et al.*, 1996).

Wastewater passes over and percolates through the reedbed and nutrients are removed below ground by a complex interaction of physical and biochemical processes. *P. australis* influences nutrient removal directly through nutrient uptake (temporary storage only) and indirectly by the below ground environment created by its extensive rhizome/root system. This facilitates microbial growth (rhizomes provide a large surface area for microbial attachment and root oxygen leakage ensures conditions are favourable for aerobic microorganisms, such as nitrifying bacteria) and water/wastewater filtration (Brix and Schierup, 1990; Gray and Biddlestone, 1995; Cooper *et al.*, 1996).

1.3.4 Reedbed restoration in Britain

There have been two driving forces behind the restoration of reedbeds in Britain. Firstly, following the Earth Summit held in Rio de Janeiro, 1992 (United Nations, 1997) the UK Government signed the Convention on Biological Diversity, committing it to the development of strategies and policies to protect and enhance wildlife and habitats in the UK. As a result, *Biodiversity: The UK Action Plan* was

launched in 1994. Included in this was a strategy for the maintenance, restoration and creation of new reedbeds under the *Fen, Carr, Marsh, Swamp and Reedbed Habitat Statement* (HMSO, 1994). In addition, an action plan for bitterns was formulated because numbers had plummeted from nearly 80 males in the early 1950s to 20 in 1995, mainly because of reedbed decline (Hawke and José, 1996). The objective of the plan was to increase the number of males, to *c.* 50 by the year 2010. To reach this target the creation of at least 1,200 ha of reedbeds in blocks greater than 20 ha was proposed.

Secondly, non-governmental organisations (NGOs), such as the Royal Society for the Protection of Birds (RSPB), produced an agenda for conservation in the UK: *Biodiversity challenge* (Wynne *et al.*, 1995). This document contained conservation objectives and detailed targets for species and habitats that included bittern and *Swamp Fen and Carr*, respectively. As a result, the conservation and creation of reedbeds by voluntary and statutory organisations reedbeds has become a priority (Hawke and José, 1996).

To successfully manage reedbeds in Britain, sufficient scientific knowledge of *P. australis* is needed to enable reedbed establishment and conservation to be carried out in an efficient and sustainable way. Furthermore, a thorough understanding of the resource will also ensure that the correct choice of plant material was used in CWSs.

The following section reviews the general biology and ecology of *Phragmites australis,* the dominant species of reedbeds and the most commonly used species for the establishment of CWS. This is followed by a review of the specific features of *P. australis* that are relevant to its use by humans. Gaps in the knowledge of the species will be highlighted, emphasising those that relate to reedbed management in Britain.

1.4 REVIEW OF THE BIOLOGY AND ECOLOGY OF *P. AUSTRALIS*

1.4.1 Taxonomy

The genus *Phragmites* belongs to the Tribe Arundineae and consists of four species, of which *Phragmites australis* (Cav.) Trin. ex Steudel is the most economically important and widespread (Clevering and Lissner, 1999). Within *P. australis* a large number of intraspecific varieties have been identified which are described as local races or ecotypes. *P. australis* belongs to the family Poaceae (Haslam, 1972). Two purported subspecies have been identified by the shape of the upper glume (much reduced leaf at the base of the grass flowering head) (Clayton, 1967; cited in Clevering and Lissner, 1999): *P. australis* ssp. *altissimus* (Benth.) (found on the shores of the Mediterranean, Iran, and some parts of Africa only) and *P. australis* spp. *australis* (Cav.) (widespread in the temperate regions of both hemispheres) (Clevering and Lissner, 1999). The other three recognised species in the genus *Phragmites* are *P. karka* (Retz.) Trin. ex Steudel found in parts of Australia, tropical Asia and Africa; *P. mauritianus* Kunth found in tropical regions of Africa and the Mascarene Islands (group of islands in the Indian Ocean); and *P. japonicus* Steudel. found in the Far East, Japan and China (Clevering and Lissner, 1999).

1.4.2 Distribution

P. australis (Cav.) Trin. ex Steudel is a cosmopolitan species: Its northern temperate limit is in Finmark, Norway (70.48° N) and from there it extends to the southern temperate zone. It is less abundant in North America, uncommon in the tropics and not native in New Zealand, parts of Australia, the Amazon Basin and extreme South America (Haslam, 1972).

1.4.3 Description

P. australis is a tall reed (*c.* 1 - 6 m high), which produces annual cane-like shoots and spreads by stout creeping perennial rhizomes (underground stem) and stolons (above ground prostrate or creeping stem) (Fig. 1.4 and Fig. 1.5). The smooth rigid

stems (culms) are erect, usually unbranched, closely sheathed and many noded. The smooth, flat, deciduous leaves (blades) are variable in colour (greyish-green/bright green), *c.* 2 - 45 mm wide and taper to long slender points (*c.* 20 - 60 cm long) (the width and length of blade is affected by ploidy level). The ligule (out growth at the inner junction of the leaf-sheath and blade) is composed of a dense fringe of short hairs.

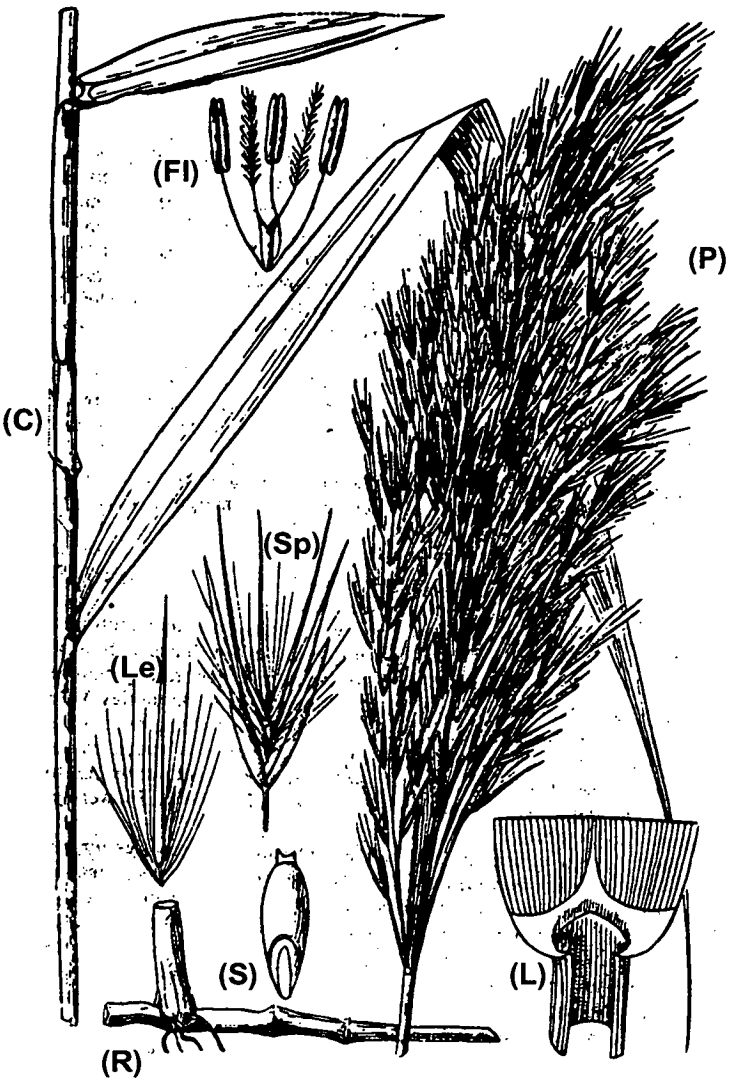


Figure 1.4 Diagram of *Phragmites australis* illustrating: (Fl) floret, bearing stamen and stigma; (C) culm; (P) panicle; (Sp) spikelet; (Le) lemmas enclosing floret; (S) seed; (L) ligule; (R) rhizome (Hubbard, 1992).

The branched flowering head (panicle) is purplish to brown in colour, *c.* 15 - 40 cm long. The branches are mainly smooth and hairy at intervals and bear lance-shaped spikelets (unit of grass flower head, bearing one or more flowers), *c.* 10 - 16 mm long, containing 2 - 6 flowers (florets). Each floret, except for the male (the lowest floret), has a basal tuft of long silky hairs and bears three stamen, *c.* 1.5 - 2 mm long, and short styles. The seed (caryopsis or grain of grasses) is smooth and enclosed by a thin lemma and palea (bracts of grasses) (Haslam, 1972; Hubbard, 1992). The seed is shed enclosed in the bracts and is plumed (i.e. has white silky hairs) (Haslam, 1972).

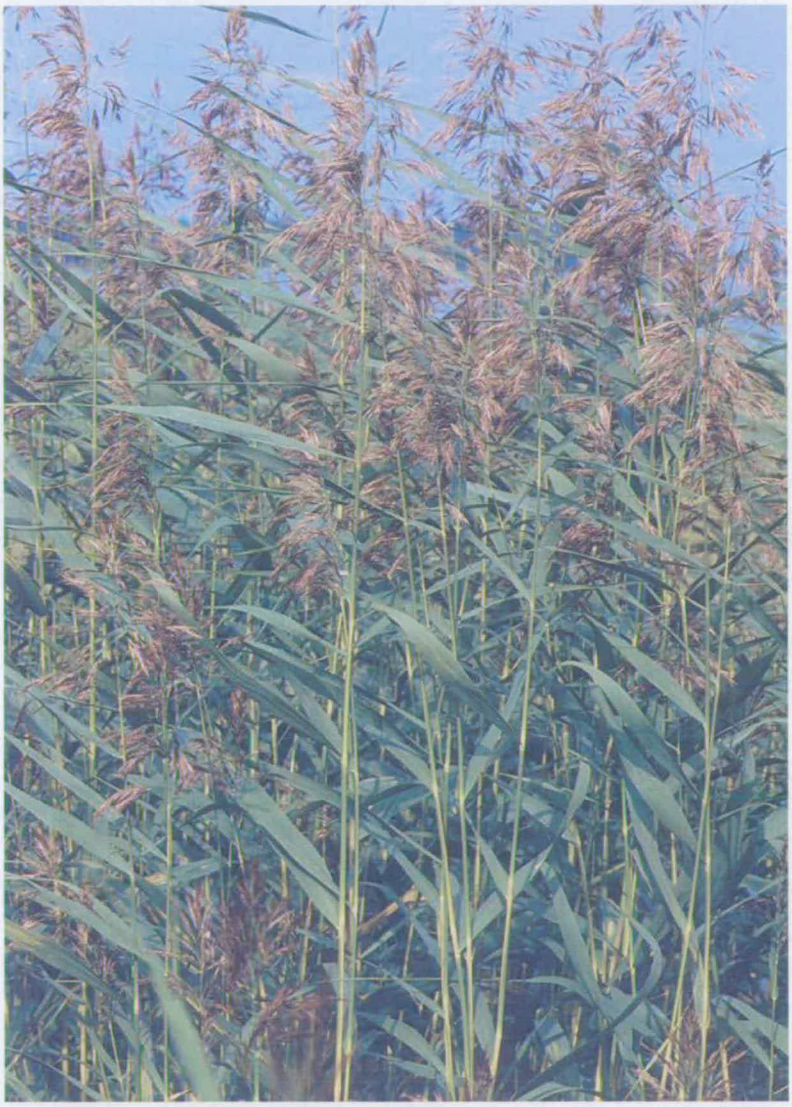


Figure 1.5 *Phragmites australis* showing culms bearing purple panicles.

1.4.4 Habitat

P. australis has a broad ecological aptitude and, although the species cannot withstand much water movement, it is tolerant of a wide range of environmental conditions (van der Toorn, 1972; Brix, 1999). *P. australis* populations are found in habitats with variable water regimes such as tidal fluctuations of *c.* 1.25 m, annual fluctuations (non-tidal) *c.* 3 m and in drier habitats in which the below ground water table is tapped by rhizomes and roots which can penetrate to depths of > 2 m (Haslam, 1970b). The above ground water depth that *P. australis* can tolerate appears to be limited by nutrient availability rather than intolerance to prolonged water logging (Haslam, 1970b). Since the leaves rot under water, the plant must grow sufficiently to maintain one third of shoot height above the water for photosynthesis (Haslam, 1972). Furthermore, the allocation of resources to increase shoot height, rather than to below ground parts may result in decreased anchorage in the sediment and a lowered carbohydrate reserve (Vretare *et al.*, 2001).

Generally the tallest reedbeds are found in warm wet and eutrophic habitats such as the Danube Delta (*c.* 6 m tall) (Haslam, 1972). There is a positive relationship between height and temperature, and the tallest reedbeds occur in habitats with the highest summer temperatures (Haslam, 1972) (although other factors such as nutrient status will also impact on shoot height). Shoots cannot grow in cold weather and are killed by severe frost. Reed stands are tall and dense in nutrient rich habitats and are short and sparse in nutrient poor habitats. *P. australis* is tolerant of a wide range of substratum pH (3.6 - 8.6), but is most productive when the substratum pH is 5.5 - 7.5 (Haslam, 1972). *P. australis* is moderately tolerant of saline water and sediment but is limited when salt (Na^+) concentrations in water exceed 670 mM (Matoh *et al.*, 1988; cited in Hellings and Gallagher, 1992). High salt concentrations decrease or prevent bud formation and stands of *P. australis* in salt marshes, although they may be extensive, often consist of a sparse cover of short shoots (Haslam, 1969). In addition, shoots may be sterile at the salt tolerance limit (Haslam, 1972).

To maintain the reed stage of the hydrosere, aerial parts must be regularly removed to prevent litter build up, which causes drying out of the reedbed. Winter removal of the aerial parts (e.g. by cutting and/or burning) for commercial (e.g. thatching) or for

conservation purposes is the least damaging because the carbohydrates from the leaves and stems have already been relocated to the rhizomes.

1.4.5 Flood tolerance

In waterlogged soils, root respirational oxygen demand is not met by soil air because of oxygen depletion (oxygen used up as a terminal electron acceptor). The anaerobic environment also causes a decrease in the redox potential (to below 200 mV) and, in these reduced conditions, potentially harmful toxins such as ethanol, methane, hydrogen sulphide and reduced forms of iron and manganese are present (Vartapetian and Jackson, 1997). Plants growing in such habitats must not only be able to transport atmospheric oxygen to the roots, but must also ensure that the immediate root environment is oxidized, to avoid uptake of toxic soil components (Gries *et al.*, 1989). *P. australis*, like other wetland plants, has physiological and anatomical attributes that enable it to grow and survive in waterlogged soils. For instance, large internal airspaces (aerenchyma) enable oxygen transport to the root and rhizome system, and oxygen leakage from the roots aerates the rhizosphere (Armstrong *et al.*, 1992; Brix, 1994).

1.4.6 Colonisation

The initial establishment of a reedbed is either by sexual recruitment from seed (wind/water/animal dispersed) or asexual through vegetative propagules (water/animal dispersed). Although germination proceeds over a wide range of temperatures (10 - 30 °C) it is often poor in the field and is inhibited by mineral poor soils, low temperatures and frost and high NaCl concentrations (such as found in sea water). For instance, van der Toorn (1972) found that there was a 50 % decrease in germination rate when seeds from different populations of *P. australis*, from the Netherlands were germinated in solutions containing > 60 % sea water.

Young seedlings may be killed by frosts, salt in any but low concentrations, flooding and desiccation or shading (often by existing vegetation) (Haslam, 1972). Soil nutrient status also affects growth and seedlings grown in peat are stunted compared to those grown on mineral soils (Haslam, 1970a). In general, young seedlings are

more likely to be killed by edaphic and climatic conditions that would be tolerated by adult plants.

1.4.7 Seed recruitment

In an established reedbed, seed recruitment is rare because of poor seedset and germination rates and competition for resources between seedlings and established clones (mainly for light) (Haslam, 1972; Clevering and Lissner, 1999). Therefore, sexual recruitment only occurs after disturbance such as clearing of existing vegetation or water drawdown. It has been suggested that clonal diversity decreases in reedbeds over time (Koppitz *et al.*, 1997), because of the paucity of sexual recruitment after initial colonization and the endurance of only those clones which are adapted to the edaphic, hydrological and climatic conditions.

1.4.8 Reproduction

1.4.8.1 Asexual reproduction

P. australis spreads within a reedbed clonally, by rhizomatous growth. Each spring, aerial shoots emerge from buds close to the substrate surface, which have grown from the base of the previous year's horizontal and vertical rhizomes. Buds are formed during the preceding late summer, when the underground carbohydrate reserves are at a maximum after translocation of nutrients from the aerial parts (Rodwell, 1995). Annual shoots, which bear the deciduous leaf blades, harden during the summer when nutrients are recycled into the rhizomes and, with the onset of senescence, become brittle and die, although they may remain standing for a number of years (Haslam, 1972). The length of the growing season is dependent on temperature and is longer in warmer climates (Haslam, 1972), where shoots can live for two years before die-back. In Britain the growth cycle is usually completed between April and September (Haslam, 1972). Rhizomes can attain considerable length (up to 15 m) and thickness (4 - 5 cm) (Nikolajevskij, 1971) and, after about three to seven seasons, the rhizome system begins to die from behind (Haslam, 1972). Numerous roots are formed in the rhizome nodes and are borne mainly on

vertical rhizomes, whilst adventitious roots are formed in the lower submerged parts of the aerial shoots (Haslam, 1972; Nikolajevskij, 1971).

1.4.8.2 Sexual reproduction

Since *Phragmites australis* is a clonal species, it is reasonable to ask why sexual reproduction is maintained. McLellan *et al.* (1997) hypothesised that a small amount of sexual reproduction allows enough recombination to preserve genotypic variation and improve fitness. In addition, wind spread seed affords a better dispersal mechanism to escape intraspecific competition and adverse environmental conditions than vegetative propagules (Eriksson, 1997).

Flowering occurs in late summer, when panicles develop mainly on the taller stems (Haslam, 1972) (e.g. from August to early September in Britain) and pollen dispersal is anemophilous. *P. australis* is partially self-incompatible (Ishii and Kadono, 2002) and therefore compatible pollen limitation may arise where large clones are found because pollen dispersal has a leptokurtic distribution (Proctor *et al.*, 1996). Seed maturation is completed by late November and wind dispersal of the seed occurs from winter to early spring (Haslam, 1972). Seed dispersal in wind and water over a distance of at least 25 km has been demonstrated in the polders in the Netherlands (van der Toorn, 1972).

1.5 ESSENTIAL FEATURES OF *P. AUSTRALIS*

The next section describes the specific features of *P. australis* which are relevant to its use by humans, and identifies what is known and the areas that still need to be investigated for the successful management of the species in Britain. Gaps in the knowledge of this species form the basis of this research.

1.5.1 Chromosomal races

Determining the chromosomal races of the resource to be utilised is essential for two main reasons. Firstly, ploidy levels affect seed production; Björk (1967) found that a

hexaploid *P. australis* population in Sweden produced only empty shrunken seeds, which he attributed to meiotic disturbance. Secondly, ploidy levels may account for large morphological and ecotype differences in plant species (Les and Philbrick, 1993). Paucã-Comãnescu *et al.* (1999) found 'morphological gigantism' in *P. australis* octoploids from the Danube Delta. Moreover this chromosomal race only occurred in freshwater habitats, whilst tetraploids dominated more saline and peat habitats.

P. australis is an allopolyploid (combining genomes from more than one ancestral species) (Leitch and Bennett, 1997) of ancient origin. The basic chromosome number of *P. australis* is $x = 12$ (Clevering and Lissner, 1999), although the original diploid form ($2n = 24$) is considered to be lost (Gorenflot *et al.*, 1979; cited in Clevering and Lissner, 1999). In *P. australis*, both euploids (more than two complete sets of chromosomes in the nucleus) and anueploids (in which whole chromosomes have been added or deleted from the balanced number of chromosomes expected) have been found (Clevering and Lissner, 1999). Across its world-wide distribution ploidy varies from $3x$ to $12x$ with tetraploids ($2n = 4x = 48$) dominating in the west and octoploids ($2n = 8x = 96$) mainly occurring in the east (Gorenflot, 1976; cited in Clevering and Lissner, 1999). The most frequently observed ploidy level is tetraploid, followed by octoploids and hexaploids ($2n = 6x = 72$) (Paucã-Comãnescu *et al.*, 1999). Mixed cytotype populations have been found in France ($3x$ - $4x$), Morocco ($4x$ - $6x$ - $8x$), Romania ($4x$ - $6x$ - $8x$) and Sweden ($4x$ - $6x$) (Gorenflot, 1976; cited in Clevering and Lissner, (1999). In North America, the native *P. australis* has been karyotyped as $3x$, $4x$ or $6x$, although ploidy levels may have been underestimated as octoploids have recently been found (Keller, 2000).

The ploidy levels of British *P. australis* populations have not been extensively researched. Only four populations have been karyotyped (River Humber, Severn Estuary and Thamesmead populations in England and one Scottish population located in the Inner Tay Estuary) (Clevering and Lissner, 1999) and all were found to be tetraploids. Therefore one of the aims of this study was to determine the chromosomal races of British population of *P. australis*.

1.5.2 Clonal structure

It is important to understand the variability in clone structure (spatial arrangement and clonal diversity) (Reusch *et al.*, 2000) among populations of *P. australis* because this will influence reedbed selection and strategies for panicle collection for seed harvesting, as well as determining the sampling strategy for panicle collection within a reedbed. In addition, reedbed clonal diversity (and hence genetic diversity) is influenced by the type of plant material (seed or vegetative propagules) used for establishment.

Clone structure varies among populations of *P. australis*. Differences in clonal diversity has been found in populations located in Germany (Neuhaus *et al.*, 1993; Zeidler *et al.*, 1994), Denmark (Koppitz *et al.*, 1997), North America (Hauber *et al.*, 1991) and China (Guo *et al.*, 2003). Individual clones can be large with the largest clone (0.3 ha) reported from a lake reed belt in northeast Germany (Neuhaus *et al.*, 1993). However in their study no statistical likelihoods were provided to judge the spurious assignments of ramets (units of a clone, separate individuals that have the same genetic composition) to genets (a clone, single genetic individual, composed of a variable number of ramets) (Clevering and Lissner, 1999; Lowe *et al.*, 2004). Koppitz *et al.* (1997) investigated clonal diversity of two reed stands at Lake Vejlerne in Denmark and observed that the diversity of clones decreased from the landward side of the stand to the water's edge. It was hypothesised that initial colonisation of lakeshores is by seed, as conditions here are favourable for seedling establishment, and only those clones adapted to deeper water, colonise the water's edge. It has also been hypothesised that clonal diversity within a population may decline over time (Ellstand and Roose, 1987; Widén *et al.*, 1994, Koppitz *et al.*, 1997), although this has never been tested.

In Britain the variability in clone structure among populations has never been investigated, and the size of clones is unknown. Indeed, the only research conducted on this topic, was by McKee and Richards (1996) who investigated the variability in genotypes of three reedbeds located in northern England. They found varying numbers of genotypes (5 - 11) among populations, although clone size was not

determined. Therefore an aim of this study is to investigate clonal size and diversity among British populations of *P. australis.*

1.5.3 Genetic diversity

A knowledge of the genetic diversity (a measure of the genetic variation found in a population or species based on the mean expected heterozygosity) (Lowe *et al.*, 2004) within and between British populations of *P. australis* will enable the genetic processes that occur in natural populations (i.e. genetic recruitment and gene flow) to be reproduced in newly created and restored reedbeds.

Genetic diversity and genetic distances (the degree of genetic similarity between a pair of individuals, populations or species) have been investigated in *P. australis* across its world-wide distribution using an array of molecular tools. For example, Koppitz (1999) reported a high degree of genetic diversity amongst world-wide populations of *P. australis* and generally found that genetic distance increased with geographic distances. Guo *et al.* (2003), found high levels of genetic diversity were maintained among 15 populations of *P. australis* sampled in the Yellow River Delta, China, in agreement with an increasing number of studies which have found that clonal plant species maintain the same levels of genetic diversity as non-clonal species (Ellstrand and Roose, 1987; Jonsson *et al.*, 1996; Reusch *et al.*, 2000; Ainsworth *et al.*, 2003).

The genetic diversity of natural populations of *P. australis* in Britain and the gene flow between them are unknown and were investigated in this study in conjunction with clonal structure among populations, using appropriate molecular marker tools.

1.5.4 Breeding system

The genetic structure and dynamics of *P. australis* populations are fundamentally affected by the balance between asexual (i.e. clonal spread) and sexual reproduction (i.e. seedling recruitment) (Ritland and Jain, 1981). It is important to determine the breeding system of *P. australis* so that reedbeds are created from the appropriate plant material and managed in a way that promotes sexual recruitment. This would

ensure that a reedbed is sufficiently genetically variable to allow for adaptation to future environmental change.

Although empirical data are not available, it is assumed that *P. australis* is highly outcrossing. (Clevering and Lissner, 1999). It is also hypothesised that in natural populations outcrossing mechanisms are compromised by extensive clonal spread (Barrett *et al.*, 1993), which accounts for the low seedset found in many populations (Gustafsson and Simak, 1963; Björk, 1967; Haslam, 1972). It is known that the species is partially self-incompatible. In a controlled pollination experiment using two populations of *P. australis* from south-western Japan, the seedset was found to be low (2.8 % - 8.9 %) when the populations were self-pollinated (Ishii and Kadono, 2002).

As the breeding system has never been investigated by genetical analysis (i.e. by using molecular tools), a further aim of this study is to use molecular marker techniques to determine the outcrossing rate of a natural population of *P. australis*.

1.5.5 Seed production

Seed sowing is one of the most commonly used techniques for reedbed creation (Hawke and José, 1996; Self *et al.*, 1996). Therefore understanding and accounting for the variability in seedset among *P. australis* populations would ensure that seed collection is both reliable and efficient.

Enormous variability in seed production amongst populations has been reported in all European studies and, in general, seed production has been found to be low. For example McKee and Richards (1996) found % seed set (measured as the proportion of seeds set out of 200 examined per population) amongst British populations varied between 0 - 100%, whilst Ishii and Kadono (2002) found the seed set rate (measured as the number of mature seeds divided by the number of florets examined for each inflorescence) amongst Japanese populations ranged from 0.1 – 59.6 %. In addition, Curran (1969) found that less than 1 % of florets contained seeds in panicles collected from Irish populations of *P. australis* and Björk (1967) found seed production (number of seeds per panicle) varied from 0 - 4325 in different

populations of southern Sweden. Numerous causes have been suggested for variable seedset including biotic factors, such as: self sterility through pollen limitation (Gustafsson and Simak, 1963; Björk, 1967; Haslam, 1972), fungal infection caused by *Claviceps purpurea* infection (Gustaffson and Simak, 1963; Björk, 1967; Curran, 1967) and meiotic disturbance (Gustaffson and Simak, 1963; Björk, 1967). Abiotic factors purported to affect seedset include climate (Gustaffson and Simak, 1963; van der Toorn, 1967; Haslam, 1972; McKee and Richards, 1996; Self, 1999), latitude (Nikolajevskij, 1971; McKee and Richards, 1996) and site size (McKee and Richards, 1996).

The only in depth study of seedset in British populations was carried out by McKee and Richards (1996) who surveyed 34 British populations of *P. australis.* While their population sample size was large, the mean seedset per population was determined by examining only five panicles from each reedbed. The seedset rate (expressed as proportion of seeds examined) was determined by a 'blender' method, which is prone to bias because the sample is derived from seeds (set and unset) which after blending of the panicle in water, sink to the bottom of a glass beaker. The floating material is discarded and since set seeds are heavier than unset seeds, seedset is overestimated. Nevertheless, variability in seedset was reported between populations and related to climatic factors (rainfall and temperature) at the time of flowering although other, unknown variables accounted for > 60 % of the variation:

Therefore, as part of this research, seed production amongst British populations of *P. australis* was reinvestigated using a different method and related to the genetic variability of each population investigated. The relationships between seedset and an endophytic fungus, *Claviceps purpurea*, which is also assumed to reduce seedset (Gustaffson and Simak; Björk, 1967; Curran, 1967) and between seedset and certain climatic factors were also assessed.

1.5.6 Adaptive genetic variation

To successfully implement management objectives for the creation of new reedbeds, decisions regarding seed source should account for the morphological variability found among British populations of *P. australis.*

Across its distribution *P. australis* is known to be highly polymorphic and numerous studies have shown variability within and between populations arising through genetic or environmental factors (Björk, 1967; Haslam 1969; Haslam 1972; van der Toorn; 1972, Véber 1981; Clevering *et al.*, 2001; Karunaratne *et al.*, 2003; Lambertini *et al.*, 2003). Intraspecific variation may be related to high plasticity, number of clones, or a combination of both (Clevering and Lissner, 1999). Variability between populations has been attributed to ploidy levels and adaptations to edaphic, hydrological or temperature differences along a latitudinal gradient (Björk, 1967; Haslam, 1969; Haslam, 1972; van der Toorn, 1972; Véber 1981; Paucã-Comãnescu *et al.*, 1999; Clevering *et al.*, 2001; Karunaratne *et al.*, 2003; Lambertini *et al.*, 2003). For instance, van der Toorn (1972) described riverine and peat ecotypes (an ecotype being the genetic adaptations of a population to the local environment (Ricklefs, 1990)) within a species located in the Netherlands. The former (which) occurred on mineral-rich clay soils and contained reeds possessing long shoots and low shoot density, whilst the latter grew on mineral poor peat soils and contained reeds with shorter shoots and a higher shoot density. It was hypothesised that the ecotypes arose through genetic differences, although this was not examined in a common garden experiment (CGE).

Genetic differences associated with latitude were examined by Clevering *et al.* (2001) in a transplant experiment in which rhizomes from nine countries ranging from northern Sweden (66.23° N) to Spain (40.66° N) were planted and grown for two years in three common environments in the Netherlands, Denmark and Czech Republic. Populations from lower latitudes showed a genetically determined longer growing season and flowered later compared to those from higher latitudes. In addition, seeds collected from six populations, ranging from southern Sweden (58.35° N) to Spain (40.66° N), were germinated and grown up in a greenhouse CGE experiment. The main experimental findings were that ten-week old *P. australis* seedlings from southern populations were taller and thicker than those from further north, although the more northerly populations produced a higher number of shoots and rhizomes than southern populations. It was concluded that populations of *P. australis* showed a clinal variation in length of growing season, time of flowering and morphology.

Paucã-Comãnescu *et al.* (1999) described phenotypic differences related to ploidy levels in *P. australis* growing in plots in Comana Lake and the Danube Delta (Romania). They found that, in fresh water habitats, octoploid ($2n = 8x = 96$) shoots were longer, thicker and bore larger panicles and leaves compared to those of tetraploids ($2n = 4x = 48$). However, as no CGE was conducted, differences due to climate, water level and other edaphic conditions may also have contributed to morphological differences. In addition, because plots were only separated by about 20 m there was a high probability that the same clone was repeatedly sampled.

The above research on European populations of *P. australis* confirms the wide scale variability in the species. However Haslam (1972) reported that the variability in morphology among British populations is much less extensive than found elsewhere.

The most detailed investigation of British populations was undertaken by Daniels (1991) who carried out a CGE using seeds collected from four different British populations (two from East Anglia and two from Dorset). Although the sample size was moderate (seven replicates from five parents per population) significant differences were found in shoot height and number of shoots between populations. A significant population x site effect on shoot production and rhizome length was also found in transplant experiments using plant material (rhizomes) collected from eight sites in England and Scotland. However the results may have been biased due to an environmental carry over effect because rhizomes from each population were planted in April and measurements were made in July and October of the same year.

In this research, the adaptive variability (measured as quantitative traits) among British populations of *P. australis* was investigated, using a CGE approach. In addition, to measure the response of populations over a range of environments (which is also under genetic control) each population was grown in full nutrient conditions, nutrient stressed conditions and treatment involving the application of synthetic sewage.

1.5.7 Genotypes for CWSs

Although *P. australis* is polymorphic (Björk, 1967; Haslam, 1972; van der Toorn, 1972; Paucã-Comãnescu *et al.*, 1999), the variability within the species has not been considered an important factor in the design or performance of CWSs and intraspecific variability in nutrient removal has never been investigated. Therefore one of the aims of this research was to compare the effectiveness in nutrient removal of different populations of *P. australis* to discover if the genetic variation found between populations affects their efficacy in water treatment.

1.6 SUMMARY OF OBJECTIVES

The broad research aims were to carry out a detailed study of *P. australis* occurring naturally in Britain, in order to 'fill the gaps' in the knowledge of this resource, so that it can be utilised in an effective and sustainable way. This involved an investigation of the species' chromosomal races, clonal structure, genetic variability (both adaptive and genetically neutral traits), breeding system, seedset, and intraspecific efficacy in nutrient removal in CWSs. A number of key questions were addressed, including:

1. What are the ploidy levels of British populations of *P. australis*?
2. How variable is the clonal structure amongst British populations?
3. What is the genetic structure of British populations?
4. What is the breeding system of *P. australis*?
5. Are there differences in seedset and germination rates amongst British populations of *P. australis*?
6. Are there quantitative genetic differences (morphological differences) within and among populations of *P. australis* in Britain?
7. Are there genetic differences in ability to remove nutrients among populations?

These questions were addressed by sampling from up to 11 populations of *P. australis*, located throughout Britain. The results of the investigation were used to

make recommendations for effectively using the resource to establish and conserve reedbeds, as well as for selection of plant material for CWSs.

1.7 THESIS STRUCTURE AND OUTLINE

The thesis is set out in eight chapters. Following this Introductory Chapter, a general materials and methods chapter describes (i) the populations chosen; (ii) molecular marker tools for genetic analysis and reasons for choosing microsatellite markers in this study; (iii) CGEs and (iv) CWS experiments. The next five chapters address the key questions outlined above (and are written in the style and follow the conventions required for a scientific paper and can therefore stand alone). Chapter Eight brings the results of the different elements of research together. Recommendations are made for the management of the British resource of *P. australis* for use in reedbed establishment and conservation and in CWSs and areas of future research are discussed.

Chapter 2 - Materials and methods

2.1 MATERIALS

2.1.1 Reedbed populations

To represent the natural resource of *P. australis* in Britain, 11 populations were selected on the basis of the following criteria. (i) Geographic location; To reflect the natural distribution of *P. australis* in Britain four populations were chosen from East Anglia (Strumpshaw Fen, Walberswick, Minsmere, North Warren), which has the highest concentration of reedbeds in Britain (Haslam, 1972), and Insh Marsh was selected as is it is one of the most northerly reedbeds in Britain. (ii) Conservation importance; Minsmere and Leighton Moss were included because they are flagship reserves of the RSPB and are managed for Red Data bird species such as Bittern and Marsh harrier (Ian Hawkins; Robin Horner, pers. comm. 2003). (iii) Size of reedbed; The Inner Tay (410 ha) and Walberswick (240 ha) reedbeds were included as they are the largest reedbeds in Britain and England respectively. (iv) Coverage of both freshwater and brackish reedbeds. (v) Examples of newly created reedbeds (Lakenheath and Mersehead). (vi) Examples of differing management practices. Sites were chosen to encompass a range of management practices, such as commercial reed harvesting, cutting and/or burning of sections of reed, water level manipulation and no active intervention. The reedbed characteristics are summarised in Table 2.1 and the locations are shown in Fig. 2.1.

Table 2.1 Details of 11 populations chosen to represent the natural resource of *P. australis* in Britain. Abbreviations of each population are given in parentheses.

Population	Latitude/ longitude	Management organisation and practices	Conservation status	Freshwater or brackish	Size (ha)
Insh Marsh (IM)	57.09° N 3.98° W	RSPB No cutting or burning	SSSI	Freshwater	26
Inner Tay (IT)	56·36° N 3.27° W	RSPB Commercial cutting	SSSI	Brackish	410
Loch Leven (LL)	56.20° N 3.35° W	SNH No cutting or burning	SSSI	Freshwater	20
St. Margaret's Marsh (SM)	56.02° N 3.42° W	SNH No cutting or burning	SSSI	Both	15
Mersehead (MH)	54.89° N 3.66° W	RSPB Newly created	SSSI	Freshwater	20
Leighton Moss (LM)	54.17° N 2.79° W	RSPB Cutting underwater	SSSI	Freshwater	79
Strumpshaw Fen (SF)	52.61° N 1.46° E	RSPB Cutting and burning	SSSI	Freshwater	82
Lakenheath (LH)	52.44° N 0·50° E	RSPB Newly created	SSSI	Freshwater	165*
Walberswick (W)	52.30° N 1 63° E	EN Commercial cutting	SSSI	Both	240
Minsmere (MM)	52.25° N 1.60° E	RSPB Cutting and burning	SSSI	Both	105
North Warren (NW)	52.18° N 1.60° E	RSPB Cutting and burning	SSSI	Freshwater	23

RSPB = Royal Society for the Protection of Birds
SNH = Scottish Natural Heritage
EN = English Nature
SSSI = Site of Special Scientific Interest
* Future projection (Norman Sills, pers. comm. 2003)

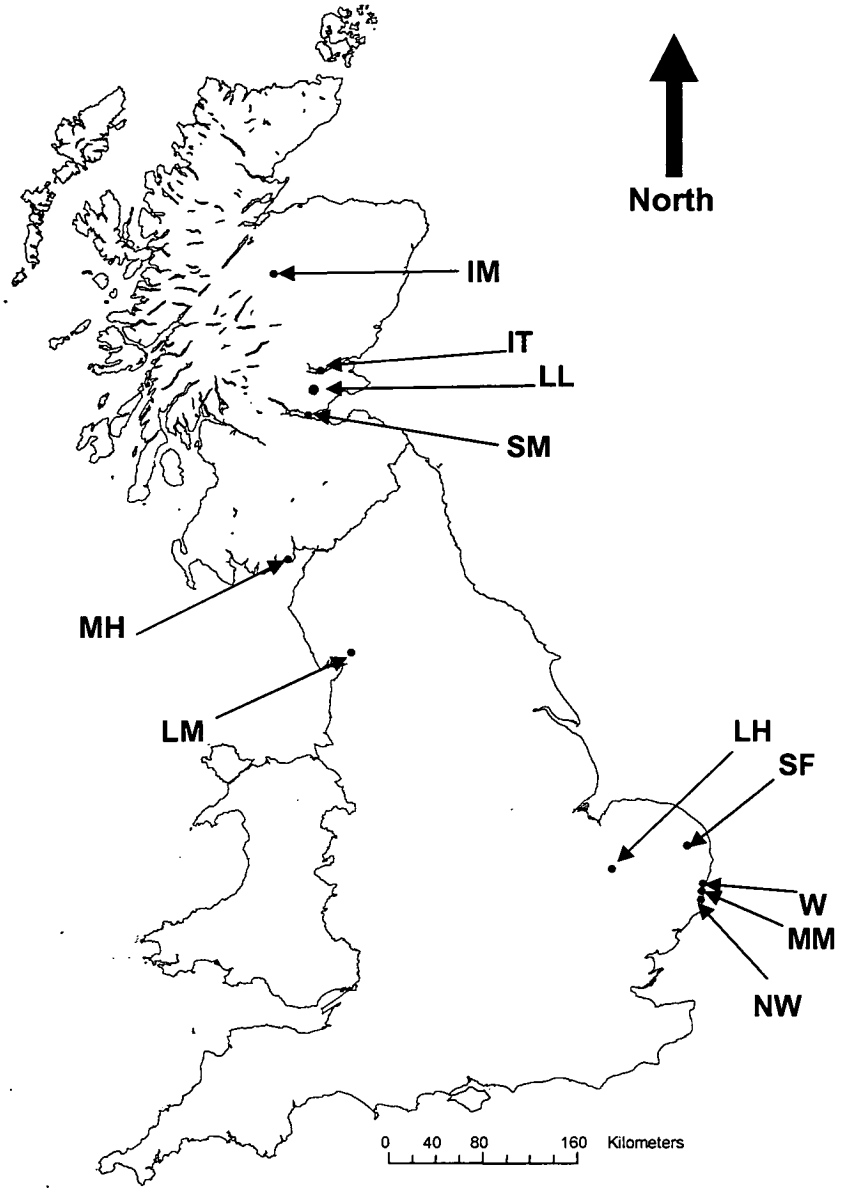


Figure 2.1 Location in Britain of the 11 reedbeds studied. Key to sites: Scottish: IM = Insh Marsh, IT = Inner Tay, LL = Loch Leven, SM = St. Margaret's Marsh, MH = Mersehead; English: LM = Leighton Moss, LH = Lakenheath, SF = Strumpshaw Fen, W = Walberswick, NW = North Warren, MM = Minsmere.

2.1.2 Site description

2.1.2.1 Insh Marsh (Grid reference: NH800020)

Insh Marsh (Fig. 2.2) is a natural wetland, covering about 1000 ha of the floodplain of the River Spey. Because of its unspoilt character it is one of the most important flood plain mires (peat forming wetlands) in Britain. The area is mainly composed of woodland, scrub (an area of dense stunted trees, bushes or shrubs), grassland and mire/reedswamp clumps associated and fringing an area of open water. There is no management of the 26 ha reedbed itself, although the floodplain is lightly grazed by cattle, ponies and sheep from the onset of spring to winter flooding. There is no hydrological control of the water level, although a ditch system, which is not connected to the Spey, ensures site drainage (Prescott, 2000).

Figure 2.2 Insh Marsh (summer 2003).

2.1.2.2 Inner Tay (NO217197)

The reedbed on the Tay Estuary (Fig. 2.3) is the largest in Britain (410 ha) and is thought to have been established in the early 1800s, when prisoners from the Napoleonic wars planted reeds (believed to have originated from the Netherlands) to protect the mudflats from erosion (Hawke and José, 1996). The reedbed is tidal, and the lower third of the reedbed (nearest the sea) is inundated twice daily with a brackish mixture of seawater and freshwater from the River Tay, whilst the upper reedbed is only flooded in high spring tides (Hawke and José, 1996). Reed has been cut for thatching for many years, but ceased from 1945 - 1970. Commercial cutting began again in the 1970s and the reed is cut in winter, on a single wale (i.e. cut every year) (58 - 80 ha $year^{-1}$). Although the reedbed produced some 20 % of the UK thatching reed output, commercial operations have recently ceased due to cheaper imports from abroad.

Figure 2.3 Inner Tay (winter 2002).

2.1.2.3 Loch Leven (NO165014)

Loch Leven (Fig. 2.4) is the largest naturally eutrophic (nutrient-rich) freshwater loch in the British Isles, with a surface area of *c.* 13.3 km^2, and is the largest loch in lowland Scotland. It was formerly larger and deeper, but lowering of the river Leven bed in the early 1800s, to ensure a regular supply of water to the downstream mills, led to a reduction in the loch's area (shoreline moved inwards by about 500 m) and depth (lowered by 1.4 m). The Loch is surrounded by farmland (80 % of the catchment is under agricultural use) and a diverse array of shoreline vegetation. Research carried out in the 1970s showed that water quality in the Loch had deteriorated, mainly through an increased phosphorus input, leading to algal blooms. Subsequent action plans have been implemented which have led to a reduction in point source phosphorus inputs and an improvement in water quality. Small patches of reedbed fringe the loch side. One of these areas was chosen for sampling and was approximately 40 m wide and 2 km long. The reedbed on the landward side is surrounded by carr (wet woodland, typically with alder or willow) and then woodland/scrub. The reedbed is not managed by burning/ or cutting and water levels of the loch fluctuate depending on rainfall and input from tributaries. In addition, water levels are affected by upstream abstraction and sluices on the Loch outflow, managed by the River Leven Trust, maintain flows for downstream water use. Usually the water level is higher in winter than summer (JNCC, 2001).

Figure 2.4 Loch Leven (winter 2002).

2.1.2.4 St. Margaret's Marsh (NT116816)

This small reedbed (Fig. 2.5) is located on the north bank of the Forth Estuary and consists of two expanses of reedbed, separated by a grassy raised bund. It is the largest reedbed in the Firth of Forth and represents *c.* 3.5 % of the Scottish coastal reedbed resource. The reedbeds are separated from the estuary's brackish water by a raised bank, but a small inlet on the east side of the reedbed allows brackish water onto this part of the site at high tides. In addition, salt from storm surges and sea spray will periodically affect the reedbed. The reedbed is relatively dry all year, although, the eastern end is considerably wetter than the western part. The reedbed is not managed by clearing of vegetation, although there may be scope to alter the water level of the bed by the inlet pipe.

Figure 2.5 St. Margaret's Marsh (summer 2003).

2.1.2.5 Mersehead (NX937561)

Mersehead (Fig. 2.6) was formerly a mixed arable grazing farm that was purchased by the RSPB in the mid 1990s. Since its acquisition, the hydrology of the site has been altered to allow flooding of certain areas using the inlet sluice gates of the Beck River. In addition, sub-surface tile drains that drew water away have been removed. A mosaic of wetland habitat has been created such as wet grassland, wet reedswamp, open waters fringed by reed and reed-bordered ditches. The annual hydrological regime consists of winter flooding, stable water levels during March-June (bird breeding season) and draw down in July to September. A reedbed has been created from reed cleared from ditches and reed seedlings grown in polytunnels (from seeds collected locally and other sources such as Inner Tay and other Scottish RSPB sites) and planted out to create a reedbed At present the establishing reed is not managed by cutting and/or burning (Fairlamb, 2000).

Figure 2.6 Mersehead (summer 2003).

2.1.2.6 Leighton Moss (SD482750)

Leighton Moss (Fig. 2.7) is a valley fen (wetland that accumulates peat and receives some water inflows from surrounding mineral soils) with succession from shallow, eutrophic mires, reedswamp and fen to scrub and carr. To prevent reed encroachment into the many pools of open water, underwater cutting (using a mower) is carried out, usually in July. Stems are cut whilst still growing and further growth is prevented by being submerged in water (as aeration of the rhizosphere is arrested). The advantage of this method of control compared to winter cutting of aerial parts is that no water draw down is necessary (as machinery access is not required), so open water pools are maintained for wildlife (Hawke and José, 1996).

Figure 2.7 Leighton Moss (summer 2003).

2.1.2.7 Strumpshaw Fen (TG341066)

The fen (Fig. 2.8) lies in the River Yare floodplain and arose due to flooding by rising sea levels of peat cuttings created in medieval times for fuel extraction. However, infilling of the open pools and reedbeds occurred because of a regular inundation of the fen and Broad by nutrient and sediment rich waters. To arrest the decline in the reedbed, a dam was created in 1978 and a bund constructed in 1979 to prevent eutrophic waters entering the reedbed at high tides (Hawke and José, 1996). The reedbed is managed by rotational cutting and burning in the winter.

Figure 2.8 Strumpshaw Fen (summer 2003).

2.1.2.8 Lakenheath (TL700860)

This reedbed (Fig. 2.9) was created from agricultural land purchased by the RSPB in 1996. The wetland area, which had been drained for carrot farming, was restored by major landform and hydrological changes involving excavation, bund and ditch creation and installation of water control structures such as sluices and pipes. Large scale planting has been conducted of *c.*30,000 seedlings per annum which have been germinated in polytunnels from distantly sourced seed (e.g. other reedbeds located in East Anglia as well a small amount from a local site) (Norman Sills, pers. comm. 2003). To date, the establishing reedbed is not managed by cutting or burning.

Figure 2.9 Lakenheath (winter 2002).

2.1.2.9 Walberswick (TM475733)

The freshwater reedbed at Walberswick (Fig. 2.10) is the largest in England and is located in a valley by the Suffolk coast. The reedbed developed on land, previously drained for agriculture, which was left to flood during World War II. The site receives a constant inflow of freshwater from springs and the higher ground that surrounds it. A shingle bank at the eastern end affords some protection against flooding from high tides. The reedbed is divided into three major compartments, separated by a series of bunds and ditches which are cleared in rotation every 8 - 15 years. Most of the reed area (> 75 %) is protected from sea water inundation by a large sea wall/bund. Water is distributed by sluice gates, enabling selective draw down between units and permitting machinery access for commercial single wale cutting (a total of 15 - 30 ha of reed is cut each year in fixed plots varying in size from 0.4 to 2.5 ha). Different water regimes exist within the separate reedbeds, which provides diverse reedbed habitats and is beneficial for wildlife and there are also open areas of standing water. Reed encroachment is kept in check by spraying with herbicide every 3 - 5 years. Long term litter accumulation throughout the reedbed is also prevented by cutting on a 20 - 25 year rotation, whilst summer cutting of fixed plots on the drier edges of the reedbed ensures these areas support a greater range of plants than the wetter areas (Hawke and José, 1996).

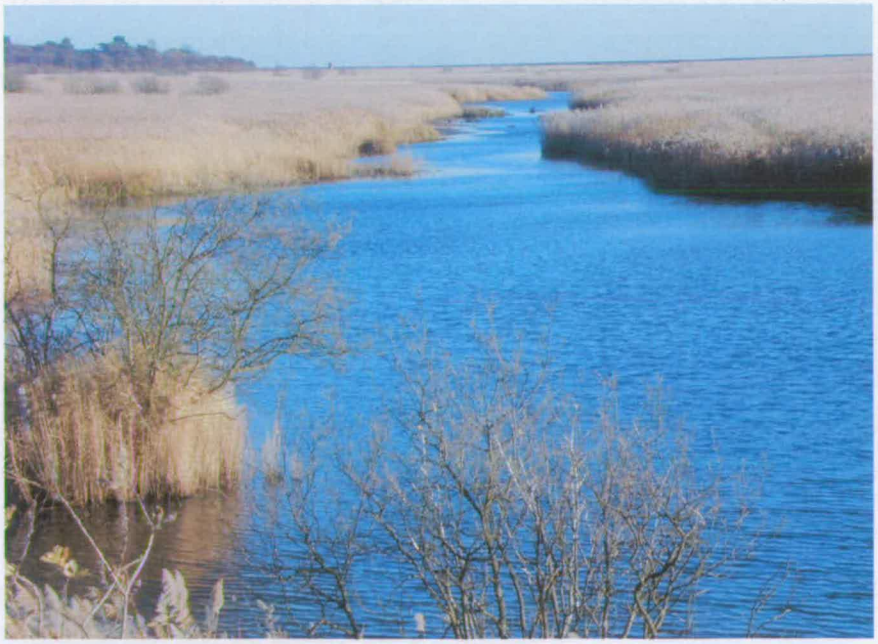


Figure 2.10 Walberswick (winter 2003).

2.1.2.10 Minsmere (TM460672)

The wetland habitats on the reserve (originally leased by the RSPB in 1947) (Fig. 2.11), have a long and complex history. Prior to *c.* 1780, the whole area was probably a wetland complex of varying habitats, however the formation of an offshore sandbar eventually blocked the mouth of the Minsmere river creating a large freshwater mere (pool or lake). In the 19th century the area was gradually drained for grazing and it was not until the advent of World War II that it was allowed to flood as an anti-invasion defence. Today the site consists of a variety of habitats with lowland wet grassland, heathland, coastal lagoons, woodland, arable land and major areas of separate reedbeds associated with meres (eutrophic ponds and lakes), ditches, mires and meadow marsh. Management of reedbeds includes a number of practices maintaining water depths at 20-30 cm from January to October (using 44 control points, mainly pipe sluices); rotational cutting of wet reed (5 ha year^{-1}) on a seven year rotation; and clearing of ditch vegetation on a 15 - 20 year rotation. The main water input to the reedbed is seepage from a woodland and heathland dominated catchment. Other inputs are rain water, runoff from arable and pastoral land and tidal exchange of seawater *via* one of the sluices (one area of reedbed only) (Green *et al.*, 2003).

Figure 2.11 Minsmere (winter 2003).

2.1.2.11 North Warren (TM460594)

This freshwater reedbed (Fig. 2.12), which had been cut on a single wale until about 1954, gradually became degraded due to successional change caused by drying out and an invasion of scrub, nettles and bramble. In the early 1990s the reedbed was regenerated by the RSPB, mainly through scrub removal, creation of new ditches and bunds and excavation. Water is supplied from the River Hundred *via* two sluices, plus seepage from several springs (Hawke and José, 1996). There is a large reed fringed pool area, as well as a small, drier more species-rich reed fen. Active management of the reedbed includes rotational burning/cutting of sections and manipulation of the water levels.

Figure 2.12 North Warren (summer 2003).

2.2 METHODS

2.2.1 Molecular markers

Molecular markers are sequences of DNA (in the nuclear, mitochondrial or chloroplast genome) or protein that can be screened to reveal neutral sites of variation (i.e. unlike morphological markers, these variations are not manifest in the phenotype) (Jones *et al.*, 1997). All molecular markers give estimates of genetic variability by sampling from regions of the genome and are widely used in systematics (the study of the diversity of organisms and their natural relationships) and ecological genetics (the investigation of the origin and maintenance of genetic variation within and between populations) (Lowe *et al.*, 2004). The ideal genetic marker has several important characteristics (Lowe *et al.*, 2004); the most relevant to this study are as follows:

(i) The marker should be either present or absent or the level of its expression should show discrete variation (which enables unambiguous scoring of alleles).

(ii) The marker should be selectively neutral (i.e. have no environmental or developmental influences).

(iii) The marker should show simple codominant inheritance (i.e. in a diploid heterozygote, both alleles should be present).

(iv) The marker set should represent a sample from the whole genome (i.e. be distributed throughout the genome), rather than located in just one region.

(v) The marker should be cheap, reliable and give reproducible results.

Unfortunately, none of the markers currently available for genetic studies encompasses all the desired characters, therefore the choice of marker for any study is a compromise between the properties of the marker system, its availability, the hypothesis to be tested and the resources available.

The next section outlines the molecular tools that are currently available and the reasons for the choice of marker in this study.

2.2.1.1 Allozymes

Allozymes are alternative forms of a particular protein (enzyme), caused by changes in amino acid sequences, that yield bands of different mobilities on electrophoretic gels. Allozymes show codominant inheritance and are relatively cheap, safe and easy to use and require no prior sequence knowledge. The main disadvantage of this technique is that, although the marker is assumed to be selectively neutral, there is evidence for selection at certain loci (Lowe *et al.*, 2004). In addition, abundance in the genome is low and low levels of polymorphism may be detected in only a limited range of enzymes. Thus, for a clonal species there may not be enough variation to discriminate between genotypes.

2.2.1.2 RAPDs

RAPDs (random amplified polymorphic DNA) are a PCR (polymerase chain reaction; a technique that increases the number of target DNA sequences by several orders of magnitude) based DNA-marker, in which PCR amplifications are carried out using arbitrary nucleotide sequences as primers. Amplification of the primer sites throughout the genome yields bands of various sizes that can be separated by electrophoresis. Bands are scored as present or absent (Silverton and Charlesworth, 2001; Lowe *et al.*, 2004). Although they are relatively cheap, simple and require no sequence information, the disadvantages of RAPDs are associated with marker dominance (i.e. homozygotes and heterozygotes cannot be distinguished), lack of reproducibility (e.g. variability in reagent concentrations, DNA quality and concentration and taq polymerase batches may produce different banding patterns for the same individual) and non-independence of loci (Lowe *et al.*, 2004).

2.2.1.3 RFLPs

RFLPs (restriction fragment length polymorphisms) use restriction (cutting) enzymes to produce DNA fragments of varying size. Each restriction enzyme recognises a specific and characteristic nucleotide sequence. Fragments are separated by electrophoresis and markers are detected by using a cloned piece of DNA from the same sample that is labelled with a radioactive or chemical tag. The cloned probe hybridises with and thus stains the DNA fragments, which can then be scored. This method shows co-dominant inheritance and is repeatable. However the technique

requires large amounts of DNA, prior sequence knowledge, access to radioisotopes and is expensive, so that in many studies only a small number of enzymes are used due to cost (Lowe *et al.*, 2004).

2.2.1.4 AFLPs

The AFLP (amplified fragment length polymorphism) method combines the use of restriction enzymes with PCR amplification to generate hundreds of informative genetic markers (fragments or bands) with a high number of polymorphisms (Mueller and Wolfenbarger, 1999). The process involves three steps. Firstly, DNA is digested with a frequently cutting and rarely cutting restriction enzyme after which the fragment ends are modified by ligation (joining) with double stranded adapters. The next stage involves pre-selective amplification by PCR. The restriction/adapter sites serve as primer binding sites for the PCR. The primer has one base pair (bp) that extends into the unknown part of the fragment, so that only the genomic fragments that have an adapter on each end and a corresponding bp will amplify during PCR. The final stage involves selective amplification by PCR. Specific primer pairs with three nucleotide extensions (3 bps) are used to reduce the number of fragments amplified by a factor of 64. The bands are then visualised by gel electrophoresis (Vos *et al.*, 1995; Mueller and Wolfenbarger, 1999; Lowe *et al.*, 2004).

This method has been shown to be cost effective (Vos *et al.*, 1995) and no prior sequence knowledge is needed. Furthermore, as long amplification sequences are used which bind perfectly to their target sequences, the results are very reliable (Vos *et al.*, 1995). The main disadvantages of this technique are the lack of codominant inheritance and the relatively large amounts of high quality DNA needed.

2.2.1.5 Microsatellite markers

Microsatellite markers (SSRs, simple sequence repeats) are tandemly repeated sequences that are randomly distributed throughout the genome (Jarne and Lagoda, 1996) and have a unit of repetition between one and five base pairs (e.g. $(AT)_n$ or $(CAG)_n$). Microsatellite regions can be detected by scanning genomic data bases or if the sequence information for a species is depauperate, then the time-consuming and expensive process of characterizing them directly is needed. This involves cloning,

detection of SSRs (i.e. repeated base pair regions) and sequencing of flanking regions for locus specific primers. Once flanking primer sites are identified, the variable SSR (i.e. the locus) can be amplified using PCR and separated by size (corresponding to alleles) using agarose, polyacrylamide or automated DNA sequencing.

Advantages of this marker system include codominant inheritance, reproducibility, selective neutrality and abundance through out the genome. In addition microsatellite markers have a higher degree of polymorphism compared to all other molecular markers which makes them ideal for genetic population studies involving clonal species (Jarne and Lagoda, 1996; Reusch *et al.*, 1998; Reusch *et al.*, 2000; Durand *et al.*, 2000; Ainsworth *et al.*, 2003). Two disadvantages in using these markers, apart from the possible requirement for sequencing and cost, are an underestimation of the number of alleles due to size homoplasy (Estoup *et al.*, 2002), although this can be discounted by sequencing of alleles, and the presence of null alleles (alleles that do not amplify). Problems associated with the latter are more likely when using primers that are designed for another species (Pemberton *et al.*, 1995). In addition, population genetic analysis of polyploid species may be difficult because the number of alleles detected per locus may be greater than two.

2.2.2 The choice of appropriate marker for this study

Previous genetic investigations of *P. australis* have used RAPDs (Neuhaus *et al.*, 1993; Koppitz *et al.*, 1997; Koppitz, 1999; Keller 1999; Lambertini *et al.*, 2003), RFLPs (Zeidler *et al.*, 1994) and allozymes (Hauber *et al.*, 1991; Guo *et al.*, 2003). Recently Saltonstall (2003) developed ten microsatellite primers to investigate variation within and among native, introduced and Gulf Coast North American lineages of *P. australis*.

After considering the potential suitability of each molecular marker outlined above, it was decided that microsatellites came closest to the ideal marker for addressing the key genetic questions posed in Chapter One (i.e. investigating the clonal structure, breeding system and genetic diversity of *P. australis*). This technique has advantages in discriminating between clonal genotypes, because of the high polymorphism of

alleles, and their codominant inheritance, allowing genetic diversity to be estimated using statistical procedures. Furthermore the disadvantages associated with microsatellite markers of start up cost and primer development have been removed because suitable primers for this species have already been developed by Saltonstall (2003).

The polyploid constitution of *P. australis* potentially complicates investigations using microsatellites, because more than two alleles may be amplified per locus. Thus detecting the presence of homozygotes or heterozygotes, as well as scoring of alleles at a locus, may be difficult. For this reason five microsatellite loci that were known to amplify one or two alleles only, and thus displayed an inheritance pattern similar to diploids, were chosen from the original ten developed by Saltonstall (2003).

2.2.3 Common Garden Experiments (CGEs)

2.2.3.1 Detecting quantitative genetic differences

Differences between populations in quantitative traits such as plant size, number of shoots and above ground biomass, show more or less continuous variation and are determined by genotype and environmental effects. Thus the characters observed in a natural population (i.e. the phenotype) arise as a result of an interaction between the genotype and the environment. These characters are heritable and are subject to selection, but the genetic basis is difficult to discern because numerous genes as well as many environmental influences are involved. To separate phenotypic variation into its two components, plants must be grown in a uniform environment ('a common garden'). Trait differences detected between plants or populations grown under the same environmental conditions are due to genetic differences, which have arisen due to differences in their genotype. Therefore the goal of genetic field tests (e.g. CGEs) is to sample the genes in a given population and to draw conclusions about the population and, in a well designed CGE, genetic differences (in adaptive traits) can be ascertained among populations. This type of field trial has been pioneered in forestry (provenance tests) and the results have been used in

commercial seed collection or for selection in breeding programmes (Loo-Dinkins, 1992). Several factors should be taken into account when designing common garden experiments:

1. *Carry over effects*

In experiments begun with seed there may be a maternal effect. For instance, variability in seed size and mineral composition between different populations may result in different rates of progeny growth (Silverton and Charlesworth, 2001). Carry over effects may also arise if vegetative propagules are planted because there may be an extended period of adjustment before the plants have outgrown the effects of the original habitat (i.e. a phenotypic effect) (Briggs and Walters, 1997).

2. *Variability within a population*

Experiments should be designed with sufficient replications of the populations or treatments to take account of the natural variability of the species.

3. *Arrangement of plant material*

Test sites are rarely uniform, even when edaphic conditions, watering and nutrient regimes are controlled. Growing populations in a greenhouse may appear a more uniform environment than a field site, but block differences in shading or micro-climate (e.g. temperature and humidity) may still arise due to a positional effect. A randomised block design reduces the impact of site variability and can be tested for in statistical analysis such as Analysis of Variance.

2.2.3.2 Genotype × environment interaction

The expression of a particular quantitative trait, such as shoot size, may vary depending on the environmental conditions in which an individual or population is grown. This is due to a genotype × environment (GE) interaction which is also under genetic control. Because of the possible presence of a GE response, genetic tests in multiple environments should be undertaken, otherwise the GE will remain undetected. A GE interaction may be detected by transplant experiments which involve growing populations and measuring traits at one field site, and then transplanting them to another site and measuring the same traits grown under differing environmental conditions. This method may be expensive and time consuming, especially if the field plots are distantly located, so a simpler method

involves presenting the populations with different environmental conditions in one garden or greenhouse.

2.2.3.3 CGE design for this study

To determine population differences in quantitative traits in this study, up to twenty samples per population were grown from seed, and arranged in a randomised block design in an unheated greenhouse. To test for a GE response, each population was grown in three different environments (nutrient-stressed, full nutrient and treatment with synthetic sewage).

2.2.4 Constructed wetland system experiments

CWSs designed for experimental purposes are either large scale CWSs (located in the field and usually designed for long-term/permanent use) or small laboratory-scale systems (e.g. greenhouse or laboratory experiments conducted over a short time-scale). In large scale CWSs monitoring is usually conducted of efficiency in nutrient (e.g. ammonium, nitrate, phosphorous and biological oxygen demand (BOD)) or metal removal and/or design performance (e.g. hydraulic loading rate, flow patterns, cost and maintenance) over time (Kern and Idler, 1999; Greenway and Woolley, 1999; Shutes, 2001; Vymazal, 2002).

Laboratory-scale mini-CWSs are usually designed for investigating parameters such as nutrient removal efficiency of different plant species (Ansola *et al.*, 1995; Rai *et al.*, 1995) or substrate (Drizo *et al.*, 1997; Yang *et al.*, 2001), hydraulic loading rate (Lin *et al.*, 2002) or plant growth and physiology (Peverley *et al.*, 1995; Clarke and Baldwin, 2002). Whilst large scale systems have the advantage of providing a model of a typical CWS, they are not easily replicated (therefore sample size is small) usually because of limitations of space and/or cost. On the other hand, small laboratory-scale systems are relatively cheap to set up and are easily replicated. The disadvantage of these systems is that they are usually investigated over a short time scale and, in addition to 'edge effects' they do not reflect the complexities of a large scale CWS (e.g. spatial and temporal heterogeneity and unpredictability due to stochastic events) (Kadlec *et al.*, 2001). Therefore the choice of CWS design is

determined by the parameters to be investigated, time constraints and the resources available.

In this study, a comparison of the efficacy in nutrient removal of five populations of *P. australis* was investigated over one year. Replication of each population was essential for statistical analysis. Therefore the experiment was performed using a total of 15 mini-CWSs (five populations x three replicates), which were grown up from rhizomes (left for a period of one year to establish, to diminish environmental carry over effects) and arranged in a randomised block design to minimise the effect of microclimate differences between mini-CWSs (e.g. variability between mini-CWSs in incoming solar radiation and evapotranspiration rates due to a positional effect in the greenhouse).

Chapter 3 – Ploidy levels and genetic marker variation of 11 British populations of *Phragmites australis*

3.1 INTRODUCTION

P. australis is a clonal species showing large variation in the extent of clonal diversity within populations (Hauber *et al.*, 1991; Neuhaus *et al.*, 1993; Zeidler *et al.*, 1994; Koppitz *et al.*, 1997; Guo *et al.*, 2003). The largest reported clone (0.3 ha) was from a lake reed belt in northeast Germany (Neuhaus *et al.*, 1993).

It is important to understand the variability in clone structure (the spatial arrangements and diversity of genets) (Reusch *et al.*, 2000) of British *P. australis* populations because this will influence reedbed selection and strategies for panicle collection for seed harvesting as well as determining the sampling strategy for panicle collection within a reedbed. In addition, seeds sourced from distant reedbeds may be the only option when local provenances are not available or viable. Management decisions regarding seed collection for reedbed establishment must therefore be based on an understanding of the extent and arrangement of genetic variation within the species. This enables seed sources to be chosen that are well adapted and sufficiently genetically variable to allow for adaptation to future environmental change.

Molecular markers have been extensively used for the genetic analysis of *P. australis* populations across its distribution. Genetic diversity, genetic distances and clonal structure of selected *P. australis* populations have been investigated using RAPDs (Koppitz *et al.*, 1997; Koppitz, 1999; Keller, 2000; Lambertini *et al.*, 2003), RFLPs (Neuhaus *et al.*, 1993; Zeidler *et al.*, 1994), and allozymes (Hauber *et al.*, 1991; Guo *et al.*, 2003). Most recently, Saltonstall (2003) developed ten microsatellite primers to investigate variation within and among North American population lineages of *P. australis*. In this study, five of these microsatellite primers were used to investigate

the clonal structure, genetic diversity and genetic structure of British populations of *P. australis*.

The polyploid constitution of *P. australis* potentially complicates investigations using microsatellites. Allo-tetraploids (combining two diploid genomes from different ancestral species) are most common and are fertile, having disomic inheritance. This is because chiasma formation (which is itself under genetic control) at first metaphase of meiosis is restricted to homologous chromosomes only, and this prevents multi-valent formation (Jenkins and Rees, 1991). Thus, using microsatellite markers, the banding patterns observed in tetraploids at a locus should show the presence of one band/allele (homozygote) or two bands/alleles (heterozygote). As only one of the populations to be used in this study has been karyotyped (Inner Tay), the ploidy level of all populations was undertaken before microsatellite analysis was conducted to provide confidence in the interpretation of the microsatellite loci.

The aims of this study were therefore to establish the ploidy levels of a widespread sample of *P. australis* populations within Britain and to use microsatellite analysis to determine the clonal structure and genetic variability and extent of differentiation among these populations. This was carried out by sampling from 11 reedbeds located in Scotland and England. Five microsatellite loci that displayed a diploid inheritance pattern (Saltonstall, 2003) were chosen for the molecular marker analysis. The results were used to make recommendations for seed collection for reedbed establishment and for managing clonal diversity within established populations.

3.2 MATERIALS AND METHODS

3.2.1 Populations

Eleven reedbeds (five from Scotland and six from England) (see Chapter 2, Fig. 2.1), were chosen for this investigation.

3.2.2 Sampling within populations

Panicles (for seed collection) and leaf samples were collected at the same 20 sampling points in each reedbed in November - December 2003 and July 2004 respectively. Sample points were located along line transects and at least 50 m apart to minimise the risk of resampling the same clone. In 2003, the sampling locations were recorded using a hand held GPS (resolution ± *c.* 9 m) to facilitate relocation in 2004. Where the reedbed was not contiguous at a site, samples were collected from separate reedbed areas using a line transect perpendicular or parallel to the water's edge, depending on the width of the reed bed. At Insh Marsh a random sampling strategy was employed because of high water levels. Sampling strategies are summarised in Table 3.1. Panicles were temporarily stored in plastic bags and were then air dried in the laboratory and stored in brown paper bags in Hessian-like sacks in an unheated greenhouse to simulate conditions in a reedbed, until required for seed germination. Leaf samples were dried in silica gel and stored at room temperature until DNA extraction.

3.2.3 Plant material for cytological investigations

One panicle was randomly chosen from three sampling points for each reedbed and twenty seeds were extracted, germinated in Petri dishes under a diurnal temperature cycle and planted out into seedling trays containing non-peat compost (Levington's Multipurpose peat free compost, Levington Horticulture Ltd., Ipswich, UK). (Protocols of seed extraction, germination and seedling growth are given in the methods section of Chapter 5). The Loch Leven reedbed was not included in the cytological investigations as none of the panicles examined (twenty panicles, one from each sampling point) produced seeds. After seven weeks growth, five root tips were collected from three randomly chosen samples from each population so that a total of 150 root tips were analysed.

Table 3.1 Summary of strategies employed for sampling panicles and leaf collection in each population of *Phragmites australis.*

Population	Sampling strategy	Access restrictions
Insh Marsh	Random	High water table and ditches
Inner Tay	4 transects from water's edge to landward side, 1 transect parallel	Physical
Loch Leven	Narrow reedbed along lakeside, parallel sampling	High water levels
St. Margaret's Marsh	5 line transects across width of reedbed	No access restrictions
Mersehead	Line transects from discrete reedbeds and ditches	Physical
Leighton Moss	Parallel and perpendicular transects to waters edge	Physical and bird disturbance
Strumpshaw Fen	Parallel and perpendicular transects to water's edge and sampling from discrete reedbeds	Physical and bird disturbance
Lakenheath	Line transects from discrete reedbeds and ditches	No access restrictions
Walberswick	Line transects along path from edges of reedbed	Bird disturbance
Minsmere	Line transects from discrete reedbeds and ditches	Physical and bird disturbance
North Warren	Sampling from discrete reedbeds (line transect). 10 samples taken from water's edge	Water's edge access by punt. Physical and bird disturbance

3.2.3.1 Cytological protocol

The protocol was adapted from Jong and Möller (2000). The harvested young white root tips (1 cm long) were cleaned with tap water, pre-treated with α-bromonaphtalene (saturated aqueous solution) and stored in glass vials in darkness at room temperature. After three hours, they were rinsed in distilled water, fixed with freshly prepared Farmer's fluid (3 ethanol : 1 glacial acetic acid) and stored over night at 4 °C. After hydrolysis for 30 minutes with 5M HCl at room temperature, followed by several changes of distilled water, the root tips were transferred to Feulgen reagent prepared according to Fox (1969; cited in Jong and Möller, 2000), and stored in darkness at room temperature for 2 hours. To facilitate squashing (to soften the root tips), the stained material was treated with an enzyme mixture of 4 % pectinase (Sigma 2401) and 4 % cellulase (BDH or Calbiochem 21947) and placed in a water bath at 35 °C for 2.5 hours. Finally the root tips were squashed in 0.4 % aceto-carmine counter-stain and chromosomes were counted using photomicrography (Zeiss Axiophot microscope; chromosome counting and image capture aided by Optimas 6.2 software).

3.2.4 DNA extraction

Genomic DNA was extracted following a modified method of Doyle and Doyle (1990). Approximately 20 mg of dried leaf material was ground in a mortar and pestle in liquid nitrogen. Grindate was poured into 1.5 ml Eppendorf tubes and 1000 µl of pre-warmed (65 °C) CTAB extraction buffer (containing 40 mg of polyvinylpyrrolidone and 20 µl of β-mercaptoethanol) was added. After vortexing, each sample was incubated in a 65 °C water-bath for 30 minutes, then mixed with 500 µl of ice-cold chloroform isoamyl alcohol (24:1) and agitated on an orbital shaker for 20 minutes before centrifuging for 10 minutes at 13,000 rpm at room temperature. The aqueous phase was transferred to another Eppendorf tube and the chloroform isoamyl alcohol extraction repeated. Ice-cold isopropanol was added to the resultant aqueous phase and the DNA was precipitated over night at - 20 °C. After pelleting the DNA by centrifuging at 13,000 rpm for 10 minutes at room temperature, the supernatant was removed and the DNA pellet was washed with 76 % ethanol. After vortexing for one minute, the pellet was stored for one hour at - 20

°C, then centrifuged at 13,000 rpm for 5 minutes, the supernatant removed and the DNA pellet dried by inverting the Eppindorf tube at room temperature for an hour. The DNA was then re-suspended in 100 μl of T. E., pH 7.6, to which 2 μl of RNAase was added, and incubated at 37 °C for an hour. The suspended DNA was washed on ice for 15 minutes with 50 μl of 7.5 M ammonium acetate and then centrifuged at 10,000 rpm at 4 °C for 30 minutes. The supernatant was precipitated with 100 μl of ice-cold 96 % ethanol and stored for one hour at - 20 °C. After centrifuging at 5,000 rpm for 10 minutes at 4 °C, the ethanol was drained off and the pellet was washed in 70 % ethanol and centrifuged again at 5,000 rpm for 10 minutes. The pellet was drained of ethanol, left to dry for an hour in the inverted Eppindorf tube and resuspended in 100 μl T.E., pH 7.6. After incubation at 37 °C for one hour, each sample was stored at - 80 °C until required for PCR analysis.

3.2.5 Microsatellite analysis

Five microsatellite loci developed by Saltonstall (2003) for *P. australis* were chosen for the analysis. All of the loci have a $(CA)_n$ dinucleotide repeat (Table 3.2) and were previously shown by Saltonstall (2003) to amplify only one or two alleles per individual in tetraploids.

3.2.5.1 PCR amplification

Reactions were performed in a total volume of 10 μl, containing ≈100 ng DNA, 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 4 pM of each primer (FAM NED or VIC end labelled) and 0.5 u Biotaq DNA polymerase (Bioline Ltd, London, UK). PCR amplifications were carried out according to Saltonstall's protocol (2003), on a thermal cycler (Gene Amp PCR system 9700, Perk and Elmer) using the following conditions: an initial denaturation at 94 °C for 12 minutes, followed by 35 iterations of 94 °C for 30 s, 52 °C (*Pa*GT13, *Pa*GT14) or 54 °C (*Pa*GT4, *Pa*GT9, *Pa*GT16) for 30 s, and 72 °C for 4 s, and finally a 2 minute extension at 72 °C. PCR products were diluted (1/100) with sterile water and all five loci of each sample were run in a single well, together with an internal size standard (Genescan 500 size standard, Applied Biosytems), on a 96 capillary 3730 DNA analyzer. Sizes were determined by automated fluorescent scanning detection. All alleles were unambiguously identified,

based on size and colour separation, and scored using GeneMapper software (v 3.5, Applied Biosystems).

3.2.6 Statistical methods

3.2.6.1 Clonal structure and diversity within populations

To formally assign samples to genetic individuals (genets or clones) or individuals possessing the same genotype (ramets), P_{gen} (equation 3.1) and P_{se} (equation 3.2) were calculated (Parks and Werth, 1993) P_{gen} calculates the likelihood that identical genotypes result from independent sexual events, whilst P_{se} calculates the likelihood of encountering a second distantly placed ramet that has been produced by an independent sexual event. The formulae for these statistics are:

$$P_{gen} = \prod_{i=1}^{k} (p_i \; q_i) 2^h \qquad \text{(Equation 3.1)}$$

where $p_i q_i$ is the product of the frequencies of the two alleles at the ith locus of the k loci represented in the multilocus genotype and h is the number of loci that are heterozygous.

$$P_{se} = 1 - (1 - P_{gen})^G \qquad \text{(Equation 3.2)}$$

where G is the number of distinct genotypes found.

Values for P_{gen} and P_{se} are conditioned on the assumption that alleles at each locus are distributed independently i.e. there is random mating and no linkage disequilibrium. For Loch Leven only one genotype was found in each of the 20 samples analysed. Therefore $p_i q_i$ in equation 1 was determined from the mean allele frequency over all populations.

One of the loci (*Pa*GT 14) did not behave in a diploid fashion, as 3 - 4 alleles were usually detected per locus. This locus was not included in the calculations, although the information on the allelic phenotype at this locus was used in the assignment of ramets or genets.

Clonal diversity within each population P_d (the proportion of distinguishable genotypes) was calculated as the number of genets detected divided by the sample size (Ellstrand and Roose, 1987).

All further statistical analysis was conducted using FSTAT software (Goudet, 2001) which estimates and tests gene diversities and fixation indices (Wright 1951). Locus *Pa*GT14 and multiple sampled ramets, identified from the results of equation 3.2, were excluded from all statistical analysis.

3.2.6.2 Gene diversity

For each locus and at the intraspecific level, number of alleles, mean number of alleles, observed (*Ho*) and expected (*He*) heterozygosity were calculated according to Nei (1987). A global test of Hardy-Weinberg equilibrium (HWE) for each locus and over all populations was tested by permuting alleles among samples and comparing the randomised data set with the observed F_{IT}. Linkage disequilibrium was tested between all pairs of loci within populations using the log-likelihood ratio G statistic and the *P*-value was estimated as the proportion of values from the randomised data sets that were larger or equal to the observed value (Goudet, 2001).

3.2.6.2.1 Differentiation statistics: F_{IS}, F_{ST} and F_{IT}

Values of Wright's (1951) fixation index F_{ST} (genetic divergence of the populations, or the reduction in heterozygosity within populations relative to the whole population due to selection or drift (Lowe *et al.*, 2004)), the within population inbreeding coefficient F_{IS} and F_{IT} (the reduction in heterozygosity within individuals relative to the total population) were estimated as θ, f and F respectively, following the ANOVA model of Weir and Cockerham (1984) in which

$$\theta = \frac{\sigma_a^2}{\sigma^2} \approx F_{ST} \qquad \text{(Equation 3.3)}$$

$$f = \frac{\sigma_b^2}{(\sigma_b^2 + \sigma_w^2)} \approx F_{IS} \qquad \text{(Equation 3.4)}$$

$$F = \frac{(\sigma_a^2 + \sigma_b^2)}{\sigma^2} \approx F_{IT}. \qquad \text{(Equation 3.5)}$$

Here σ_a^2 is the diversity between populations, σ_b^2 is the diversity of individuals within populations and σ_w^2 is the diversity of alleles within individuals. Global testing of θ and f and F could not be calculated by bootstrapping or jackknifing as only four loci were included in the analysis.

The degree of genetic similarity between pairs of populations (genetic distance) was calculated using the multilocus Weir and Cockerham estimator of F_{ST} (θ) between all pairs of populations. The hypothesis that populations were differentiated according to the isolation by distance model (Wright, 1943) was tested using linear regression of pairwise θ against the pairwise geographic distance (Rousset, 1997).

3.3 RESULTS

3.3.1 Ploidy

Chromosome counts for each of the three samples from the ten British populations of *P. australis* confirmed that all populations had 48 chromosomes and were tetraploid ($2n = 4x = 48$). Fig. 3.1 shows the tetraploid nature of the chromosomes in a typical root tip squash from St. Margaret's Marsh.

3.3.2 Microsatellite analysis

3.3.2.1 Amplification of alleles

Each of the five loci could be scored unambiguously. At loci *Pa*GT4, *Pa*GT9 *Pa*GT13, and *Pa*GT16 one or two alleles were detected (Table 3.2) per individual as expected for a diploid inheritance pattern. Fig. 3.2 shows the banding pattern for three samples from the Inner Tay population for locus *Pa*GT16. The diploid allele number shown in Fig. 3.2 was not seen at locus *Pa*GT14 which produced between 1 - 4 alleles per individual, with three alleles per individual being the most frequent over all populations. All loci were polymorphic with the number of alleles varying

between 2 (*Pa*GT13) and 15 (*Pa*GT14) (Table 3.3). The total number of alleles scored from all individuals over all five loci was 45.

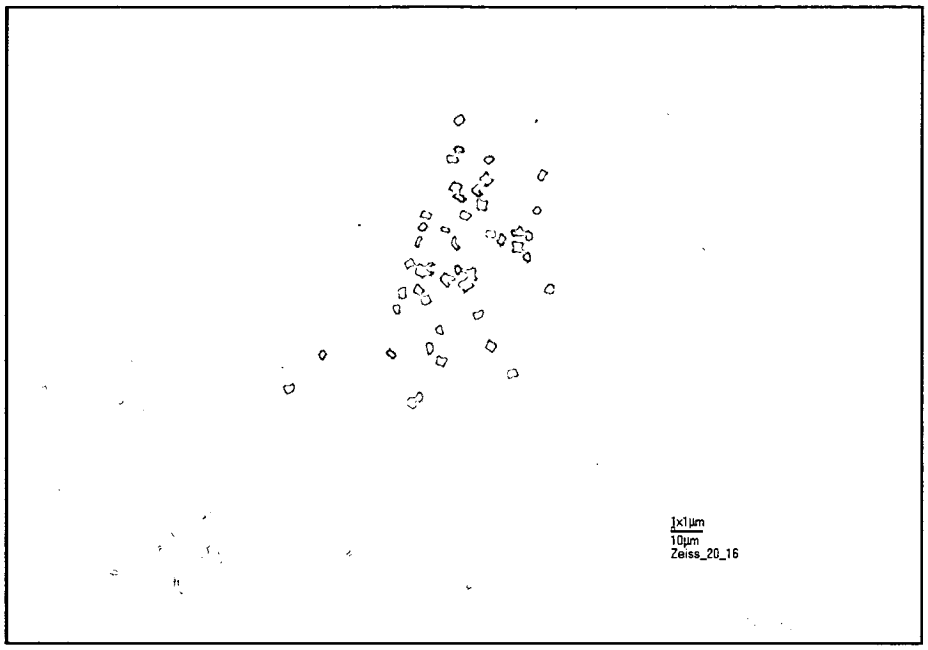


Figure 3.1 Root tip mitotic chromosomes (metaphase) of *P. australis*, 2*n* = 48. Plant material from St. Margaret's Marsh.

Linkage disequilibrium was tested for each pair of loci within populations. Out of 32 tests (the test was not applicable to Loch Leven) there were only three significant results ($P < 0.05$, using Bonferroni corrections) which were found for loci *Pa*GT4 x *Pa*GT16 (Mersehead), *Pa*GT9 x *Pa*GT16 (Mersehead) and *Pa*GT4 x *Pa*GT9 (North Warren).

Table 3.2 Primer sequences, number of repeat units, overall population allele size range and maximum (max.) number of alleles per sample of five microsatellite loci of 11 British populations of *P. australis*.

Locus	Primer sequences (5' - 3')	No. repeat units	Allele size range (bp)	Max. no. of alleles per sample
*Pa*GT4	*F*: TGCTCCCTGCCAGTTTCTTG *R*: TATCCACCCTTCGAAGGCAC	$(CA)_9$	272-287	2
*Pa*GT9	*F*: CCATGTGTTAATGTTGTCC *R*: TTGAATCCACACGTTTCCG	$(CA)_{10}$	187-226	2
*Pa*GT13	*F*: CTCATGCATCACTTCACAGG *R*: ACACGGACCTAACATCAACC	$(CA)_9$	209-211	2
*Pa*GT14	*F*: GTTGCAGCAAGTATTTGG *R*: CAAGCATTCTAGTAGTAGC	$(CA)_7$	168-192	4
*Pa*GT16	*F*: ACCAATCAGTCAGACTAGCC *R*: GTTCTCATGTTGGAGAAGCC	$(CA)_{10}$	233-292	2

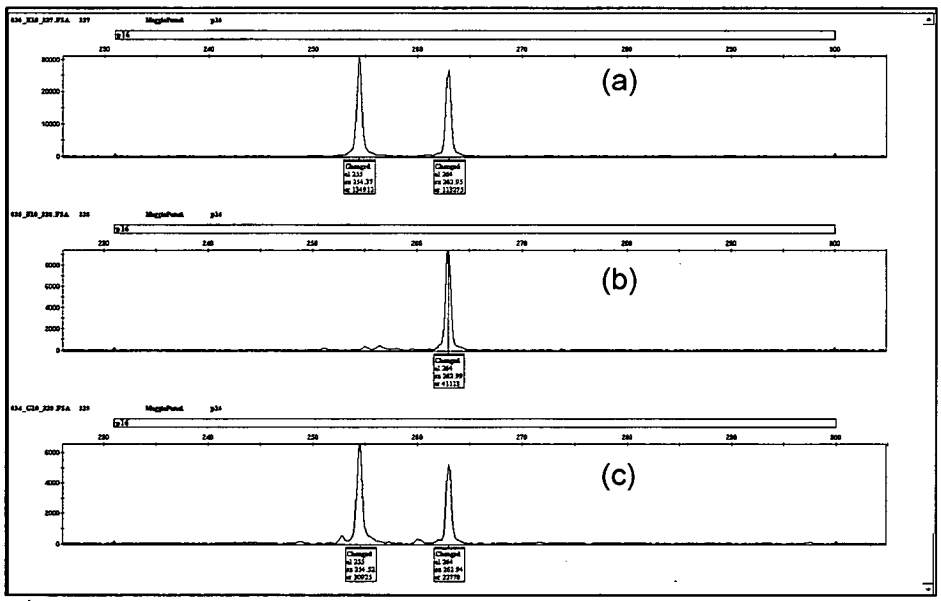


Figure 3.2 Banding pattern of three samples from the Inner Tay population for loci *Pa*GT16. (a) and (c) samples show the presence of a heterozygote, whilst (b) shows the presence of a homozygote.

Table 3.3 Total number of alleles, *Ho*, *He* (observed and expected heterozygosity) and *f* (inbreeding coefficient) of 11 British populations of *P. australis*. * *Pa*GT14 results not used in calculations see text for explanation.

Locus	Total no of alleles detected	*Ho*	*He*	*f*
*Pa*GT4	7	0.688	0.648	- 0.108
*Pa*GT9	11	0.725	0.777	- 0.037
*Pa*GT13	2	0.07	0.068	- 0.176
*Pa*GT14	15	*	*	*
*Pa*GT16	10	0.65	0.696	- 0.156
Overall loci	45	0.533	0.547	- 0.098

3.3.2.2 Clonal structure and diversity within populations

Within each population the number of unique genotypes varied between one and twenty and the total number scored over all populations was initially 121 out of a possible 220. Putative clones within populations were determined by calculating P_{gen} and P_{se} and those genotypes with $P_{se} < 0.05$ were assumed with greater than 95 % confidence to comprise a single genet (clone) (Parks and Werth, 1993). The genotypes, P_{gen} and P_{se} values of all possible clones for each population are shown in Table 3.4.

Each of the 20 samples from Loch Leven had the same genotype (including locus *Pa*GT14, which was not used in the calculations) and there was a very high probability that all 20 samples were ramets of one clone ($P_{se} = 0.0008$). Of the three potential clones of Insh Marsh, only one could be assumed with greater than 95 % confidence to comprise a single genet, whilst the three putative clones at Inner Tay were confirmed to be present. Parks and Werth's (1993) estimation of P_{se} is problematic because of circular reasoning as it relies on an estimation of G (equation 3.2), the number of genotypes encountered, but the actual number cannot be known until after the calculation. After formally assigning clones within populations, the number of unique genotypes amongst the 11 populations was increased to 185 out of a possible 220.

Table 3.4 Genotype (base pair size) and number of individuals sampled (ramets, R) of putative clones (C) from 11 populations (Pop) of *P. australis* and the estimated probability P_{gen} and P_{se}. Locus *Pa*GT14 was not used in the calculations. Population abbreviations are given in Table 2.1.

Pop	R	*Pa*GT4	*Pa*GT9	*Pa*GT13	*Pa*GT14	*Pa*GT16	P_{gen}	P_{se}
IM								
C 1	2	274,274	198,198	209,209	176,181,187	255,264	0.059	0.35^{ns}
C 2	11	274,274	198,209	209,209	176,181,187	255,264	0.0072	0.05*
C 3	2	274,274	198,198	209,209	176,185,187	255,261	0.031	0.20^{ns}
IT								
C 1	4	274,285	187,204	209,209	176,183,187	255,282	0.00033	0.003**
C 2	7	274,279	198,204	209,209	176,187,190	255,261	0.0011	0.010*
C 3	2	274,285	198,198	209,209	168,176,185	261,264	0.0089	0.017*
LL								
C 1	20	274,277	198,200	209,209	176,183,190	264,290	0.0008	0.0008***
SM								
C 1	2	274,285	187,198	209,209	168,176,183,187	255,255	0.039	0.41^{ns}
MH								
C 1	2	274,288	198,198	209,209	176,181,187	264,264	0.039	0.41^{ns}
C 2	5	274,274	198,200	209,209	181,187	261,264	0.017	0.20^{ns}
C 3	2	274,282	187,191	209,209	176,187,190	264,290	0.00005	0.0007***
LM								
C 1	7	274,285	204,206	209,209	168,176,187,190	259,264	0.076	0.51^{ns}
C 2	3	274,285	204,206	209,209	187,190	259,264	0.076	0.51^{ns}
SF								
C 1	2	274,277	198,198	209,209	176,183,190	261,264	0.0032	0.06^{ns}
C 2	2	274,277	200,200	209,209	168,190	261,264	0.0001	0.002**
MM								
C 1	2	277,277	202,206	209,209	168,176,179,187	261,264	0.0021	0.04*
NW								
C 1	5	274,277	200,206	209,209	176,190	264,264	0.02	0.23^{ns}
C 2	3	274,277	198,198	209,209	168,176,187,192	264,264	0.076	0.64^{ns}
C 3	2	274,277	198,209	209,209	168,176,187,192	264,264	0.025	0.28^{ns}

ns, not significant; * $P < 0.05$; ** $P < 0.001$, *** $P < 0.001$

The spatial distribution of 10 genets of the Inner Tay and 1 genet of the Loch Leven population are shown in Fig. 3.3 and 3.4 respectively. The largest genet was found in the monoclonal population of Loch Leven with a length of ≈1.9 km. The clone was not continuous, but separated into reed 'clumps' of varying sizes forming chains along the shore line. Inner Tay clone 1 stretched from the estuary's tidal mud flats to ≈230 m inland, perpendicular to the water's edge. Clone 2 was the largest clone in the population and potentially covered an area of ≈5.1 ha. (The estimate of the size

of both clones assumes that individuals between samples are ramets of the same genotype as clones 1 and 2), Clone 3, was parallel to the estuary's edge and ≈114 m in length.

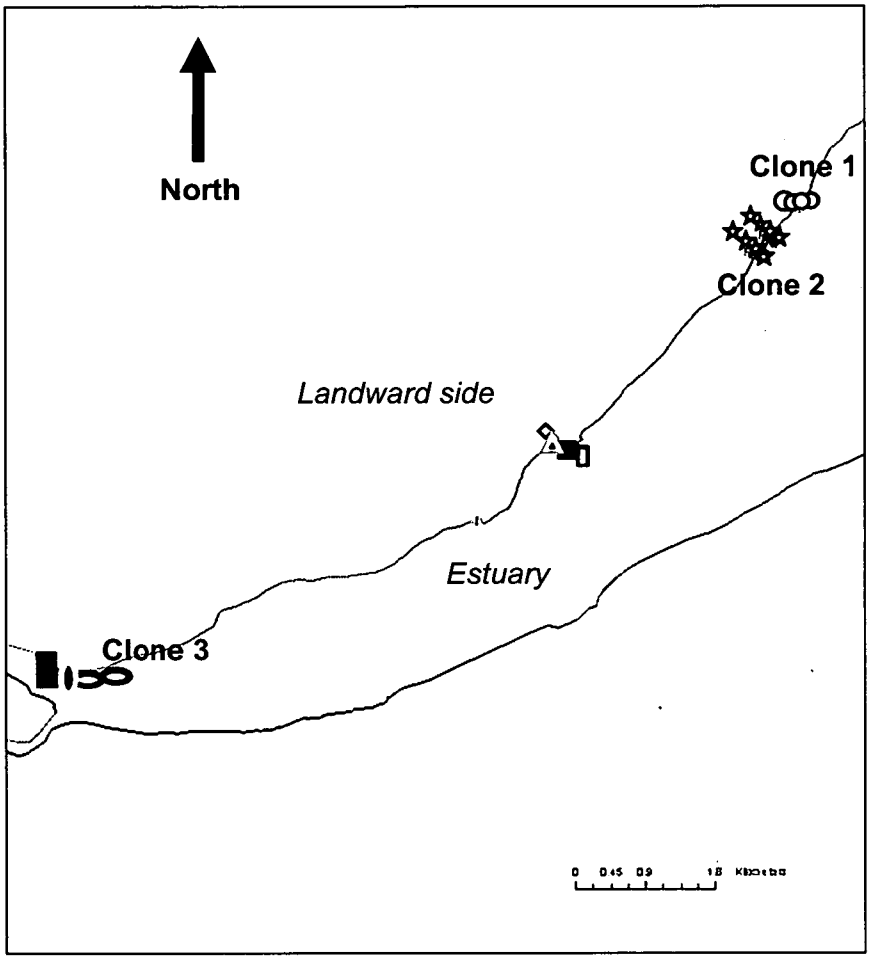


Figure 3.3 Map of spatial distribution of multilocus genotypes of the Inner Tay population. Each clone is assumed with greater than 95 % confidence to comprise a single genet. Samples were > 50 m apart and the location of each sample was recorded using a hand held GPS (horizontal resolution ± *c.* 9 m). Each clone has the same symbol and is not drawn to scale. Only clones 1, 2 and 3 are labelled on the figure.

The clonal diversity per population was calculated as P_d (proportion of distinguishable genotypes) and was high for each population except Loch Leven and Inner Tay (Table 3.5), where P_d was 0.05 and 0.5 respectively.

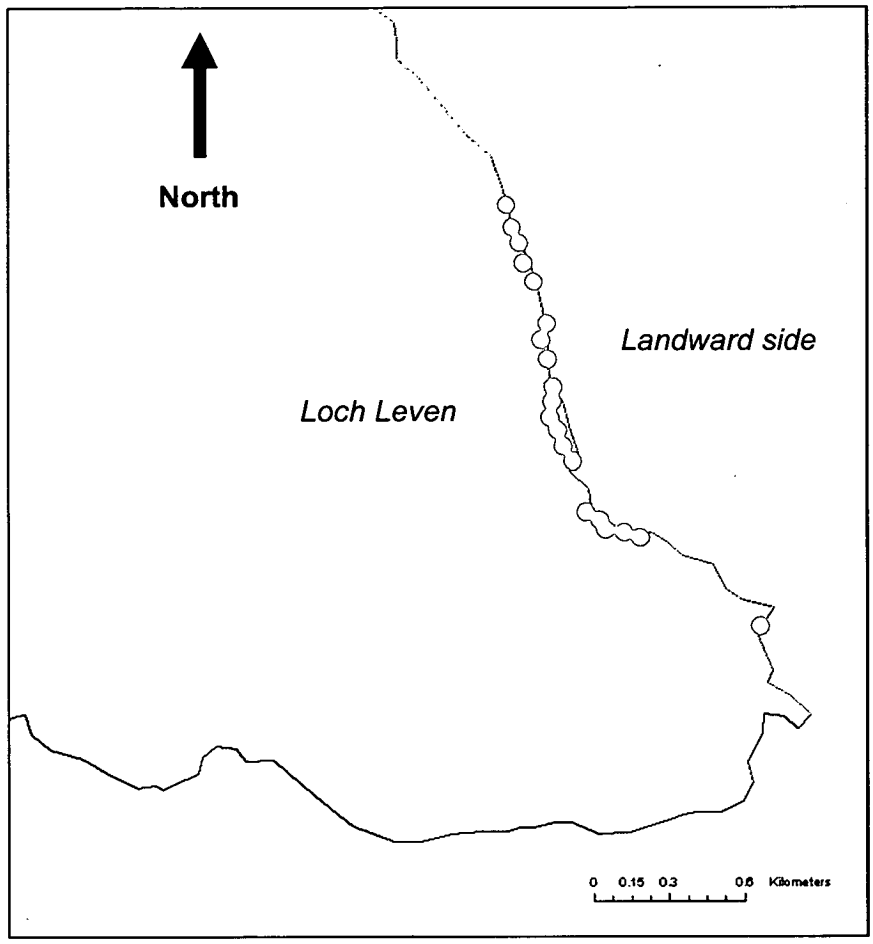


Figure 3.4 Map of spatial distribution of multilocus genotypes comprising one genet (clone) of the Loch Leven population. Samples were > 50 m apart, and the location of each sample was recorded using a hand held GPS (horizontal resolution ± *c.* 9 m). Length of clone was ≈1.9 km. Ramets are not drawn to scale.

3.3.2.3 Genetic diversity

The genetic diversity displayed within populations of *P. australis* was high. Mean allele numbers varied between 1.5 (Loch Leven) and 5.25 (Walberswick) per population (Table 3.6). For all populations, *Pa*GT13 displayed a maximum of two alleles and eight of the 11 populations had a fixed homozygote (209 bp), whilst three of the southern populations (Strumpshaw Fen, Walberswick and North Warren)

contained the rare heterozygote (209, 211). Private alleles were found at Inner Tay (locus *Pa*GT16, 233 bp) Lakenheath (*Pa*GT4, 287 bp; *Pa*GT16, 292 bp), Walberswick (*Pa*GT9, 213 bp) and Minsmere (*Pa*GT16, 233 bp).

Table 3.5 Genotypic diversity, measured as the proportion of distinguishable genotypes (P_d), in 11 *P. australis* populations. Population abbreviations given in Table 2.1

Population	No. of genets detected g	No. of ramets sampled R	P_d
IM	19	20	0.95
T	10	20	0.50
LL	1	20	0.05
SM	20	20	1.00
MH	19	20	0.95
LM	20	20	1.00
SF	19	20	0.95
LH	20	20	1.00
W	20	20	1.00
MM	19	20	0.95
NW	20	20	1.00

Ho within populations was generally high for all loci (Table 3.6). The monoclonal Loch Leven population was heterozygous at all loci except *Pa*GT13. Over all populations *Ho* per loci varied between 0.07 (*Pa*GT13) and 0.725 (*Pa*GT9) (Table 3.3) and was high for three out of four loci. This was due to several loci within populations being nearly fixed for certain heterozygous genotypes (Table 3.6). For example *Ho* = 1 for locus *Pa*GT16 at Leighton Moss and for North Warren *Ho* = 0.945 at locus *Pa*GT4. *He* averaged over all four loci ranged from 0.213 (Insh Marsh) to 0.605 (Walberswick) per population (Table 3.6). The mean *Ho* across all loci and populations was 0.533, whilst *He* was slightly higher than *Ho* being 0.547 (Table 3.3).

Table 3.6 Within population genetic diversity in 11 British *P. australis* populations characterised by number of alleles detected (*A*), observed (*Ho*) and expected (*He*) heterozygosity and the inbreeding coefficient (*f*) for each loci and each population (mean values). Multiply sampled ramets and *Pa*GT14 locus have been excluded from the calculations. Population abbreviations are given in Table 2.1.

Population	Microsatellite loci				
	*Pa*GT4	*Pa*GT9	*Pa*GT13	*Pa*GT16	Mean
IM					
A	2	4	1	3	2.5
Ho	0.05	0.15	0	1	0.3
He	0.053	0.247	0	0.55	0.213
f	0	0.148	NA	- 0.819	- 0.487***
IT					
A	5	6	1	4	4
Ho	1	0	0	0.88	0.47
He	0.683	0.756	0	0.722	0.54
f	- 0.463	- 0.059	NA	- 0.246	- 0.249
LL					
A	2	2	1	2	1.5
Ho	1	1	0	1	0.75
He	NA	NA	NA	NA	NA
f	NA	NA	NA	NA	NA
SM					
A	3	5	1	4	3.25
Ho	0.737	0.85	0	0.35	0.484
He	0.615	0.692	0	0.313	0.405
f	- 0.197	- 0.228	NA	- 0.118	- 0.195
MH					
A	4	6	1	4	3.75
Ho	0.421	0.63	0	0.45	0.375
He	0.66	0.56	0	0.458	0.41
f	0.411	- 0.09	NA	- 0.028	0.124
LM					
A	4	4	1	2	2.75
Ho	0.85	0.95	0	1	0.7
He	0.65	0.55	0	0.5	0.425
f	- 0.308	- 0.727	NA	- 1	- 0.647*
SF					
A	4	8	2	4	4.5
Ho	0.778	0.684	0.105	0.632	0.550
He	0.633	0.784	0.102	0.7	0.555
f	- 0.164	0.127	- 0.029	0.098	0.028
LH					
A	6	7	1	3	4.25
Ho	0.700	0.700	0	0.75	0.538
He	0.647	0.821	0	0.554	0.506
f	- 0.081	0.147	NA	0.026	0.039
W					
A	6	8	2	5	5.25
Ho	0.4	0.789	0.45	0.5	0.535
He	0.592	0.836	0.355	0.636	0.605
f	0.324	0.119	- 0.267	0.292	0.158
MM					
A	4	6	1	3	3.5
Ho	0.737	0.737	0	0.5	0.494
He	0.682	0.774	0	0.554	0.503
f	- 0.1	- 0.034	NA	- 0.083	- 0.07
NW					
A	4	6	2	2	3.5
Ho	0.947	0.631	0.211	0	0.447
He	0.618	0.725	0.193	0.105	0.410
f	- 0.532	0.129	- 0.091	1	- 0.09

Significant departure from Hardy-Weinberg expectations * $P < 0.05$, *** $P < 0.001$

Within populations, the mean inbreeding coefficient (*f*) was negative for six out of ten populations (Loch Leven was excluded from the analysis as only one genotype was found) (Table 3.6). Significant negative *f* values were found at Leighton Moss (- 0.647) and Insh Marsh (- 0.487) (Table 3.6). Over all populations, *f* was a very slightly negative (- 0.098), indicating that there was a slight heterozygote excess, although the significance of the result could not be ascertained as only four loci were examined. Over all loci and populations, the correlation of genes within individuals (inbreeding), measured by *F* (Weir and Cockerham, 1984) was 0.096, tests for Hardy-Weinberg equilibrium (HWE) for each loci and over all populations using *F* showed that two of the four loci (*Pa*GT9 and *Pa*GT16) did not conform to HWE ($P < 0.01$). Over all loci and populations, HWE was rejected ($P < 0.01$) indicating that there was evidence of structuring amongst populations. This was confirmed by the over all measure of population differentiation θ (0.177) which is an estimation of Wright's (1951) fixation index F_{ST} (Weir and Cockerham, 1984). Although the significance of this result could not be tested it indicates a degree of genetic differentiation amongst populations.

3.3.2.4 Isolation by distance model

Pairwise estimates of F_{ST} for all populations are given in Table 3.7. Loch Leven was excluded as only one genotype was found. St. Margaret's Marsh had the largest mean genetic distance ($F_{ST} = 0.302$), whilst Inner Tay had the least ($F_{ST} = 0.102$).

Regression analysis of pairwise estimates of F_{ST} versus geographical distance showed a significant positive association ($F_{1,60} = 12.39$, $P < 0.01$), although the R^2 value was low (0.157) (Fig. 3.5). Excluding Inner Tay, on the assumption that this population originated from The Netherlands, did not substantially alter the weak association ($F_{1,52} = 15.88$, $P < 0.001$, $R^2 = 0.219$) (Fig 3.6).

Table 3.7 Pairwise F_{ST} values, mean F_{ST} and geographical distances (km) between 10 British populations (Pop) of *P. australis* populations, excluding Loch Leven.

Pop	IM	SM	IT	MH	LM	SF	MM	LH	W	NW
IM		0.293	0.191	0.216	0.447	0.286	0.322	0.247	0.230	0.315
SM	221		0.126	0.300	0.349	0.238	0.290	0.207	0.254	0.360
IT	187	39		0.061	0.205	0.050	0.056	0.030	0.055	0.141
MH	341	126	165		0.284	0.058	0.064	0.099	0.103	0.050
LM	430	209	246	97		0.203	0.212	0.131	0.183	0.250
SF	699	494	517	422	331		0.016	0.029	0.042	0.082
MM	739	533	557	456	363	363		0.057	0.050	0.093
LH	685	472	499	386	291	291	97		0.042	0.137
W	734	528	552	452	360	360	452	78		0.098
NW	745	538	563	460	367	367	7	80	13	
MEAN F_{ST}	0.290	0.269	0.102	0.140	0.252	0.112	0.130	0.109	0.123	0.170

Upper sector F_{ST}. Lower sector, distances in kilometres

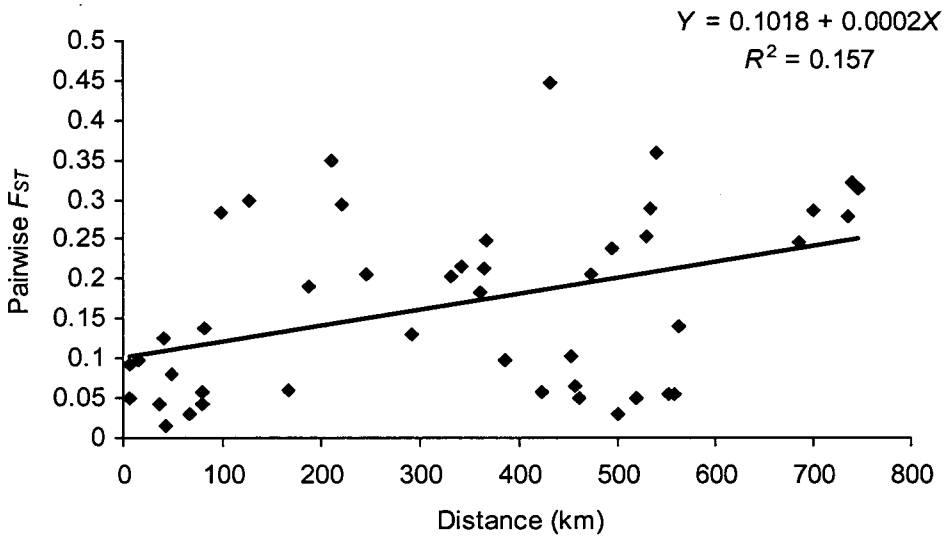


Figure 3.5 Regression analysis of pairwise F_{ST} and geographical distance among 10 British populations of *P. australis*. Loch Leven was excluded from the analysis.

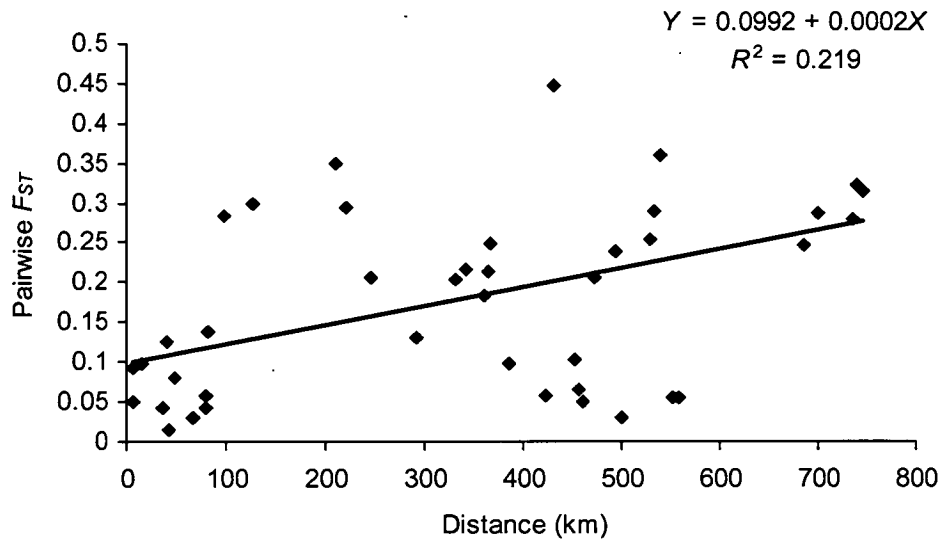


Figure 3.6 Regression analysis of pairwise F_{ST} and geographical distance among 9 British populations of *P. australis.* Inner Tay and Loch Leven were excluded from the analysis.

3.4 DISCUSSION

3.4.1 Ploidy Levels

The ploidy level of all ten British populations of *P. australis* examined in this study was found to be tetraploid. The same ploidy level was also found by Clevering and Lissner (1999) who examined four British populations of *P. australis* (including the Inner Tay) using flow cytometry. Although the ploidy level of the Loch Leven population could not be ascertained due to lack of plant material, field observations suggested that the population phenotype was similar to that of the other ten *P. australis* populations examined in this study. There was no evidence of the 'gigantism' (increase in shoot height, diameter and leaf area) that is associated with *P. australis* from higher ploidy levels (Paucã-Comãnescu *et al.*, 1999). So it is likely that this population was also a tetraploid.

For microsatellite analysis it was assumed that all populations were allo-tetraploid and showed strict bivalent formation at meiosis, resulting in a diploid inheritance

(Jenkins and Rees, 1991). Furthermore, it was assumed that the allelic dosage followed fixed parental inheritance patterns and that the presence of two alleles at a locus were a result of two copies of each allele rather than one copy of one and three of the other (Saltonstall, 2003). (The latter assumption is supported by the investigation of the breeding system of *P. australis* in Chapter 4 that showed diploid segregation of microsatellite markers in open-pollinated families from the Inner Tay population.)

3.4.2 Genetic analysis using microsatellite markers

The data presented in this study, is one of the few to use microsatellite markers to analyse variation in a polyploid species. The polymorphism displayed by the four microsatellite loci (excluding locus *Pa*GT14) was high. A total of 30 alleles were scored over all populations and the mean allele number within populations was 3.52.

3.4.2.1 Genotypic diversity

Multilocus genotypes based on four polymorphic microsatellite loci revealed that there was a wide variation in clonal diversity amongst the 11 populations of *P. australis* sampled. Whereas two populations displayed a low number of clones or complete monoclonality, other populations had maximal genotypic diversity among the 20 ramets sampled. The variability in clonal diversity was also found by Neuhaus *et al.* (1993) and Lambertini *et al.* (2003) who, using RAPDs to identify genotypes, found both monoclonal and multiclonal populations of *P. australis* in the Po Plain region of Italy and the northern German Lake district respectively. Guo *et al.* (2003), using allozymes, found both low and high numbers of genotypes within 15 populations of *P. australis* in the Yellow River delta, China, but none that were monoclonal. Variability in clonal diversity among populations has also been found for other species. Reusch *et al.* (2000) reported a dramatic range in clonal diversity in 12 populations of eelgrass (*Zostera marina*), detected by microsatellite markers, whilst Aspinwall and Christian (1992) and Jonsson *et al.* (1996) using allozymes, recorded both high and low levels of clonal diversity in 25 populations of Queen of the prairie (*Filipendula rubra*) and three populations of the Arctic sedge (*Carex bigelowii*), respectively.

Initially, clonal diversity in a reedbed is determined by the number of founding clones, arriving as seed (wind, water or animal dispersed), vegetative propagules (rhizome fragments spread by water, wind, animals or man) (Haslam, 1972; Clevering and Lissner, 1999; Keller, 2000) or recruitment from the seedbank (ter Heerdt and Drost, 1994). Thereafter seedling recruitment into a reedbed is rare because seedlings are out competed for resources, such as light, by the established *P. australis* population (Haslam, 1972) and sexual recruitment only occurs after disturbance (pulse recruitment) (Eriksson, 1997) such as water drawdown and possibly after burning or cutting of the vegetation. Colonisation of space within a population is usually by rhizomatous (clonal) growth only. It has been suggested that clonal diversity declines in reedbeds over time (Koppitz *et al.*, 1997), as clones which are adapted to the edaphic, hydrological and climatic conditions out compete less well adapted individuals. Furthermore *P. australis* has been found to be self-incompatible (Ishii and Kadono, 2002). Where large clones are present, or a population is monoclonal, compatible pollen limitation may occur (pollen dispersal is anemophilous), which may contribute to low seedset (Ishii and Kadono, 2002). Therefore genetic diversity within a population is dependent on founder effects, the number of clones that persist and on disturbance events which may provide the window of opportunity for sexual recruitment. Unless there is gene flow (by seed, pollen or vegetative propagules) between populations, genetic structuring will occur and, in general, genetic distances of European populations have been found to increase with geographic distance (Koppitz, 1999).

In this study, Loch Leven was the only population found to be monoclonal and the P_{se} value (Parks and Werth, 1993) suggests that it was unlikely that the same multilocus genotype arose through many independent sexual events ($P < 0.001$). The monoclonal reedbed at Loch Leven was not continuous but arranged in discrete clumps of disparate size that formed a narrow chain ≈20 m wide and ≈1.9 km long by the loch shore (Fig. 3.4). Each clump had spread into the water and had successfully colonised to a water depth of ≈0.8 m. The monoclonal structure of the population accords with the hypothesis that clonal populations, after an initial burst of sexual recruitment when the population is founded, show a decrease in genotypic diversity over time, after which only a few genets remain (Widén *et al.*, 1994). Since

seed germination is inhibited and small seedlings are intolerant of flooded conditions (Haslam, 1972; Amsberry *et al.*, 2000), initial reedbed establishment, the so-called *stage of settlement* (Koppitz *et al.*, 1997), must have occurred on the landward side, by seed or possibly vegetative propagules. During this phase the reedbed would exhibit high genetic diversity, especially if founded by seed. Thereafter, competition between expanding clones would lead to a decrease in clonal diversity and only those clones that were successfully adapted to the deep water could spread into the loch (Koppitz *et al.*, 1997). Indeed, Amsberry *et al.* (2000) observed that ramets of *P. australis* that invade harsh habitats (such as deep water) are supported by ramets in more physically benign habitats (e.g. the landward side). As the loch side appeared to have homogeneous hydrological and edaphic conditions (although not formally tested), one clone may have out competed all of the others. This *stationary stage* (Koppitz *et al.*, 1997) has been observed for other populations of *P. australis* (Zeidler *et al.*, 1994). The discrete clumps may have arisen due to the disappearance over time of clones not tolerant of deep water such that only one clone was left. In addition, the increased eutrophication of the Loch may have been a contributory factor to clonal decline (the water quality in Loch Leven deteriorated in the early 1970s, see section 2.1.2.3). Moreover, sexual recruitment (*via* an external seed source) into the established reedbed would be unlikely because the reedbed is not managed (by burning, cutting or drawdown) and therefore disturbance does not occur.

The Inner Tay population was the only other population studied which had low clonal diversity. Three clones were detected, two of which were large and located at the eastern edge of the reedbed (nearest to the North Sea) (Fig. 3.3). Similar to Loch Leven, the possibility that repeatedly sampled genotypes had arisen independently could not be discounted as four microsatellite loci may not have been sufficient to differentiate genotypes (even though the likelihood of this was small, $P < 0.05$). Although the P_{se} values for each genotype did not include the fifth locus of *Pa*GT14, the same three alleles were found in each individual that was analysed (Table 3.4) at this locus, for each putative clone (this was also found for the Loch Leven population), which further supports the assignment of ramets.

The largest clone at Inner Tay was located towards the eastern end of the reedbed and was ≈5.1 ha in extent (Figure 3.3). The clone stretched from the estuary's tidal mudflats, which are inundated twice daily by each high tide, to the landward side. The other clone in the most eastern part of the reedbed stretched ≈240 m from the mudflats to the landward side. The third clone was the smallest (114 m in length) and was located at the western edge of the reedbed, parallel to the waters edge. This upper section of the reedbed is only flooded by high spring tides. Generally in the Tay reedbed, clonal diversity increased from east to west, perhaps as a result of a decrease in salinity from east to west. The western part of the reedbed is located father away from the North Sea and is possibly less brackish than the eastern part because of greater mixing of freshwater from the River Tay with the tidal waters of the North Sea. Indeed, sediment sodium concentration (a measure of salinity) was found to decrease from east to west (Reynolds, 2004). (Sediment samples were taken from the same 20 points as leaf collection in this study).

Salt tolerance in *P. australis* varies, with some ecotypes being more tolerant than others (van der Toorn, 1972). The development of a reed stand may be adversely affected by high salinity (Hellings and Gallagher, 1992; Hanganu *et al.*, 1999) and it is possible that only those clones adapted to high saline concentrations colonised the eastern end of the reedbed. Coupled with this, seed germination is inhibited by high sodium chloride concentrations in the sediment (Haslam, 1972). Therefore sexual recruitment into the established reedbed may not be possible in the eastern part. These two hypotheses could be tested by: (i) transplanting rhizomes from the western into the eastern part of the reedbed (ii) germinating seeds from panicles harvested from both the eastern and western parts of the reedbed in saline conditions typical of the sediments in the eastern and western parts of the reedbed.

The Inner Tay reedbed has been cut for thatching for many years, apart from 1945 - 1974. Commercial cutting restarted in 1974 and produced approximately 20% of the UK thatching reed output (TayReed Company, pers comm. 2002; Hawke and José, 1996). Large sections of the reedbed (≈56 - 80 ha) are cut in the winter months on a rotational basis resulting in regular disturbance. However, harvesting the aerial parts does not remove the perennial rhizome system and it is likely that new shoots from

apical buds outcompete any seedlings trying to establish. Coupled with this, twice daily inundation with sea water at the eastern end of the reedbed would inhibit seedling establishment, particularly at the water's edge.

Since the reedbed at Inner Tay is continuous and not separated into clumps, the age of each clone was estimated. The average rhizome growth rate for this population is $\approx$0.6 m year^{-1} (this was estimated from the CGE, Chapter 6). Dividing half the maximum distance between ramets of each clone by the annual growth rate yields an estimated age of each clone of 200 years (Clone 1, 240 m long), 218 years (Clone 2, 5.1 ha) and 98 years (Clone 3, 114 m long). Whilst the age can only be estimated approximately by this method, the age of the oldest clones agrees with the postulated date of establishment of the reedbed by prisoners during the Napoleonic wars *c.* 1798 - 1815 (F. Keegan pers. comm. 2005; Hawke and José, 1996). The method for planting this population can only be speculated about, but there is no reason to suggest that it differed from the simplest and cheapest method still employed today, namely planting of seeds. Seed sowing must have occurred only on the landward side and, over time, the reedbed would have colonised the tidal and more saline mudflats by vegetative growth.

The clones found in the Inner Tay and Loch Leven populations are the largest reported in genetic studies of *P. australis* populations. The previous largest clone (0.3 ha) was found by Neuhaus *et al.* (1993), but, no statistical likelihoods were reported to assess the spurious assignments of ramets to genets. Furthermore, the lack of genetic variability found within the monoclonal population may have been an artefact due to the low number of primers (e.g. 2) used in the RAPD analysis (Keller, 2000).

Since large clones were found in only two of the eleven populations studied here, and it is hypothesised that genotypic diversity should decay over time within a population of a clonal species (Ellstand and Roose 1987; Widén *et al.*, 1994), the question remains as to how clonal diversity has been maintained in the other populations. It is probable that a combination of a heterogeneous environment (Minsmere, Leighton Moss, North Warren, Strumpshaw Fen), recent establishment (Lakenheath,

Mersehead) and disturbance by manipulation of water levels, burning or cutting of the reedbed (Walberswick, Strumpshaw Fen, North Warren, Minsmere, Leighton Moss) accounts for the maintenance of clonal diversity as well as allowing periodic sexual recruitment in the other populations studied.

3.4.2.2 Genetic diversity within populations

High levels of genetic diversity were observed within most populations, with *He* ranging from 0.213 (Insh Marsh) to 0.605 (Walberswick). Over all populations, mean *He* was 0.547, which is similar to the findings of Saltonstall (2003) and is in accordance with an increasing number of studies which have found that clonal plant species maintain the same levels of genetic diversity as non-clonal plant species (Ellstrand and Roose, 1987; Jonsson *et al.*, 1996; Reusch *et al.*, 2000; Ainsworth *et al.*, 2003).

For six out of ten populations the inbreeding coefficient was negative, but only two populations had significant negative values (Leighton Moss and Insh Marsh) which could indicate non-assortative mating (Lowe *et al.*, 2004) such that an individual was less likely to mate with another individual of the same genotype, causing an heterozygote excess. However, for these two populations, the calculated f value could also be caused by the predominance of certain genotypes that were sampled multiple times but were not found with > 95 % confidence to be the same clone under the probability tests of Parks and Werth (1993) (Table 3.4). However, it may be the case that heterozygous individuals were at a selective advantage over the long term period of selection. Heterozygous excess, possibly due to over dominance (Silverton and Charlesworth, 2001), was seen in the monoclonal population of Loch Leven as well as the three large clones of the Inner Tay population (Table 3.4) where three of the four loci were heterozygous. Over all populations, f was slightly negative which was not unexpected for a clonal, predominantly self-incompatible species.

3.4.2.3 Genetic diversity among populations

In the multilocus estimate of genetic differentiation, $\theta = 0.177$, which indicated a moderate degree of differentiation among populations. Coupled with this, HWE over all loci and populations was rejected ($P < 0.01$). The value of θ was slightly lower

than that found by Guo *et al.* (2003) (F_{ST} = 0.226) but their study may have overestimated genetic structure in *P. australis* as multiple sampled ramets were not excluded from the analysis.

In this study 83 % of the genetic diversity found using neutral markers was maintained within populations. This suggests that the genetic diversity detected using neutral markers is maintained in the different clones found within each population, although the possibility of somatic mutations maintaining clonal diversity (Widén *et al.*, 1994; Klekowski,. 1997, Clevering and Lissner, 1999) cannot be ruled out. However, if this was the case, it would be expected that the Loch Leven would contain more clones.

3.4.2.4 Pairwise estimates of F_{ST}

In the pairwise estimates of F_{ST} (Table 3.7), the value was low (F_{ST} = 0.042) for populations of Walberswick and Minsmere which are located within 7 km of each other, in the south east of England, suggesting that there is a small but significant amount of gene flow (either pollen, seed or vegetative propagules) between the two populations. Since every clone genotyped in this study was unique for each population (i.e. no clone was found in another population), gene flow between populations is unlikely to occur by vegetative propagules and is brought about either by pollen or seed dispersal only. The latter was also found in Koppitz's (1999) investigation of genetic diversity among world-wide populations of *P. australis* in which none of the clones detected in the study had settled in different lakes away from the original population. Lakenheath and Strumpshaw Fen had a low pairwise F_{ST} (0.029) even though they are 67 km apart. Lakenheath is a newly created reedbed and seed for planting has been obtained from Strumpshaw Fen and Minsmere giving rise to the low F_{ST} values, (pairwise F_{ST} = 0.029 and 0.057 respectively), as well as from other populations not investigated in this study. Likewise, the Inner Tay population has been used as a seed source for Mersehead, located 165 km away, and once again the pairwise F_{ST} was lower (0. 061) than would be expected by geographic location

Whilst it was found that the northern populations of Insh Marsh and St. Margaret's Marsh showed the greatest genetic distance compared to all of the other populations (mean pairwise F_{ST} = 0.288 and 0.269 respectively), the other Scottish populations Mersehead and Inner Tay, did not have high values. Indeed, Mersehead was found to be similar to Strumpshaw Fen located 422 km away (F_{ST} = 0.058). The mean F_{ST} for the Inner Tay population (0.102) did not support the hypothesis that this population's origin was allopatric (i.e. from The Netherlands), since it would be expected that the mean pairwise estimate of genetic distance would be large. In fact this population had the lowest mean F_{ST} of all the 11 populations. Although a rare allele was found in this population (locus *Pa*GT16, 233 bp) private alleles were also found at low frequency at Walberswick (locus *Pa*GT9, 213 bp) and Lakenheath (locus *Pa*GT4, 285 bp; locus *Pa*GT16, 292 bp). It is possible that the low F_{ST} for Inner Tay has arisen because the seed has been dispersed long distances by anthropogenic means, either as a seed source for reedbed establishment (e.g. Mersehead) or the commercial harvesting and distribution of reed stems to support the thatching industry in southern England.

3.4.2.5 Isolation by distance model

Regression analysis (with and without Inner Tay) showed a significant but weak association between genetic distance and geographic distance (R^2 = 0.157 and R^2 = 0.219, respectively). In agreement with the findings of other authors (Koppitz, 1999; Gou *et al.*, 2003) the populations did fit the isolation by distance model, but the degree of differentiation was low. Therefore it can be concluded that a small amount of gene flow is maintained (mainly by seed) between some British populations. This most probably occurs by anthropogenic activities such as commercial thatching (Inner Tay and Walberswick) and during reedbed establishment (e.g. Lakenheath and Mersehead) and conservation when a non-local seed source is used for planting.

3.5 SUMMARY

All British populations of *P. australis* investigated in this study were tetraploids. The clonal structure among populations was variable and although most populations exhibited high levels of genotypic diversity, two populations had low diversity or monoclonal populations. In addition, there was evidence of genetic structuring among populations and, in general, gene flow was limited between populations that were located far apart. The practical implications of this research will be examined in the final thesis chapter.

Chapter 4 – The breeding system of *Phragmites australis* revealed by microsatellite markers

4.1 INTRODUCTION

The breeding system fundamentally affects the genetic structure and dynamics of populations (Ritland and Jain, 1981). There are three basic breeding mechanisms: outcrossing (the fusion of male and female gametes from different genetic individuals), selfing (fusion of gametes from a single genetic entity) and apomixis (reproduction without fertilization, which can be by production of vegetative parts or seeds without sexual fusion) (Jarne and Charlesworth, 1993; Briggs and Walters, 1997). Outcrossing introduces new combinations of existing alleles into a population as well as novel alleles recruited from other populations by gene flow *via* pollen.

An effective mechanism for ensuring outcrossing is self-incompatibility, which acts at the pre-zygotic stage (e.g. rejection of self-pollen) or less commonly at the post-zygotic stage, to reject self-fertilization. For some plant species self-incompatibility is more cryptic. For instance, self-pollen is capable of achieving fertilization, but is out competed by pollen from another source which has a faster growth in the pollen tube (Jarne and Charlesworth, 1993). Outcrossed species maximize the advantages associated with sexual reproduction whilst limiting the reduced fitness effects of deleterious mutations (Brennan *et al.*, 2005).

Selfing is an assured method of producing offspring and is advantageous in extreme or marginal habitats, ensuring that well adapted genotypes can be replicated with little change (Briggs and Walters, 1997). However, long term disadvantages include inbreeding depression and inability to adapt to environmental change. Apomixis produces well adapted plants of maternal genotype. In polyploid species, with unbalanced chromosome numbers, this method of reproduction may be the only option because meiotic disturbance prevents viable seed production. Disadvantages of apomixis include lack of genetic variability and susceptibility to pests and diseases.

There has recently been a shift in thinking regarding the mating systems of plant species from the traditional view of plants as being either entirely outcrossing or selfing. It is now thought that plant species are typically much more mixed mating in nature (Brennan *et al.*, 2005).

In grasses, all three breeding mechanisms are found. However, many species are wind pollinated and highly outcrossing (Wang *et al.*, 2004) and in perennial species apomixis by vegetative spread, through the production of rhizomes, stolons or plantlets, is common. Self-compatibility is found in some grass species (e.g. wild rice, *Oryza perennis*) and is more common in grasses of extreme habitats and/or with high ploidy level. For example, *Festuca microstachys* is an endemic hexaploid grass that occurs in dry rocky exposed sites in western North America from sea level to *c.* 1800 m and has a selfing rate of *c.* 99 % (Adams and Allard, 1977). Other grasses such as the large tussock grasses which dominate the subalpine zone in the South Island of New Zealand (genus *Chionochloa*, family Poaceae) have a mixed mating system. Eight species have been found to be self-compatible, although outcrossing still occurs, so that a new generation is predominantly outcrossed because inbreeding depression reduces the seedling survival of selfed individuals (McKone *et al.*, 1997).

Phragmites australis is a typical perennial grass species that mainly spreads by vegetative growth (rhizomes), but also produces seed. As *P. australis* has been found to be mainly self-incompatible, by hand-pollination experiments (Ishii and Kadono, 2002), the clonal structure (i.e. size and spatial distribution of the genets) influences the patterns of pollen dispersal and mating opportunities (Ruggiero *et al.*, 2005). Therefore seedset may be reduced by compatible pollen limitation. In fact, numerous studies have found immense intraspecific variability in seedset (Gustafsson and Simak, 1963; Björk, 1967; Curran, 1969; McKee and Richards, 1996) and seedling recruitment in an established reedbed is a rare event (Haslam, 1972).

The balance between the two kinds of sexual reproduction influences the population's genetic diversity (Ruggiero *et al.*, 2005) and, in general, clonal diversity of a reedbed may decrease over time (Koppitz *et al.*, 1997). This has implications for conservation of *P. australis* populations (reedbeds), since the latter must be

sufficiently genetically variable to allow for adaptation to future environmental change and to decrease the conflict between self-incompatibility and seedset. Even a limited amount of sexual reproduction may enhance the genetic diversity of the offspring compared with the parents, by producing new combinations of existing alleles. The extent to which seedling recruitment increases the genetic variability is also determined by the difference in genetic variability between the parent clones and the progeny produced. In addition novel alleles may be introduced from other populations by gene flow (pollen or seed).

In this study the breeding system of *P. australis* was determined using four of the ten microsatellite markers developed by Saltonstall (2003) to investigate genetic variation within and among North American population lineages of *P. australis*.

The study aims were to investigate the breeding system and progeny genetic diversity, using families sampled from a large reedbed (Inner Tay, 410 ha) with high clonal spread (see Chapter 3). (Individuals in a family have the same maternal genotype; i.e. the seeds collected from a panicle constitute a family.) The following questions were addressed: (i) Does the evidence for self-incompatibility, found by hand pollination, equate to a high outcrossing rate? (ii) Does the latter explain low seedset in natural populations? (iii) How does the genetic diversity of the progeny compare to the adult population? (iv) Would higher seedling recruitment enhance the genetic diversity of reedbeds?

Seeds were collected from 20 families of *P. australis* sampled throughout the Inner Tay reedbed and up to ten offspring from each family were assayed. Research results were used to make management recommendations for enhancing seedset and increasing the genetic diversity of reedbeds in a natural manner.

4.2 MATERIALS AND METHODS

4.2.1 Seed collection

Panicles containing mature seeds of *P. australis* were collected from 20 families in the Inner Tay reedbed, Scotland (for reedbed location see Chapter 2, Fig 2.1). Each sampling point (one sample represents one family) in this population were the same as those used for the cytological and genetic diversity investigations (Chapter 3, Table 3.1) as well as for seedset and germination experiments (Chapter 5) and the CGEs (Chapter 6). Samples of families were located along five line transects (Table 4.1), perpendicular to the water's edge and at least 50 m apart to minimise the risk of resampling the same clone. For each family, 20 seeds were extracted from one panicle and germinated in Petri dishes following the protocol of Self (1999) as described in Chapter 5 section 5.2.4.

Table 4.1 Details of the twenty families of *P. australis* sampled from the Inner Tay reedbed for estimation of the mating system. The number of progeny available for assay and the transect from which each family was sampled are shown.

Family	No of progeny for assay	Transect	Family	No of progeny for assay	Transect
1	5	1	11	6	3
2	7	1	12	10	3
3	6	1	13	10	4
4	5	1	14	10	4
5	8	2	15	6	4
6	10	2	16	7	4
7	3	2	17	7	5
8	7	2	18	0	5
9	8	3	19	0	5
10	5	3	20	6	5

Once the seeds had germinated, the Petri dishes were transferred to a laboratory bench and placed under halogen lights. After a week, all germinated seedlings were planted into trays (22 cm x 52 cm) consisting of 20 cells (4 cm x 4 cm x 6 cm), each filled with non-peat compost (Levington's Multipurpose peat free compost, Ipswich, UK). Progeny were grown under halogen lamps for three weeks and then DNA was extracted from leaf samples collected from up to ten progeny per family and assayed

for four microsatellite loci. For some families it was not possible to grow 10 progeny and the number of progeny per family varied between 0 and 10, with an overall mean of 6.3 (Table 4.1).

4.2.2 DNA extraction

Genomic DNA was extracted following a modified method of Doyle and Doyle (1990) detailed in Chapter 3, section 3.2.4.

4.2.3 Microsatellite analysis

Four microsatellite loci (*Pa*GT4, *Pa*GT9, *Pa*GT13 and *Pa*GT16) developed by Saltonstall (2003) were chosen for the analysis. All of the loci have a $(CA)_n$ dinucleotide repeat and have been shown by Saltonstall (2003) and by this study (Chapter 3, section 3.3.2) to amplify only one or two alleles per individual in tetraploids. Details of primer sequences, number of repeat units and the protocol for PCR amplification are given in Chapter 3 section 3.2.5.

4.2.4 Statistical methods

FSTAT software (Goudet, 2001) was used to determine genetic diversity within the population. The number of alleles and observed (*Ho*) and expected (*He*) heterozygosity were calculated according to Nei (1987) for each locus and for the population.

4.2.4.1 Genetic structure within populations

Since families were collected from transects that were between 0.35 km and 9 km apart and because this study had detected three large clones (at transect 1, 2 and 3 and 5e) within this population (see Chapter 3, section 3.3.2.2), using the same sampling points that were used in this study, it was decided to test for population substructure, using Wright's (1951) *F*-statistic, F_{ST}, (the fixation index). This was carried out by pooling some of the families: families 1 - 4 (transect 1, only one clone found so each family had same maternal genotype) constituted one population, families 5 - 12 were another population (transect 2 and 3, only one maternal genotype for the eight families), families 13 - 16 (transect 4, each family had a

different maternal genotype) were another population and family 17 (transect 5, one genotype found here for families 17 and 18) and family 20 (transect 5, two genotypes found for families 19 and 20) were treated as two separate populations.

F_{ST}, was estimated following the ANOVA model of Weir and Cockerham (1984) (see Chapter 3, section 3.2.6.2.1 and equation 3.3). Global testing of θ could not be calculated by bootstrapping or jackknifing as only four loci were included in the analysis.

4.2.4.2 Outcrossing rate

Estimates of mating system parameters based on the mixed mating model were carried out using the statistical procedures and computer software developed for multilocus estimation of mating systems (MLTR 2.4 software) by Ritland and Jain (1981) and Ritland (2002).

In the mixed mating model, a fraction of the progeny are derived from self-fertilization (s) and the remainder derived from outcrossing (t) at random, so that $t = 1 - s$. Estimates of the selfing rate were found by maximising the likelihood of the data with respect to the selfing rate, using the expectation-maximisation method (Cheliak *et al.*, 1983). The model assumes that all plants have the same outcrossing rate, randomly sample the same pollen pool and have n unlinked loci (i.e. no linkage disequilibrium) (Ritland, 2002).

The MLTR estimates of inbreeding parameters were: F = single locus inbreeding coefficient of maternal parents (± SE); t_m = multilocus population outcrossing rate (± SE); t_s = single locus population outcrossing rate (± SE); r_p = the correlation of paternity (fraction of siblings that share the same father, i.e. the proportion of full-sibs among outcrossed sib-pairs) (± SE). The standard errors were based on 100 bootstraps, where the unit of resampling was the progeny array. If the single locus selfing rate is greater than the multilocus selfing rate (such that $t_m > t_s$), then this indicates biparental inbreeding (mating between relatives), which can be calculated from $t_m - t_s$.

4.3 RESULTS

4.3.1 Amplification of alleles

Each of the four loci could be scored unambiguously. At all loci, one or two alleles were detected per individual as expected for a diploid inheritance pattern (Table 4.2). All loci were polymorphic and the total number of alleles per locus varied between 2 (*Pa*GT13) and 14 (*Pa*GT9) (Table 4.2)

Table 4.2 Details of four microsatellite loci of *P. australis:* Allele size range, total number of alleles scored, observed and expected heterozygosity (*Ho*, *He*) for each locus and over all loci in the 18 families studied.

Locus	Allele size range (bp)	Maximum no. of alleles per sample	No of alleles scored within the seedlings	*Ho*	*He*
*Pa*GT4	271 - 287	2	12	0.611	0.798
*Pa*GT9	187 - 222	2	14	0.633	0.721
*Pa*GT13	209 - 211	2	2	0.008	0.166
*Pa*GT16	250 - 290	2	12	0.613	0.743
Over all loci			40	0.466	0.607

The genetic diversity of *P. australis* seedlings (progeny) in the Inner Tay population was high. The total number of alleles scored was 40. Observed heterozygosity (*Ho*) was generally lower (mean 0.466) than expected heterozygosity (*He*) (mean 0.607) both within and over all loci (Table 4.2).

Calculation of θ (= 0.038) using the pooled families, detailed in section 4.2.4.1, provided evidence of population substructure (differentiation).

4.3.2 Maternal inbreeding and outcrossing rate

The single locus inbreeding coefficient of maternal parents, F, was negative (- 0.181 ± 0.041), which indicates that there was a slight, but significant heterozygote excess (Table 4.3)

Table 4.3 Mating system parameters estimated using MLTR (Ritland and Jain, 1981; Ritland, 2002): F, single locus inbreeding coefficient of maternal parents; t_m, multilocus population outcrossing rate; t_s single locus population outcrossing rate; r_p, the correlation of paternity. Errors are ± 1 SE of 100 bootstraps.

F	t_m	t_s	t_m-t_s	r_p
- 0.181 ± 0.041	0.966 ± 0.060	0.908 ± 0.046	0.057 ± 0.040	0.199 ± 0.117

The multilocus estimate of the outcrossing rate of *P. australis* was high (t_m = 0.966 ± 0.060) and not significantly different from 1.0, providing no evidence for self-fertilisation (Table 4.3). The single locus estimate was slightly lower (t_s = 0.908 ± 0.046) and just significantly different from 1.0. Since $t_m > t_s$, this suggests that mating between relatives is likely to have occurred, although the difference between the two outcrossing estimates was not significant (Dole and Ritland, 1993; Ritland, 2002). Therefore, in the mixed mating model, *P. australis* was found to be outcrossing with no evidence for selfing. Approximately 20 % of the progeny shared the same father (r_p = 0.199 ± 0.177), although the correlation was not significantly different from zero.

4.4 DISCUSSION

4.4.1 Breeding system

In the Inner Tay reedbed the estimated multilocus outcrossing rate for *P. australis* was very high (t_m = 0.966), indicating that self-fertilisation is limited. This is in

agreement with the results of controlled pollinations conducted by Ishii and Kadono (2002) who found a high degree of self-incompatibility in *P. australis*, though some seed was set by self-fertilisation (2 - 8%). However, the estimated outcrossing rate in the present study most likely showed an upward bias due to differential mortality of selfed offspring prior to assay. This is suggested by the fact that, for some of the families, seeds either did not germinate or died soon after germination and were therefore excluded form analysis. Furthermore, in families sampled from areas containing a widespread single clone, mortality amongst progeny was often high, and those seedlings that did grow were often smaller and of poorer quality compared to those grown from families collected where many genets were present. Thus comparing the average number of progeny assayed from each transect, the lowest mean number of seedlings was obtained from transect 5 (3.25) which contained one clone (140 m long), followed by transect 1 (5.75) in which all four families belonged to the same clone (240 m long) (see Chapter 3, Fig. 3.3). The families with the highest mean number of offspring (8.25 seedlings) belonged to transect 4 in which each family was obtained from a genetically different clone. It is therefore probable that where widespread single clones were present, some selfing occurred.

The single locus outcrossing estimate was lower than the multilocus estimate suggesting the presence of biparental inbreeding (although the result was not significant). This could be caused by clone structure in which related clones are in closer proximity than would be expected by chance. The presence of such genetic substructuring was detected in the Inner Tay reedbed. Genetic differentiation (θ = 0.038) was found among samples grouped by geographic location in the population. Where large clones are present the chance increases that seeds within a family are derived by fertilisation with pollen coming from the same donor. This means that many offspring in a family are full sibs (i.e. share the same maternal and paternal genotypes) as demonstrated by the correlation of paternity estimate which suggests that $\approx$20% of siblings share the same father.

In this study the mating system of *P. australis* was determined in only one population and, since it has been found that there can be intraspecific variability in outcrossing rate (Sun and Ritland, 1998; Brennan *et al.*, 2005), it would be useful to examine

other populations of *P. australis* to determine if the outcrossing rate is affected by clonal structure. Despite the limitations of examining only one plant population, this is the first study to show, by using microsatellite markers, that *P. australis* is mainly an outcrossing clonal species.

4.4.2 Genetic diversity of the progeny

As well as investigating the mating system this study also demonstrated that the genetic diversity of the sexually reproduced progeny was high, with a total of 40 alleles scored over four loci. In contrast a total of only 16 alleles were detected from the 10 adult genotypes from Inner Tay (see Chapter 3, Table 3.5). Although this discrepancy could have arisen due to sample size difference (i.e. 125 progeny *vs* 10 adults), a total of only 30 alleles were found from 185 adult genotypes detected in 11 British populations of *P. australis* (see Chapter 3, Table 3.2). This was less than the 40 alleles detected in the progeny from this one population. In agreement with these findings, gene diversity, as measured by *He*, was higher in the progeny (0.607) compared to the Inner Tay adults (0.540) and 11 British populations (0.547) (Chapter 3, Table 3.2). However, unlike the number of alleles scored, the difference was not large, reflecting the relative insensitivity of *He* to the presence of rare alleles.

The finding of higher diversity in offspring than in adult *P. australis* contrasts with results reported in the literature in which *Calluna vulgaris* seedlings (obtained from the seed bank) have been found to have the same allozyme diversity as the adult population (Mahy *et al.*, 1999). On the other hand, Aspinwall and Christian (1992) found ten unique genotypes among seeds of a single infructescence of *Filipendula rubra* which was obtained from a homogenous adult cluster possessing only one genotype. Although an explanation for the unique genotypes was not given, it was noted that there was compatible pollen available from two disjunct populations located *c.* 1.5 km from the study site. In this study, it is probable that the extra alleles detected in the progeny of the Inner Tay population have arisen due to allopatric gene flow (i.e. from an external pollen source) or from numerous, possibly small, clones that were not identified when sampling the adult population.

The disparity of genetic diversity between the progeny and adult population is remarkable and reflects the fact that, although there is a profusion of potential genetic diversity within the Inner Tay population, it is locked up in a limited number of vegetatively spreading genotypes. Sexual reproduction in *P. australis* is frequent and the population has a high seedset (mean number of seeds per panicle over two years *c.* = 1700) (see Chapter 5, section 5.3.1), so there is no shortage of potential sexual recruits. However, due to competition (mainly for light) from the established clones, there is limited opportunity for sexual recruitment in an established reedbed (Haslam, 1972; Clevering and Lissner, 1999). The importance of competition in preventing sexual recruitment has been found for other clonal species with abundant seedset such as *Filipendula rubra* (Aspinwall and Christian, 1992) and *Ranulculus repens* (Lovett Doust, 1981). In addition, the fitness of the novel genotypes of the progeny may be less than the clones already present, since the latter have already survived inter-clonal competition (Ellstrand and Roose, 1987; Eriksson, 1997). Unless there is a disturbance event such as drawdown, burning or cutting of the vegetation, clonal diversity of the reedbed is unlikely to increase over time.

4.4.3 Implications for reedbed management and conservation

This result has implications for the creation of new reedbeds and the conservation of old ones. The only way to establish and maintain the genetic diversity in a reedbed is by using seed recruitment. Initial reedbed establishment must be from seeds and not vegetative propagules, whilst in old reedbeds genetic diversity can be encouraged through anthropogenic disturbance, such as drawdown or vegetation clearing which will promote sexual recruitment. However, it is unknown whether new genotypes will endure over time because selection and drift, as well as competition from locally adapted genotypes, may exclude them, particularly in a stable environment (Clevering and Lissner, 1999).

It is also apparent that only a small amount of gene flow *via* seedling establishment would be needed to increase the genetic and genotypic diversity of the population (Ellstrand and Roose, 1987). In the long-term an increase in diversity may be

essential as new clones may be better able to withstand future environmental changes, such as increased surface air temperature, changing hydrological conditions and new pests and diseases (Callaghan *et al.*, 1992).

Chapter 5 – Variability in seedset and germination of British populations of *Phragmites australis*

5.1 INTRODUCTION

In Britain, wetlands dominated by *P. australis* (reedbeds) are a rare and declining habitat (Gilbert *et al.*, 1996). Accordingly, several action plans have been implemented by the UK Government's statutory nature conservation advisors and non governmental organisations to reverse this decline through reedbed creation and restoration.

Several techniques may be employed for reedbed establishment. These include seed sowing, seedling planting, and rhizome transplants. Usually the first or second method is favoured because labour and transport costs make rhizome planting uneconomical for all but the smallest projects (Hawke and José, 1996; Self *et al.*, 1996). However, establishment from seed can be problematic as it may be difficult to obtain a local viable seed source.

Enormous variability in seed production amongst populations has been reported in all European studies and, in general, seed production has been found to be poor (Gustafsson and Simak, 1963; Björk, 1967; Curran, 1969; Haslam, 1972; McKee and Richards, 1996). Seedset amongst Swedish populations varied from zero to *c.* 4000 fertile fruits per inflorescence (Björk, 1967), whilst panicles collected from Irish populations were depauperate in flower fertility, such that in all population < 1 % of florets contained seeds (Curran, 1969). Moreover, McKee and Richards (1996) found seedset amongst British populations to be highly variable, ranging from 0 - 100 %.

Numerous causes have been suggested for variable seedset including biotic factors such as: self sterility through compatible pollen limitation (Gustafsson and Simak, 1963; Björk, 1967; Haslam, 1972), fungal infection caused by *Claviceps purpurea* infection (Gustaffson and Simak, 1963; Curran, 1967; Björk, 1967) and meiotic disturbance (Gustaffson and Simak, 1963; Björk, 1967). Abiotic factors purported to

affect seedset include climate (Gustaffson and Simak, 1963; van der Toorn, 1972; Haslam, 1972; McKee and Richards, 1996; Self, 1999), latitude (Nikolajevskij, 1971; McKee and Richards, 1996) and site size (McKee and Richards, 1996).

P. australis has been shown to be partially self-incompatible (Ishii and Kadono, 2002). In addition this study has found, using genetic analysis, that *P. australis* is highly outcrossing (see section 4.3.2). Therefore compatible pollen limitation may arise where large clones are found (e.g. Neuhaus *et al.*, 1993; and this study: Chapter 3, section 3.3.2.2) thus reducing the potential seedset of a population. Furthermore it has been found that large reedbeds produce more seed than small sites (McKee and Richards, 1996), probably because there is more opportunity for cross pollination to occur in larger populations, even if genetic structuring is present.

Seedset in *P. australis* may be reduced by the presence of *Claviceps purpurea* infection (Gustaffson and Simak, 1963; Björk, 1967; Curran, 1969). *C. purpurea* is an endophytic fungus (Ascomycete family Clavicipitaceae; tribe Balansieae) that infects and destroys the floral structures in a range of grass species throughout Europe, America and Australasia (Clay, 1990a). Its effect on fecundity can be measured directly as every floret possessing an ergot has lost the potential to develop a seed. The ergots are easy to differentiate from seeds as they form long cigar-shaped purplish-black structures which protrude from the spikelet. Although ergot infection has often been observed in *P. australis,* its distribution in British populations and effects on fecundity has not been evaluated.

Poor fertility in *P. australis* is also attributed to meiotic disturbance caused by high polyploid levels (Gustaffson and Simak, 1963). For example, the occurrence of empty shrunken seeds in *P. australis* in Sweden was attributed to the presence of a hexaploid population (Björk, 1967). Since each population sampled here was a tetraploid (see section 3.3.1) this factor would not affect fertility.

Climatic factors may affect seedset. For instance, high summer rainfall may reduce cross-pollination, whilst low temperatures during flowering and embryo development may decrease potential seedset (Gustaffson and Simak, 1963; McKee and Richards, 1996). In addition, McKee and Richards (1996) found a slight latitudinal trend in

Britain with southern sites being more productive than those from the north. This trend has also been reported in Russia (Nikolajevskij, 1971), but has not been found in the Netherlands (Björk, 1967) or Sweden (van der Toorn, 1972).

For successful reedbed establishment from seed, the germination rate must be maximised. However, seed germination in the field is variable and often poor (Haslam, 1972) due to factors that include: soil nutrient status (poor on organic soils), water levels (poor germination in standing water), water salinity (e.g. sea water and concentrations of sodium chloride > 5 % have been reported to be inhibitory) (Haslam, 1972) and temperature (slow at low temperatures). Björk (1967) found maximum laboratory germination (80 - 90 %) at 30 °C and poor germination (< 5 %) between 10 - 20 °C. The optimal conditions for germination in *P. australis* have been identified by Self (1999).

The overall aims of this research were to determine and account for the variability in seedset, *C. purpurea* infection and germination rate amongst British *P. australis* populations by addressing the following questions: (i) Are there differences in seedset, *C. purpurea* infection and germination rates amongst British populations of *P. australis*? (ii) Which of the biotic and abiotic factors described above influence seedset and *C. purpurea* infection? (iii) Is there a relationship between seedset and *C. purpurea* infection and seedset and germination rate?

To answer these questions, samples were collected in two consecutive years from 11 reedbeds located in Scotland and England. The results were used to make recommendations for sourcing seeds and harvesting within a population and for conservation of old reedbeds.

5.2 MATERIALS AND METHODS

5.2.1 Site details

Eleven reedbeds, five from Scotland and six from England were chosen for sampling (for location of reedbeds see Chapter 2, Fig. 2.1) taking account of conservation importance, latitude, site size, ease of access and coverage of both freshwater and brackish habitats (for site details see Chapter 2, Table 2.1).

5.2.2 Sampling

Panicles were collected at the same 20 sampling points at each site in November - December of 2002 and 2003. Sample points were located along line transects and at least 50 m apart to minimise the risk of resampling the same clone. In 2002 the locations were recorded using a hand held GPS (resolution ± *c.* 9 m) to facilitate relocation in 2003. At some sites, access was restricted because of a physical barrier. Where the reedbed was not contiguous samples were collected from each separate reedbed using a line transect perpendicular or parallel to the water's edge, depending on the width of the reed bed (for further details of within population panicle sampling, see Table 3.1). At each sample point, *c.* 20 panicles were cut from the reed stems and temporarily stored in plastic bags. After collection, the panicles were air dried and stored in brown paper bags in Hessian-like sacks in an unheated greenhouse until analysis.

5.2.3 Seedset and *Claviceps purpurea* infection

For each sampling point within a population, a cut panicle was randomly selected and its length measured (distance between base of the panicle and the tip) (Fig. 5.1). Using forceps, 20 spikelets were removed and the florets they contained were separated using dissection pointers. Seeds were removed in the same way. Each floret was scored for presence/absence of a seed or replacement of the seed by an ergot of *C. purpurea* (Fig. 5.2) with the aid of a binocular microscope (x 10). For each population a sample size of 20 panicles resulted in the examination of 400 spikelets, containing 2 - 6 florets (800 - 2400 florets).

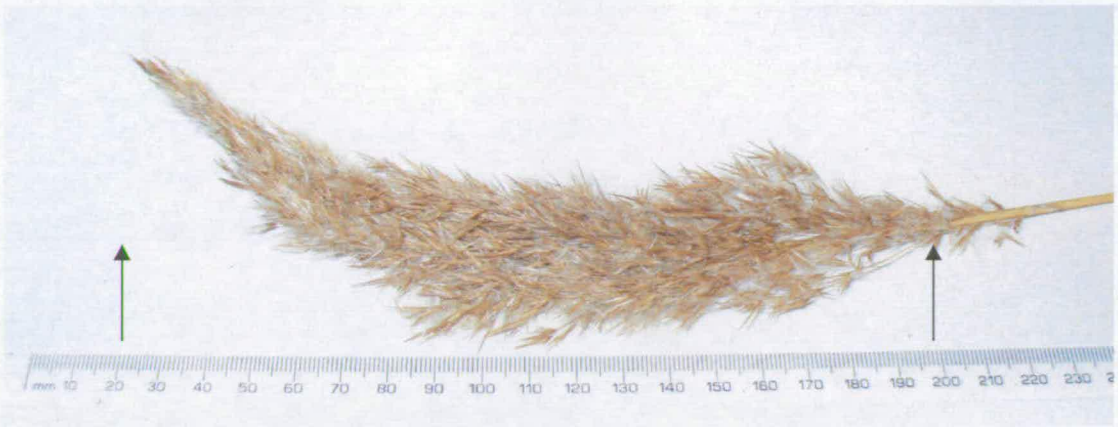


Figure 5.1 Cut panicle from Walberswick population; length of panicle is measured between two points indicated by arrows.

Figure 5.2 *a* spikelet containing 4 florets, *b* spikelet containing an ergot of *Claviceps purpurea, c* one seed removed from floret.

From the 2002 results, the relationship between mean seedset per 20 spikelets and 5 spikelets per population was found to be statistically significant (Pearson's correlation coefficient, $r = 0.99$, $P < 0.001$). Therefore in 2003, only 5 spikelets were examined per panicle (100 spikelets per population). The unit of measurement for each sampling point was seedset per spikelet and *C. purpurea* infection per spikelet (as number of ergots), so for each point the total number of seeds and *C. purpurea* infection recorded was divided by the number of spikelets examined (i.e. 20 in 2002 and 5 in 2003).

From this, the mean seedset per spikelet and mean *C. purpurea* infection per spikelet was determined for each population. In addition, the mean seedset per panicle per population was calculated by finding the number of spikelets per unit length of

panicle. The latter was estimated by randomly selecting one panicle per population and recording its length and the total number of branches present. Then a sub-sample of five branches was examined to assess the mean number of spikelets per branch. The number of spikelets per unit length of panicle was calculated as:

$$\frac{\textit{Mean no. of spikelets per branch} \times \textit{no. of branches per panicle}}{\textit{length of panicle}} \qquad \text{(Equation 5.1)}$$

Over all populations, the mean number of spikelets was 5.10 ± 0.38 spikelets cm^{-1}. This value was then used to calculate the seedset per panicle for each sampling point from:

$$\textit{Seedset per spikelet} \times 5.1 \times \textit{panicle length of each sample (cm)} \qquad \text{(Equation 5.2)}$$

By applying equation 5.2 to each sampling point, the mean seedset per panicle (which was calculated as the average of the 20 sampling points) was determined for each population.

5.2.4 Germination trials

For each population, material for the germination trials comprised 20 seeds taken from each of the 20 panicles previously assessed for seedset. Since the Loch Leven panicles contained no seed it was excluded from the experiment. In addition, panicles from Insh Marsh and Mersehead contained few seeds, so the sample size was five and 12 panicles respectively. All other populations had sample sizes between 17 and 20. The trials were conducted for seed collected in 2002 only.

The protocol for estimating the germination rate followed that of Self (1999). Twenty seeds from a single panicle were added to a 9-cm diameter Petri dish half filled with sterile sand, overlaid with filter paper, moistened with sterile water and sealed with para-film. The Petri dishes were placed under a halogen lamp for 16 hours then incubated in a diurnally fluctuating temperature regime of *c.*12 hours at 37 °C and *c.* 12 hours at 15 °C by transferring Petri dishes between incubators for six days, after which time observation of germination was stopped. Germination was defined as protrusion of the radicle from the seed coat. Each Petri dish was examined and the

number of seeds germinated from the total number present was counted. From these results, the mean percentage germination rate for each of the 20 sampled panicles in each population was determined.

5.2.5 Reproductive potential

Reproductive potential was defined as the product of seedset per spikelet and mean percentage germination per population. Once seedset and germinability were found empirically, the reproductive potential of each of the 20 samples per population was calculated for 2002. From this, the mean reproductive potential of each population was determined.

5.2.6 Weather data

To test for an association between seed production and antecedent weather conditions, data were obtained for each site either from an onsite weather station or from the nearest station (from the British Atmospheric Data Centre) for 2002 and 2003. The monthly mean maximum daily temperature and total monthly rainfall for August (when pollination occurs), September and October (when seedset and maturation occurs) were obtained. A list of sites and nearest weather stations is given in Table 5.1. Since Walberswick and North Warren are located close to Minsmere, weather data from Minsmere was used for these sites.

5.2.7 Statistical analysis

All statistical tests were carried out using MINITAB 14 for Windows. Each variable (i.e. seedset per spikelet and per panicle; *C. purpurea* infection per spikelet; percentage germination rate; reproductive potential per spikelet and panicle length) was tested for normality and homoscedasticity among populations using Kolmogorov-Smirnov and Bartlett's test, respectively (Sokal and Rohlf, 1995). As there was a significant departure from normality and homogeneity of variance for all variables except panicle length, statistical analysis was conducted on transformed data. Fourth-root transformations were performed on seedset per spikelet, seedset per panicle and *C. purpurea* infection per spikelet; a square-root transformation was

performed on productivity per spikelet; and an arcsine transformation was performed on percentage germination (Sokal and Rohlf, 1995).

Table 5.1 Weather stations recording temperature and rainfall data and approximate distance from each population.

Population	Nearest weather station	Approximate distance from population (km)
Insh Marsh	Aviemore	14
Inner Tay	Strathallan School	12
Loch Leven	Kinross	4
St Margaret's Marsh	Braefoot Bay	7
Mersehead	Drum Mains	6
Leighton Moss	Beetham Hall	4
Strumpshaw Fen	Lingwood, Strumpshaw Hill	2
Lakenheath	Santon Down	12
Walberswick	Minsmere	6
Minsmere	On site	
North Warren	Minsmere	6

To determine if there was a significant difference in seedset per spikelet, seedset per panicle amongst populations and between years, a Two-Way Analysis of Variance (ANOVA) was performed (incorporating Tukey's test for seedset per spikelet and *C. purpurea* infection per spikelet, for 2002 and 2003), using the General Linear Model (GLM). The model was used to test for the main effects of population and year and the interaction between these two factors. Pearson's correlation was used to test for association of mean seedset per spikelet, per panicle and mean *C. purpurea* infection per spikelet between years.

One-Way ANOVA, incorporating Tukey's test, using GLM was employed to test the significance of differences in germination rate amongst populations and also inter-population differences in reproductive potential per spikelet.

Subsequent analysis involving tests for relationships between seedset and other variables were performed without using seedset data from Loch Leven. This population was excluded because there is strong evidence to suggest that the absence of seed production in both years was caused by compatible pollen limitation alone (this study found the population to be monoclonal, see section 3.3.2.2) and not some other factor.

Pearson's correlation was used to test for association between mean seedset per spikelet and the following variables: mean *C. purpurea* infection per spikelet mean percentage germination, mean panicle length and clonal diversity. In addition the association between mean *C. purpurea* infection per spikelet and clonal diversity and percentage germination were also tested. (The values of clonal diversity per population determined in Chapter 3 were used in the analysis, see Table 3.5)

Finally, single and multiple regression analysis was performed on data collected in both years to determine if any of the other parameters associated with population (i.e. latitude, site size, and weather) could account for the significant amount of among population variation in seedset per spikelet and *C. purpurea* infection per spikelet.

5.3 RESULTS

5.3.1 Seedset

Seedset per spikelet between populations was highly variable in 2002 and 2003 (Fig. 5.3). The lowest seedset occurred at Loch Leven (0 seeds in both years), whilst the highest was from Walberswick in 2002 (1.27 ± 0.19 seeds per spikelet) and Inner Tay in 2003 (1.63 ± 0.21 seeds per spikelet). A Two-Way ANOVA showed a significant difference in mean seedset between populations ($P < 0.001$), but no difference between years (Table 5.2).

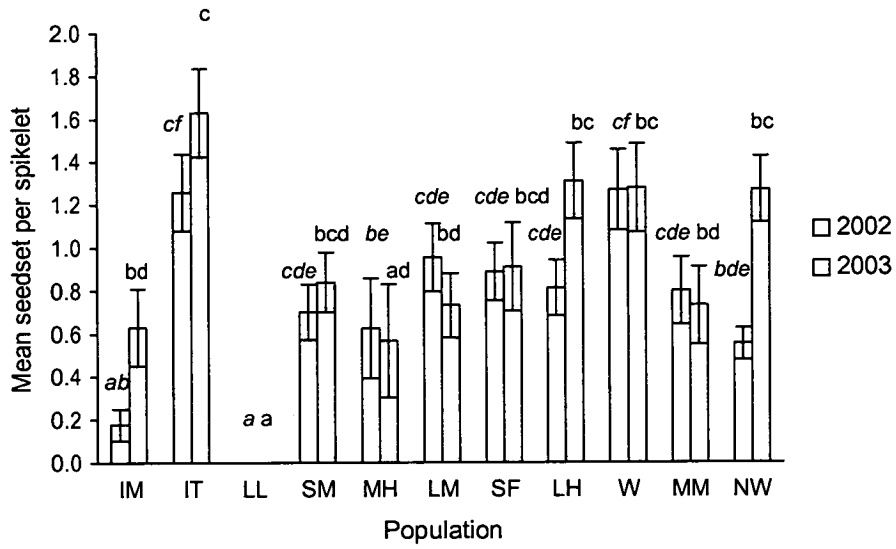


Figure 5.3 Mean seedset per spikelet ± SE for 11 populations (untransformed data). *n* = 20. Populations arranged from left to right in order of decreasing latitude. Populations with the same letter (italics for 2002, normal for 2003) are not significantly different for mean seedset per spikelet. Population abbreviations are given in Table 2.1.

Table 5.2 A Two-way ANOVA of seedset per spikelet, seedset per panicle and *C. purpurea* infection per spikelet amongst populations in 2002 and 2003.

Source of variance	d.f.	MS seedset per spikelet	MS seedset per panicle	MS *C. purpurea* infection per spikelet
Population	10	0.304***	90.678***	0.433***
Year	1	0.073^{ns}	1.697^{ns}	0.066^{ns}
Population x Year	10	0.035*	8.170**	0.014^{ns}
Error	408	0.018	3.233	0.009

ns not significant, *P< 0.05, **P < 0.01, ***P < 0.001

However there was a significant population × year interaction (P < 0.05). An interaction plot (Fig. 5.4) showed that populations behaved differently in different years. Six of the eleven populations had an increase in mean seedset per spikelet in

2003 compared to 2002 (Inner Tay, Lakenheath, North Warren, St. Margaret's Marsh, Insh Marsh and Strumpshaw Fen) whilst four decreased slightly (Minsmere, Mersehead, Walberswick and Leighton Moss) and one showed no change between years (Loch Leven). Seedset in 2002 and 2003 was positively correlated but the significance of the correlation was marginal ($r = 0.630$, $P = 0.051$). The total seedset per panicle was also highly variable amongst populations in both years (Fig. 5.5). After Loch Leven, the lowest mean seedset was found in 2002 at Insh Marsh (218 ± 91 seeds per panicle) and in 2003 at Mersehead (684 ± 328) whilst the highest was recorded at the Inner Tay in both years (1504 ± 239 in 2002, 1981 ± 263 in 2003).

There was a highly significant difference in mean seedset per panicle amongst populations ($P < 0.001$), but no difference between years (Table 5.2). However there was a significant population × year interaction ($P < 0.01$) with each population showing the same underlying trend in mean seedset per panicle between years as was found for seedset per spikelet. Once again, correlation of seedset between years was positive and marginally significant ($r = 0.625$, $P = 0.053$).

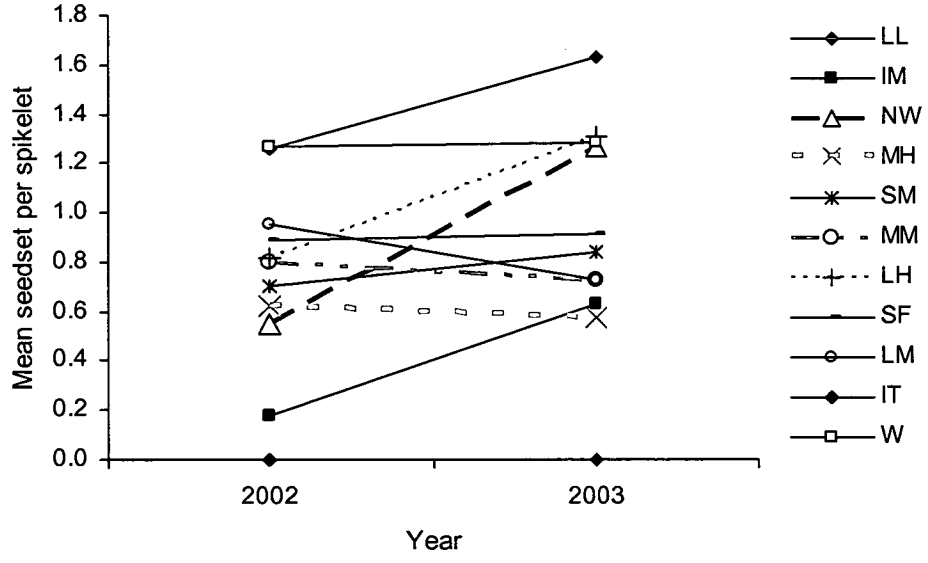


Figure 5.4 Interaction plot of mean seedset per spikelet per population in year 2002 and 2003 (untransformed data). Population abbreviations are given in Table 2.1.

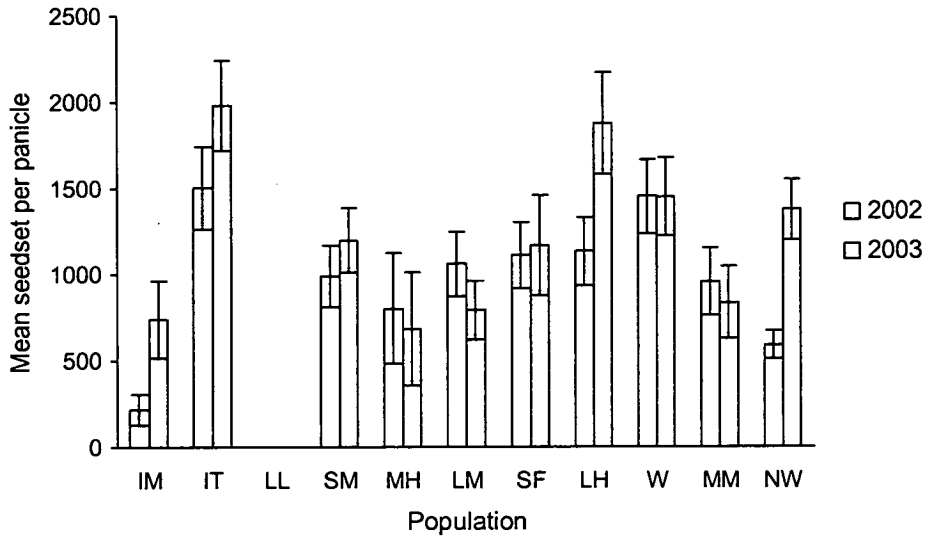


Figure 5.5 Mean seedset per panicle ± SE in 11 populations of *P. australis* (untransformed data) n = 20. Populations arranged from left to right in order of decreasing latitude. Population abbreviations are given in Table 2.1.

5.3.2 Panicle length

Among populations, there was a highly significant difference in panicle length ($F_{10, 204} = 12.10, P < 0.001$). The shortest panicles were found at North Warren (210 ± 8 mm), whilst the longest were found at Loch Leven (308 ± 7 mm) (Fig. 5.6).

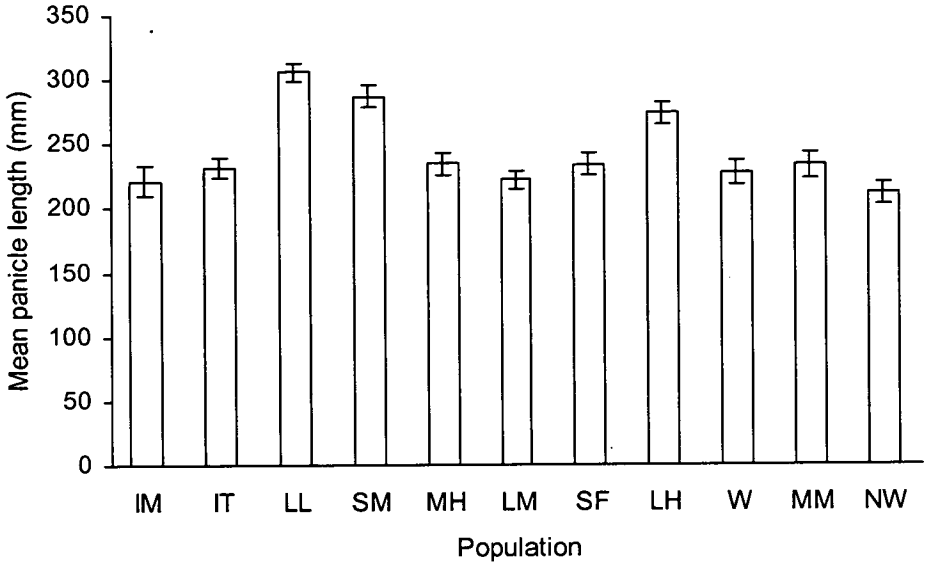


Figure 5.6 Mean panicle length ± SE of 11 populations of *P. australis* in 2002 *n* = 20. Populations arranged from left to right in order of decreasing latitude. Population abbreviations are given in Table 2.1.

5.3.3 *Claviceps purpurea* infection

Claviceps purpurea infection per spikelet amongst populations was highly variable in both 2002 and 2003 (Fig. 5.7). In 2002, the lowest mean infection was found at Lakenheath (0.025 ± 0.011 *C. purpurea* infection per spikelet) and in 2003 at Walberswick (0 infection), whilst the highest was recorded at Insh Marsh in both years (1.51 ± 0.20 in 2002, 1.74 ± 0.28 in 2003). There was a significant difference in *C. purpurea* infection per spikelet amongst populations ($P < 0.001$), and only a marginally significant difference between years ($P = 0.055$). In addition, there was no significant population x year interaction ($P = 0.128$) (Table 5.2). That populations showed the same trend in *C. purpurea* infection in both years was confirmed by a positive and highly significant correlation coefficient ($r = 0.943$, $P < 0.001$).

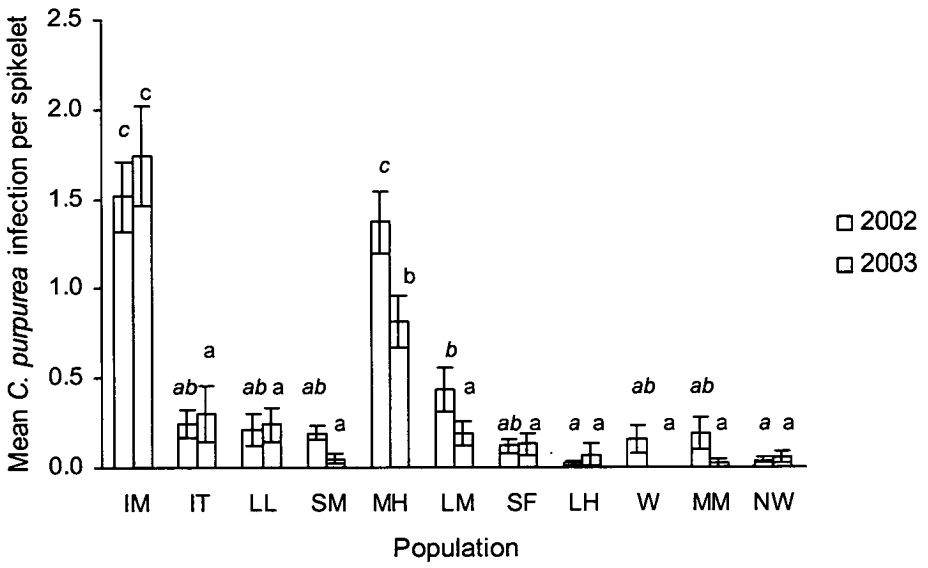


Figure 5.7 Mean *C. purpurea* infection per spikelet ± SE in 11 populations of *P. australis* (untransformed data) *n* = 20. Populations arranged from left to right in order of decreasing latitude. Populations with the same letter (italics for 2002, normal for 2003) are not significantly different for mean *C. purpurea* infection per spikelet. Population abbreviations are given in Table 2.1.

5.3.4 Germination trials

Germination rate amongst populations varied, with all populations having a germination rate > 50 % apart from Insh Marsh (12.4 ± 8.7 %) (Fig. 5.8). The highest germination rate was recorded at the Inner Tay (84.7 ± 2.5 %). A One-Way ANOVA confirmed a significant difference in mean percentage germination amongst populations ($P < 0.001$) (Table 5.3).

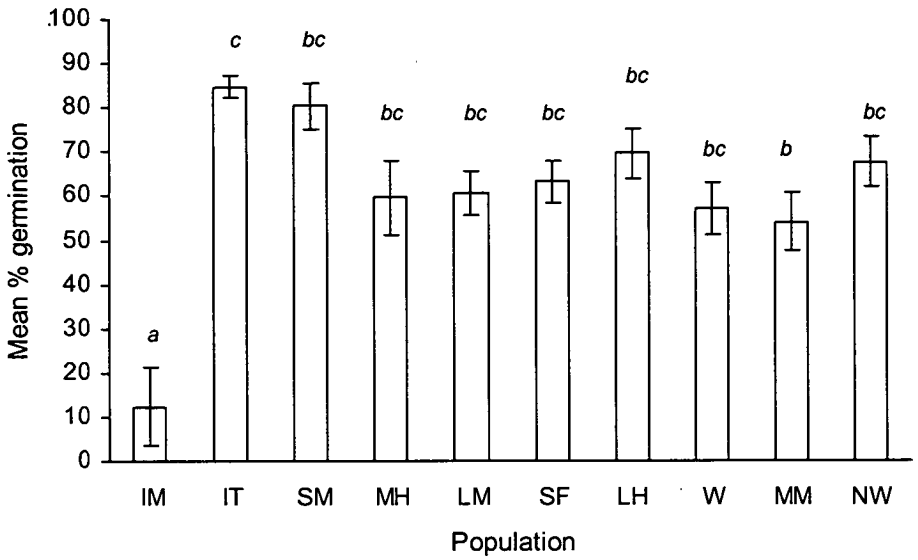


Figure 5.8 Mean percentage germination rate ± SE of 10 populations. (IM *n* = 6; MM *n* = 17; W *n* = 19; LM *n* = 20; SF *n* = 19; MH *n* = 12; NW *n* = 17; LH *n* = 19; IT *n* = 20; SM *n* = 18). Populations arranged from left to right in order of decreasing latitude. Populations with the same letter are not significantly different for mean % germination rate. Population abbreviations are given in Table 2.1.

Table 5.3 A One-Way ANOVA comparing mean percentage germination (arcsine transformed) amongst populations.

Source of variation	d.f.	MS
Population	9	5.723***
Error	165	0.099

***$P < 0.001$

There was a significant positive correlation between mean seedset per spikelet and percentage germination within populations ($r = 0.701$, $P < 0.05$), implying that as seedset increases so does the germination rate. In addition, there was a significant negative relationship between percentage germination and mean *C. purpurea* infection per spikelet ($r = -0.653$, $P < 0.05$), implying that germination rate is decreased by the presence of *C. purpurea* infection.

5.3.5 Reproductive potential per spikelet

The population with lowest reproductive potential per spikelet (apart from Loch Leven which did not produce any seeds and was not included in the analysis) was Insh Marsh (0.07 ± 0.02 reproductive potential per spikelet), whilst the most productive was Inner Tay (1.07 ± 0.15) (Fig. 5.9). A One-Way ANOVA showed a highly significant difference in reproductive output between populations ($P < 0.001$) (Table 5.4). Since germination rates were fairly similar and high for most populations, the main driver of reproductive potential appears to be seedset.

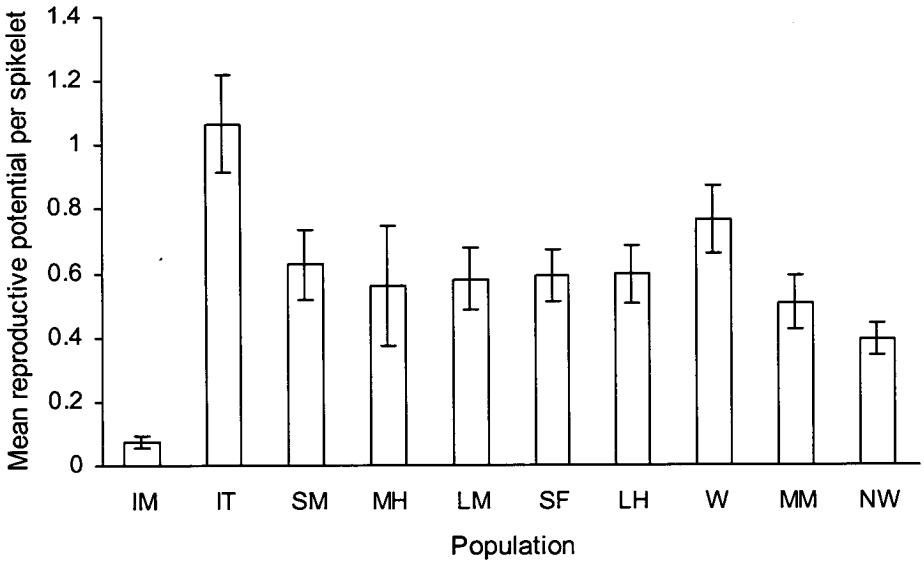


Figure 5.9 Mean reproductive potential per spikelet ± SE of 10 populations (Loch Leven excluded as no seedset). (IM *n* = 6; MM *n* = 17; W *n* = 17; LM *n* = 20; SF *n* = 19; MH *n* = 12; NW *n* = 19; LH *n* = 19; IT *n* = 20; SM *n* = 18). Populations arranged from left to right in order of decreasing latitude. Population abbreviations are given in Table 2.1.

Table 5.4 A One-Way ANOVA comparing mean reproductive potential (square-root transformed) amongst populations.

Source of variation	d.f.	MS
Population	9	0.363***
Error	157	0.076

*** $P < 0.001$

5.3.6 Factors affecting seedset

5.3.6.1 Biotic factors

There was a negative association between mean seedset per spikelet and mean *C. purpurea* infection per spikelet in both years, although only 2002 showed a significant result (r = - 0.697, $P < 0.05$, 2002; r = - 0.552, P = 0.098, 2003). In addition, correlations between seedset per spikelet and *C. purpurea* infection among panicles calculated separately for each population were significant in four populations (Mersehead, 2002; Minsmere, 2002; Walberswick, 2002 and Insh Marsh 2003) (Table 5.5). This test was not applicable to Walberswick in 2003 as none of the spikelets examined were infected with *C. purpurea.* As 19 tests were performed, it would be expected that 1/19 tests would show a significant result due to chance alone (Type I Error). However, the number of significant results was higher (4/19) and the correlation coefficient was negative in 15/19 tests, implying a trend for seedset to decrease if *C. purpurea* infection is present in the spikelet.

No significant correlation was found between mean seedset per spikelet and mean panicle length within (r = 0.082, P = 0.821) and among populations (r = - 0.387, P = 0.252). Therefore seedset cannot be assessed simply from panicle length. No significant correlation was found between mean seedset per spikelet and clonal diversity (as measured by proportion of distinguishable genotypes) per population in 2002 and 2003 (r = 0.283, P = 0.428 in 2002; r = 0.260, P = 0.468 in 2003).

Table 5.5 Within population Pearson's correlation coefficient (*r*) of mean seed set per spikelet versus mean *C. purpurea* infection per spikelet in 2002 and 2003 for 10 populations of *P. australis*. (Loch Leven excluded as no seedset). At Walberswick in 2003 none of the spikelets examined were infected with *C. purpurea*. Population abbreviations are given in Table 2.1.

Population	2002	2003
SF	- 0.039^{ns}	0.144^{ns}
MH	- 0.543*	0.007^{ns}
NW	- 0.205^{ns}	- 0.069^{ns}
LM	0.031^{ns}	- 0.038^{ns}
LH	- 0.085^{ns}	- 0.241^{ns}
MM	- 0.460*	- 0.236^{ns}
SM	- 0.261^{ns}	- 0.166^{ns}
W	- 0.641**	NA
IT	0.139^{ns}	- 0.084^{ns}
IM	- 0.124^{ns}	- 0.489*

ns not significant, *$P < 0.05$, ** $P < 0.01$

5.3.6.2 Abiotic factors

Table 5.6 shows the results of regression analysis of mean seedset per spikelet and latitude, site size and various weather parameters in 2002 and 2003. There was a positive association between mean seedset per spikelet and mean maximum October temperature although only 2003 shows a significant result ($R^2 = 0.177$, $P = 0.143$ in 2002; $R^2 = 0.459$, $P < 0.05$ in 2003). There was also a significant positive association between mean seedset per spikelet and site size in 2002 and 2003 ($R^2 = 0.498$, $P < 0.05$ in 2002; $R^2 = 0.520$, $P < 0.05$ in 2003).

Table 5.6 Regression analysis of mean seed set per spikelet (fourth root transformed) in 2002 and 2003 *vs* latitude, site size, and mean maximum temperature and total monthly rainfall in August, September and October.

Relationship	**2002**		**2003**	
Mean seedset per spikelet vs:	*F value*	R^2 †	*F value*	R^2 †
Latitude	1.25	0.027^{ns}	1.11	0.012^{ns}
Site size	9.94	0.498*	10.74	0.520*
Mean maximum temperature August	1.59	0.069^{ns}	0.71	0.000^{ns}
Mean maximum temperature September	1.46	0.055^{ns}	1.27	0.033^{ns}
Mean maximum temperature October	2.72	0.177^{ns}	7.79	0.459*
Total rainfall August	0.03	0.000^{ns}	0.52	0.000^{ns}
Total rainfall September	0.65	0.000^{ns}	0.04	0.000^{ns}
Total rainfall October	0.48	0.000^{ns}	0.51	0.000^{ns}

† adjusted R^2
ns not significant, * $P < 0.05$

There was no significant association between mean seedset per spikelet and other weather parameters or latitude. Using multiple linear regression, the model that accounted most of the variability in mean seedset per spikelet in 2002 and 2003 included the factors mean maximum October temperature and site size in 2002 and 2003 ($R^2 = 0.799$, $P < 0.01$ in 2002; $R^2 = 0.794$, $P < 0.01$ in 2003) (Table 5.7).

Table 5.7 Multiple regression analysis of mean seedset per spikelet (fourth root transformed) in 2002 and 2003 *vs* mean maximum October temperature and site size.

	2002		**2003**	
Model variables	*F value*	R^2 †	*F value*	R^2 †
Mean maximum temperature October + site size	16.86	0.799**	16.45	0.794**

† adjusted R^2
** $P < 0.01$

5.3.7 Factors affecting *C. purpurea* infection

There was a significant negative association between mean *C. purpurea* infection per spikelet and clonal diversity per population in 2002 and 2003 (r = - 0.730, $P < 0.05$ in 2002; r = - 0.727, $P < 0.05$ in 2003).

Table 5.8 shows the results of regression analysis of mean *C. purpurea* infection per spikelet in 2002 and 2003 and latitude and various weather parameters. There was a significant negative association between mean *C. purpurea* infection per spikelet and latitude (R^2 = 0.338, $P < 0.05$ in 2002; R^2 = 0. 385, $P < 0.05$ in 2003) as well as a significant negative association between mean *C. purpurea* infection per spikelet and mean maximum temperatures in August, September and October of 2002 (R^2 = 0.574, $P < 0.05$ in August; R^2 = 0.747, $P < 0.01$ in September; R^2 = 0.785, $P < 0.01$ in October) and October 2003 (R^2 = 0.950, $P < 0.001$). Using multiple linear regression, the model that accounted for most of the variability in mean *C. purpurea* infection per spikelet in 2002 and 2003 included the factors mean maximum temperature in August and October (R^2 = 0.814, $P < 0.01$ in 2002; R^2 = 0.971, $P < 0.001$ in 2003) (Table 5.9).

Table 5.8 Regression analysis of mean *C. purpurea* infection per spikelet (fourth root transformed) in 2002 and 2003 *vs* latitude, mean maximum temperature and total monthly rainfall in August, September and October.

Relationship	**2002**		**2003**	
Mean C. purpurea infection per spikelet vs	*F value*	R^2 †	*F value*	R^2 †
Latitude	5.59	0.338*	6.63	0.385*
Mean maximum temperature August	11.79	0.574*	2.17	0.128^{ns}
Mean maximum temperature September	24.63	0.747**	0.68	0.000^{ns}
Mean maximum temperature October	30.13	0.785**	153.21	0.95***
Total rainfall August	1.69	0.080^{ns}	0.16	0.000^{ns}
Total rainfall September	0.07	0.000^{ns}	0.01	0.000^{ns}
Total rainfall October	0.05	0.000^{ns}	0.23	0.000^{ns}

† adjusted R^2

ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 5.9 Multiple regression analysis of mean *C. Purpurea* infection per spikelet (fourth root transformed) in 2002 and 2003 *vs* mean maximum temperature in August and October

	2002		2003	
Model variables	*F value*	R^2 †	*F value*	R^2 †
Mean maximum temperature August + October	18.47	0.814**	135.48	0.971***

† adjusted R^2
** $P < 0.01$, *** $P < 0.001$

5.4 DISCUSSION

5.4.1 Seedset

In this study highly significant differences in seedset were found among 11 British *P. australis* populations. Seedset ranged from 0 - 1.63 seeds per spikelet and 0 - 1981 seeds per panicle among populations. The huge disparity in seedset across populations has been reported in other European research (Björk, 1967; van der Toorn, 1972; McKee and Richards, 1996). At one extreme, the Loch Leven population was entirely devoid of seed in both years of this study. Lack of seedset at this site was also found by McKee and Richards (1996). The most probable explanation is that there is compatible pollen limitation caused by the presence of a monoclonal population *c.* 2 km long, fringing the shoreline of the lake (see Chapter 3, section 3.3.2.2). On the other hand, the ploidy level of Loch Leven was not determined. Therefore an alternative hypothesis for the lack of seedset may be meiotic disturbance caused by another cytotype (e.g. 3*x*, 5*x*, 6*x*) (see Chapter 1, section 1.5.1) being present in this population instead of the tetraploid (4*x*) cytotype which was found in the other ten British populations karyotyped in this study (see Chapter 3, section 3.3.1). Although if this was the explanation, it would be expected that the disomic inheritance pattern of the four loci examined using microsatellite analysis would not occur in this population (see Chapter 3, section 3.3.2.1).

The total lack of annual seed production by Loch Leven has consequences for biodiversity. For instance, the bearded tit is a sedentary bird species of reedbeds and is dependent, for a great part of the year, on an abundant supply of reed seeds as part of its diet (Jonsson, 1999). There is no resident population found at Loch Leven. The possibility that this species is at the edge of northern limit of its range can be discounted as the Inner Tay reed bed located *c.* 30 km further north, hosts a breeding population. It is interesting to note that a survey of the other nine *P. australis* populations in this study revealed that the bearded tit was resident at every reedbed except Mersehead and Insh Marsh, which both recorded low seedset in this study. However Insh Marsh is possibly beyond the bearded tit's northern range and, as the Mersehead reedbed is newly established (created in 1996), a colonisation event may not yet have occurred.

The Inner Tay population produced the greatest seedset per panicle in both years (mean seedset in 2002, 1504; mean seedset in 2003, 1981), a result found previously by McKee and Richards (1996), who recorded a high seedset over a two year period despite its northern location. However, the maximum seedset per panicle for this population was considerably lower than that recorded in the Netherlands by van der Toorn (1972) (5800 seeds per panicle) and in southern Sweden by Björk (1967) (4325 seeds per panicle). Nikolajevskij (1971) and McKee and Richards (1996) found that populations from lower latitudes produced more seeds. No association with latitude was found in this study, which agrees with the findings of Björk (1967) and van der Toorn (1972). On the other hand, the most northerly populations in Britain, such as Insh Marsh, may flower so late in the year that there is insufficient time for seeds to mature before the onset of senescence occurs (Haslam, 1972).

Since this study has shown *P. australis* to be an outbreeding species (see Chapter 4, section 4.3.2) and it is thought to be partially self-incompatible (Ishii and Kadono, 2002) it was surprising that no relationship was found between seedset per spikelet and clonal diversity in populations possessing more than one clone. In large populations compatible pollen limitation may not be a factor, even if clone size is substantial, provided the population is not monoclonal, which may explain why the Inner Tay population, the largest reedbed in Britain (410 ha) maintained a high seed

production, despite the presence of at least three large clones, the largest found being *c*.5.1 ha in extent (see section 3.3.2.2). This may account for the significant relationship which was found between site size and seedset per spikelet (R^2 = 0.498 in 2002; R^2 = 0.520 in 2003), because site size may be a surrogate measure for clonal diversity. Consequently, although large clones were found in the Inner Tay population, smaller clones, not detected in sampling, ensured that cross-pollination occurred.

Regression analysis also showed that seedset was affected by mean maximum October temperature in 2003 (R^2 = 0.459) although the relationship was weak in 2002 (R^2 = 0.170). Increased seed production associated with unusually high summer temperatures was found by Gustaffson and Simak (1963), van der Toorn (1967), and Haslam (1972), although the relationship was not formally tested. McKee and Richards (1996) found that seed production among populations was positively associated with combined high temperatures in August, September and October. During October, seed maturation occurs (Haslam, 1972). In this study, there was a 5.5 °C difference in mean maximum October temperature in 2003 between the northern population of Insh Marsh (8 °C) with that of the southern population of Walberswick (13.5 °C). At the latter site, seed production was twice that of Insh Marsh in 2003. Differences in weather conditions (between years) may explain the significant population x year interaction effect on seedset per spikelet and seedset per panicle among populations. This implies that populations behaved differently in different years such that six of the eleven populations showed an increase in seedset in 2003, whilst the others either decreased slightly or stayed the same. However, the mean maximum October temperature in 2003 was only higher in one of the six populations (Inner Tay).

From the results of the regression analysis of abiotic factors, it appears that site size was more important than maximum October temperature in determining seedset. However, the model that accounted for most variability in seedset amongst populations incorporated both of these parameters (R^2 = 0.799 in 2002 and R^2 = 0.794 in 2003).

5.4.2 *C. purpurea* infection

The distribution of *C. purpurea* infection was variable and significantly different amongst the 11 populations. The most northerly population of Insh Marsh had the highest infection rate in both years, whilst the southern populations of Lakenheath and Walberswick had the lowest infection rates in 2002 and 2003 respectively. Regression analysis showed, that unlike seedset, there was a negative latitudinal trend in infection (R^2 = 0.33 in 2002; R^2 = 0.38 in 2003). It is probable that the later flowering of the northern populations, provided more favourable conditions for ergot infection, as this has been observed for other species such as late flowering varieties of ryegrass (Jenkinson, 1958). Coupled with this, climate has been found to be an important factor in the prevalence of *Claviceps* spp infection. For instance the sclerotia (ergot) may require a period of low temperature before germinating in May/June and spore release is dependent on water availability and soil temperature (Gray *et al.*, 1990). In addition, high relative humidity and relatively low temperatures increase infection rate (Workneh and Rush, 2002)

Regression analysis revealed a highly significant negative association between mean maximum October temperature and the incidence of *C. purpurea* infection among populations (R^2 = 0.79 in 2002; R^2 = 0.95 in 2003) and mean maximum temperature in August in 2002 but not 2003 (R^2 = 0.574 in 2002; R^2 = 0.128 in 2003). Workneh and Rush (2002) similarly found a significant relationship between the prevalence of *Claviceps africana* infection in sorghum and climate factors in Texas, USA. Infection rates increased during cooler, wetter summers which created humid conditions that were favourable for ergot development. In this study, total rainfall was not found to be an important factor associated with incidence of *C. purpurea* infection.

Mean maximum October temperature was more important than August temperature in determining the incidence of *C. purpurea* infection amongst populations, but the best model incorporated both of these parameters (R^2 = 0.814 in 2002 and R^2 = 0.971 in 2003). For both months, a lower temperature was associated with an increased incidence of infection. It is possible that low temperatures in August were associated

with decreased pollen production or limited pollen tube formation (outgrowth of the pollen grain that transports the male gametes to the ovule) (Martin and Hine, 2000). This would cause a decrease in the number of fertilized ovaries, which are immune to infection (Workneh and Rush, 2002). Therefore low pollination of flowers may result in an increased infection rate. In October, a secondary infection by conidia (asexual spores), which is manifest as a sticky honey dew, occurs (Gray *et al.*, 1990). It is hypothesised that conidia infection may be temperature or humidity dependent but these relationships were not tested in this study.

The distribution of *C. purpurea* is also affected by genetic uniformity of the host population (Gray *et al.*, 1990) because the former is a systemic pathogen which spreads through the whole clone. It is an axiom of plant epidemiology that genetic uniformity makes host populations more vulnerable to disease (Parker, 1988). In this study, there was a significant negative relationship between clonal diversity and *C. purpurea* infection (r = - 0.730 in 2003; r = - 0.727 in 2004), implying that the least diverse populations had a higher incidence of infection.

It is unclear whether the infection is transmitted through seeds (although it appears unlikely, since a fertilized ovary is immune to infection). If seeds carry infection and successfully germinate, then sexual recruitment will increase the genetic diversity, but not decrease the incidence of *C. purpurea* infection in a population. In addition, it has been found that ergot sclerotia buried in the soil, may overwinter there and form ascocarps (fruiting body of Ascomycete fungi) in the early summer, before the onset of flowering, which ripen and release spores that land and infect unfertilised open florets (Dabkevičius and Mikaliūnaitė, 2005).

It has been suggested that pathogens such as *C. purpurea* promote a genetically uniform host population by suppressing sexual reproduction (i.e. seedset) in favour of asexual reproduction (i.e. vegetative spread) (Clay, 1990a). Furthermore, research has shown that increasing levels of endophyte infection may occur with increasing age of the host population (Bradshaw, 1958), although infection must confer some positive effect to the host, otherwise the most heavily infected populations would not persist.

Since *P. australis* spreads within an established reedbed mainly through rhizomatous growth (Haslam, 1972), loss of seed production may not reduce fitness in the short term, provided the environmental conditions to which the genotype is adapted prevail, since sexual reproduction and seed dispersal are the only way to escape adverse environmental conditions (Eriksson, 1997). Lack of seed production may contribute to vegetative vigour of the plant as well as affording some defence from herbivory, through alkaloid production by the pathogen (Clay, 1990b). Since the Poaceae family in general is relatively devoid of anti-herbivory protection, the ergot alkaloids produced through *C. purpurea* infection fill an empty defensive niche that would otherwise exist (Clay, 1990b). However, for conservation purposes, it is not desirable to have a reedbed devoid of seeds and insect herbivores, as this could decrease faunal biodiversity. For instance, passerine species such as the reed warbler (*Acrocephalus scirpaceus*) and reed bunting (*Emberiza schoeniclus*), whose main breeding ground is reedbed, are dependent on a range of insect species as part of their diet (Poulin *et al.*, 2002).

C. purpurea infection within a population may also be affected by reedbed management. At some sites, a portion of the reedbed is cut each year and the reed stems collected are used for thatching (e.g. Walberswick and Inner Tay, although at the latter site commercial harvesting ceased in 2005) or burnt (e.g. Strumpshaw Fen, Minsmere, Leighton Moss and North Warren). Management of the reedbed in this way is essential to prevent successional change. The ensuing reedbed disturbance may allow seed recruitment during these 'windows of opportunity' (pulse-recruitment) (Eriksson, 1997) and the new genotypes that arise may be resistant to fungal infection (provided the seeds do not transmit infection). Conversely at sites where no cutting or burning occurs (e.g. Mersehead and Insh Marsh) the *C. purpurea* infection may spread across the reedbed unless some clones are resistant to infection.

5.4.3 Seedset *vs C. purpurea* infection

A negative correlation was found between seedset per spikelet and *C. purpurea* infection amongst populations although this relationship was only significant in 2002 (r = - 0.697). Within population correlation for both years found that this negative

relationship was significant at four sites. Surprisingly, Insh Marsh and Mersehead, although heavily infected and with low seed production, did not show a significant relationship in both years and when the result was significant, the correlation was found to be weak. An explanation for this disparity may be that where fecundity is inherently low in a population, direct measures of the effect of ergot on seedset are difficult (Clay, 1990a). This is supported by results from Walberswick and Minsmere. Both sites produced a relatively high seedset in both years and *C. purpurea* infection was greatest for each population in 2002. In this year, for each population, a significant negative correlation was found between seedset and ergot infection, suggesting that when other environmental factors are favourable to seed production, but *C. purpurea* is present, it may lower the potential seedset.

5.4.4 Germination

Germination rates were significantly different among populations with all populations except Insh Marsh having a germination rate greater than 50 %. The highest germination rate was recorded at Inner Tay, with over 80 % of seeds germinating. These rates are similar to those found by van der Toorn (1972) in the Netherlands and Nikolajevskij (1971) in Russia and are considerably higher than recorded by McKee and Richards (1996) in Britain. The low germination rate found in Insh Marsh (< 15 %) intimates that seed maturation was not complete. Insh Marsh was the most northerly population examined in this study and therefore climatic factors, such as occasional frosts during the period of seed maturation, may have resulted in a quantity of unviable seed being produced (e.g. over three consecutive days, in October 2002, the nocturnal temperature recorded was < 0 °C).

In addition a significant negative relationship was found between % germination rate and *C. purpurea* infection per spikelet, and Insh Marsh recorded the highest incidence of infection out of the eleven populations. Therefore systemic infection by *C. purpurea*, could have contributed to a reduced germination rate. Although the mechanism by which germination is inhibited is not known, production of less sexual recruits would ensure that the host population (i.e. the *P. australis* clones) maintains the genotypes that are susceptible to infection. Although, if this was the only

explanation, it would be expected that Mersehead would have a lower germination rate because this population also had a very high incidence of *C. purpurea* infection compared to the other populations (Fig. 5.7).

A further contributory factor could be that although, this study has found *P. australis* to be a highly out-crossing species (see Chapter 4, section 4.3.2), a certain amount of selfing may occur if no other pollen is available (Ishii and Kadono, 2002). At Insh Marsh a large putative clone was found (see Chapter 3, section 3.3.2.2) and, since wind-borne pollen dispersal follows a leptokurtic distribution (Proctor *et al.*, 1996), it is unlikely that a large clone, would receive pollen from another genet, particularly in a small reedbed. Consequently, in the germination trials for Insh Marsh inbreeding depression (i.e. reduced fitness of the selfed progeny relative to the outcrossed progeny) (Charlesworth and Charlesworth, 1987) could prevent germination of the selfed seeds, even under benign laboratory conditions. However the probability that the ramets of this clone occurred by independent sexual events could not be ruled out. Further evidence for inbreeding depression associated with seeds from large clones was suggested by the performance of families grown from seeds collected from the Inner Tay reedbed, as part of the breeding systems study (Chapter 4). It was observed that seedlings grown from samples collected where large clones were found (see Chapter 3, section 3.3.2.2) grew less vigorously than those where clone size was smaller.

Haslam (1972) reported that whilst *P. australis* germinates easily under laboratory conditions, field germination is more difficult. Therefore the germination rates found in this trial for all populations would be much lower in the field. In fact seedlings are only common in open habitats, where they are free from competition from other macrophytes (Haslam, 1972).

5.4.5 Reproductive potential

The Inner Tay population had the greatest reproductive potential, whereas the Insh Marsh population had the least. (Loch Leven was excluded from the analysis since no seeds were produced). These results are not surprising since the reproductive potential of a population takes account of both seedset and germination. In addition,

a positive correlation was found between seedset and germination rate, so that a population with a high seedset per spikelet is likely to have a high germination rate. For reedbed establishment, a high reproductive potential of the source population is of utmost importance when the scale of planting is large. For instance, a 165 ha reedbed has been created at Lakenheath in Cambridgeshire from agricultural land purchased by the RSPB in 1996. Approximately 30,000 seedlings have been successfully germinated each year in polytunnels (which produces the necessary diurnal temperature cycle necessary for germination) from distantly sourced seed and then transplanted in a 'plug' of soil. In total *c.* 250,000 seedlings have been planted since 1996 (Norman Sills, pers. comm. 2003).

5.5 SUMMARY

Large and significant differences in seedset, germination rate and incidence of *C. purpurea* infection were found amongst British populations of *P. australis*. Numerous factors (e.g. weather, *C. purpurea*, and genotypic diversity) have been found to be associated with each of these variables and will be discussed in more detail, together with the practical implications of the findings of this research, in Chapter 8.

Chapter 6 – Quantitative genetic differences in British populations of *Phragmites australis*

6.1 INTRODUCTION

P. australis is a highly polymorphic species and numerous studies have shown that variability within and between populations arises through genetic or environmental factors (Haslam, 1969; Björk, 1967; Haslam, 1972; van der Toorn, 1972; Véber, 1981; Clevering *et al.*, 2001; Karunaratne *et al.*, 2003; Lambertini *et al.*, 2003). Variability between populations has been attributed to: (i) ploidy levels (Hanganu *et al.*, 1999; Paucã-Comãnescu *et al.*, 1999); (ii) adaptations to temperature differences along a latitudinal gradient (Haslam, 1972; Clevering *et al.*, 2001; Karunaratne *et al.*, 2003); (iii) adaptation to edaphic and hydrological conditions (Daniels, 1991). Some authors separate *P. australis* into ecotypes such as riverine or peat (van der Toorn, 1972), littoral or limosal (Dykyjová and Hradecká, 1973), halophytic or glycophytic ecotypes (Waisel and Rechav, 1971) or numerous biotopes (a biotope is " a place of life; the totality of the environmental conditions under which a biocoenose exists") (Björk, 1967).

P. australis in Britain has been described as having a narrower range of variation than found elsewhere (Haslam, 1972). This is an important assumption, since the creation of new reedbeds often requires the planting of non local seed provenances. Until now, the decision regarding seed source has often been biased towards populations with high seed viability rather than considering the growth form of a population. Furthermore, intraspecific variability in *P. australis* has not been considered an important factor in the design or performance of CWS.

Therefore the aim of this study was to achieve a better understanding of the quantitative genetic variability of *P. australis* in Britain and its implications for reedbed establishment for conservation purposes and for its use in CWSs.

Differences due to ploidy level can be discounted because all populations sampled for this experiment are tetraploids (see Chapter 3, results section 3.3.1).

The following questions were addressed: (i) Are there differences between populations of *P. australis* in Britain and should this be considered when sourcing reeds for conservation and use in CWSs? (ii) Which characters show genetic variation? (iii) What is the distribution of genetic variation between and within different populations? (iv) What are the geographic patterns of variation for the characters? (v) Can populations be classified into distinct ecotypes, or is character variation continuous? (vi) Are the patterns of variation constant under different environmental conditions (e.g. in respect to edaphic conditions)?

To answer these questions, three common garden experiments (CGEs) were conducted in which seeds were collected from ten British *P. australis* populations and grown up in nutrient-stressed conditions, full-nutrient conditions and in synthetic sewage to simulate growth conditions in constructed wetlands. A variety of both above ground and below ground morphological traits were measured for the plants, to provide a complete picture of the morphological variation and response to different environmental conditions.

6.2 MATERIALS AND METHODS

6.2.1 Plant material for experiments

P. australis populations from ten reedbeds (four from Scotland and six from England) were chosen for the common garden experiments (Loch Leven was excluded from this study, as no viable seed was collected) (for location of populations, see Chapter 2, Figure 2.1). Panicles containing mature seeds were collected from 20 sampling points at each site from November - December 2002. The same sampling strategy detailed in Chapter 3 section 3.2.2 was used for each population. At each sample point, *c.* 20 panicles were cut from the reed stems and

temporarily stored in plastic bags. After collection, the panicles were air dried and stored in brown paper bags in Hessian-like sacks in an unheated greenhouse until analysis.

For each sampling point within a population, a cut panicle was randomly selected from which 20 seeds were extracted and stored in glass vials in a dark room at 4 °C until required for germination. Since *P. australis* is an outbreeding species, seeds removed from the same panicle were considered to be half-sibs (Clevering, 1999). In April 2003, the 20 seeds extracted from a single panicle were germinated in a Petri dish following the protocol of Self (1999). The method for seed germination is given in Chapter 5, section 5.2.4.

The Petri dishes containing the germinated seedlings were transferred to a laboratory bench and placed under halogen lights. After a week, all germinated seedlings from each panicle were planted into trays (22 cm × 52 cm) consisting of 20 cells (4 cm × 4 cm × 6 cm) each filled with non-peat compost (Levington's Multipurpose peat free compost, Ipswich, UK). An outer tray containing tap water of 1 - 2 cm depth ensured that the compost in the cells was permanently moist. Seedlings were grown under halogen lamps for two weeks and then in a natural light regime. Once every two weeks, each tray was fertilised with 3 l of Tomorite fertiliser (NPK: 4 % : 4.5 % : 8 %, plus seaweed extract; Levington Horticulture Ltd., Ipswich, UK).

6.2.2 Experimental Design

6.2.2.1 Experiment 1: nutrient-stressed conditions

In June 2003, 20 seedlings from each of the ten populations, consisting of two seedlings per panicle (i.e. two half-sibs) x ten panicles per population, randomly chosen from ten different sampling points in each population, were transferred to individual pots (21 cm diameter; 5.7 l volume) containing a non-peat compost (Golden Grow potting and bedding peat alternative, William Sinclair Horticulture Ltd, Lincoln, UK). The sample size for each population ranged from $n = 22$ to $n = 18$, except for Insh Marsh where $n = 3$, because a combination of poor seedset and

germination resulted in few seedlings being produced. The pots were randomly distributed among five wooden trays (each 120 cm width x 12 cm depth x 185 cm length), lined with plastic and arranged in a randomised block design in an unheated greenhouse. From June to October, the water level in each tray was maintained at 3 cm depth and the seedlings were fertilised once every two weeks with 5 l of Tomorite fertiliser per tray.

After two weeks in the peat free compost, the seedlings developed chlorosis (leaves and shoots turned yellow) because the compost was found to have a low available nutrient content (most probably iron and nitrogen). Consequently, high nitrogen containing fertilizer was applied weekly (5 l of Vitax foliar feed per tray: NPK: 35 % : 5% : 10 % : micronutrients, Steely Minerals Limited, Ormskirk, UK), but the chlorosis did not diminish. Since each population experienced the same conditions, it was decided to continue with morphological measurements in August 2003 and acknowledge that the experiment proceeded under 'stressed' environmental conditions (i.e. low available nitrogen). Symptoms of chlorosis did not diminish until autumn 2003, when the plants were repotted.

To determine quantitative genetic differences among and within the ten populations of *P. australis*, four morphological measurements were made for each plant at the beginning of August 2003: length of the longest shoot (measured from near the shoot base, using the inner margin of the pot as a reference, to the shoot tip immediately below the apical leaf sheaf); basal shoot diameter of the longest shoot (measured with dial callipers at the same basal point as shoot length); number of leaves on the longest shoot and the total number of shoots present.

6.2.2.2 Experiment 2: full-nutrient conditions

In November 2003, 20 seedlings per population (two half-sibs x 10 panicles) that had been grown in seedling trays but not used in Experiment 1, were planted into pots (22.5 cm diameter, 7.2 l volume) containing a mixture of 50 % peat free compost (Levington's Multipurpose peat free compost), 25 % sand and 25 % loam. Only nine populations were used for the experiment as there were no seedlings left from Insh

Marsh. Each pot was placed in a round tray (25 cm diameter, 5 cm depth) and distributed amongst five blocks arranged in a randomized block design. Throughout the growing season (April - October 2004) the plants were regularly watered, so that each plant was standing in a tray containing 1 - 2 cm of water. Once a fortnight, 5 l of Tomorite fertilizer was applied per block.

To determine quantitative genetic differences amongst and within the nine populations, above and below ground measurements were made on each plant at the end of the growing season in October 2004.

The above ground measurements were: mean shoot length of the five longest shoots (shoot length was determined in the same way as Experiment 1), mean basal shoot diameter of the five longest shoots (diameter measured in the same way as in Experiment 1), mean number of leaves of the five longest shoots, width and length of the longest leaf blade on the longest the shoot (from which the width/length ratio of the longest leaf was determined), and total number of shoots per plant. The sample size for each population was n = 18 - 22. The above ground biomass was found by harvesting the shoots and leaves from each pot and oven drying at 80 °C for 72 hours before weighing.

Below ground measurements were made on a sub-sample of each pot after harvesting of above ground biomass. Each pot and contents (plant material consisting of roots and rhizomes, soil and pot) was weighed and then the pot was removed. Using a sharp knife, a quarter sector of the pot contents was separated and weighed. The sector fraction was calculated by dividing the weight of a quarter of the pot contents by the weight of the total pot contents minus the weight of the empty pot. The plant material was thoroughly washed to remove all soil particles, and roots were then separated from rhizomes (Figure 6.1).

Figure 6.1 Washed rhizomes of *P. australis*, separated from roots.

The below ground measurements of each plant sub-sample were: total rhizome length, total number of rhizome internodes, length between two internodes of the thickest rhizome, mean diameter of the two internodes of the thickest rhizome and total number of developed buds. The sample size for each population was n = 18 - 22. Roots and rhizomes were oven dried separately at 80 °C for 72 hours and then weighed. To determine total rhizome length, total number of rhizome internodes, total number of buds and total root and rhizome biomass, each value was multiplied by 1/sector fraction.

6.2.2.3 Experiment 3: synthetic sewage

In November 2003, each plant from Experiment 1 was transferred to pots (22.5 cm diameter, 7.2 l volume) containing a mixture of 50 % peat free compost (Levington's Multipurpose peat free compost), 25 % sand and 25 % loam and replaced in one of five blocks (wooden trays) over winter. In March 2004 each of the five wooden trays from Experiment 1 was divided into two compartments by a wooden barrier (compartment A = no treatment; compartment B = treatment with synthetic sewage) which isolated one from the other (Fig. 6.2). The two half-siblings of the samples from each population (that now exhibited no chlorosis symptoms of the previous

year) were randomly assigned to treatment with synthetic sewage or no treatment (compartment A or B) and distributed throughout the five blocks. It was decided to exclude the Insh Marsh population from the experiment as the sample size (n = 3) was too small to partition between treatment and non treatment. Therefore the experiment was composed of nine populations of 15 - 20 plants, up to ten half-siblings receiving synthetic sewage and up to ten half-siblings receiving no treatment.

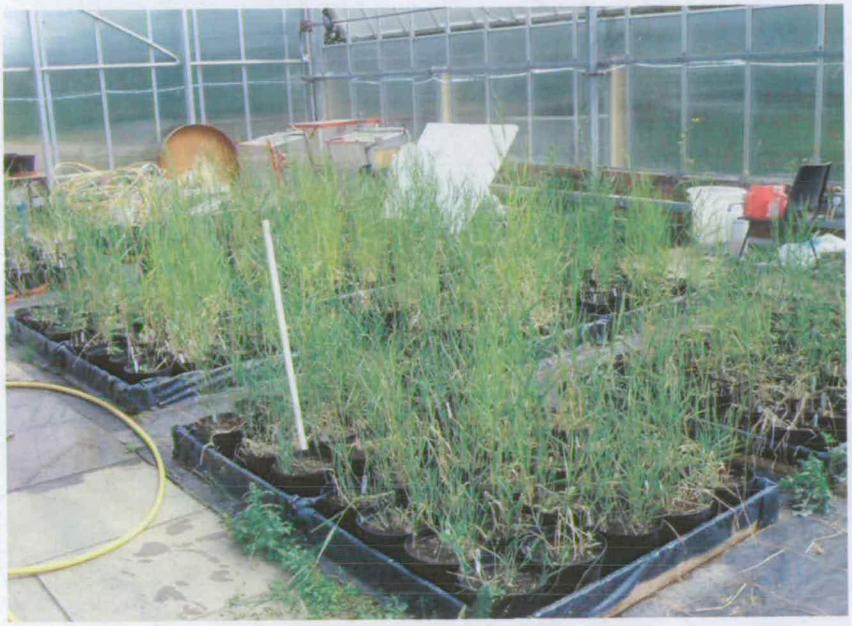


Figure 6.2 Experiment 3. *P. australis* populations arranged in randomised block design, August 2004. Each block is divided in two and received treatment with synthetic sewage or no treatment. Metre rule shown for scale.

From mid-May 2004 until mid-August, 20 l of synthetic sewage was applied fortnightly to compartment B of each block, whilst compartment A received 20 l of tap water. For practical reasons it was decided to apply synthetic sewage in this experiment rather than real sewage. The composition of synthetic sewage followed the guidelines set out by the Department of the Environment (1981) and contained in 1 litre of tap water: 160 mg peptone, 110 mg meat extract, 30 mg urea, 7 mg sodium chloride, 4 mg calcium chloride dehydrate, 2 mg magnesium sulphate heptahydrate, and 8 mg dipotassium hydrogen phosphate. Samples of diluted synthetic sewage

were periodically analysed for nitrate (NO_3-N), ammonium (NH_4-N), phosphate (P), measured as orthophosphate, and BOD concentration.

The concentrations of NO_3-N, NH_4-N and P were determined colorimetrically using a continuous flow autoanalyser (Bran and Luebbe). BOD_5 concentration was determined with the OxiTop® system (Wissenschaftlich-Technische Werkstätten (WTW) GmbH) in which piezoresistive electronic sensors detect changes in dissolved oxygen concentration in the sample, due to microbial oxidation of organic compounds, as a change in pressure. Samples were first seeded with a nitrification inhibitor, N-allylthiourea (WTW), and then incubated in the dark at 20 ± 1 °C for five days.

The mean concentrations of Biological Oxygen Demand (BOD_5) nitrate, ammonium and orthophosphate in the synthetic sewage were: BOD_5 = 194 ± 7.36 mg l^{-1}; NO_3^- = 1.31 ± 0.09 mg l^{-1}; NH_4^+ = 5.59 ± 1.71 mg l^{-1}; P = 4.83 ± 0.29 mg l^{-1}. The synthetic sewage was made in batches of 200 times the concentration and could be stored for up to a week at 4 °C until required for dilution. From the middle of August to the end of October 2004 the synthetic sewage was applied in weekly batches of 20 l to compartment B of each block. During this period the water in compartment A of each block was maintained at the same level as B. In addition to water or synthetic sewage, each block received monthly applications of 20 l of Tomorite fertilizer.

At the end of the experiment in October 2004 three above ground measurements were recorded: mean shoot length of the five longest shoots (shoot length was determined in the same way as Experiment 1); mean basal shoot diameter of the five longest shoots (diameter measured in the same way as in Experiment 1) and total number of shoots. The sample size for each population was n = 15 - 20. Above and below ground biomass were determined in a sub-sample of ten randomly selected plants from each population; five that had received synthetic sewage and five that had no treatment. The above ground biomass was determined by weighing the shoots, including leaves, of each plant that had been harvested and oven dried at 80 °C for 72 hours. The total below ground biomass was determined by washing and

drying a sub-sample (quarter sector) of each plant, as in Experiment 2. The fraction of the sub-sample was determined by weight, and the total below ground biomass of each plant was calculated by multiplying each value by 1/sector fraction.

6.2.3 Statistical analysis

All statistical tests were carried out using MINITAB 14 for Windows. Each variable in Experiments 1, 2 and 3 was tested for normality and homoscedasticity among populations using Kolmogorov-Smirnov and Bartlett's test, respectively (Sokal and Rohlf, 1995). A significant departure from normality and homogeneity of variance resulted in $\log_{10}$ transformation of the following variables in Experiment 2 for statistical analysis: total number of shoots, mean shoot diameter, mean number of leaves of the five longest shoots and total rhizome length.

6.2.3.1 Experiments 1 and 2

A Nested Analysis of Variance (ANOVA) was performed, using a General Linear Model (GLM), to determine if there were significant quantitative genetic differences amongst and within populations in each of the measured variables. To determine if populations could be separated into ecotypes, principal components analysis (PCA) was performed using a correlation matrix on the four above ground variables of Experiment 1 and ten combined above and below ground variables (excluding biomass) of Experiment 2. Values of the first two principal components were plotted against each other and a One-Way ANOVA was performed, using a GLM to determine if there were significant differences among and within populations in values of the first two principal components.

Pearson's correlation was used to test for association among variables both between and within populations. Regression analysis was performed to determine if the latitude of the population could account for the significant amount of among population variation in each of the variables measured (i.e. was there a north-south difference?).

6.2.3.2 Experiment 3

To test for a significant effect of synthetic sewage on morphology and biomass of *P. australis* populations a Two-Way ANOVA, using a GLM, was performed. The model was used to test for the main effects of population and treatment and the interaction between these two factors (genotype x environment).

6.3 RESULTS

6.3.1 Experiment 1: *P. australis* grown in a nutrient-stressed environment

Significant differences were found in shoot length, shoot diameter, leaf number and number of shoots among populations grown under nutrient-stressed conditions (Table 6.1). The variability among populations for each character was substantial. For instance, Minsmere had the shortest shoots (285.15 ± 17.4 mm) which were only 0.66 the length, on average, of the longest shoots from Insh Marsh (430.67 ± 44.5 mm) (Table 6.2). North Warren had the thinnest diameter of shoots (1.05 ± 0.15 mm) which were only 0.57 the diameter of the thickest shoots from Mersehead (1.83 ± 0.10 mm). There was no significant variation within populations for each of the variables ($P > 0.05$) (Table 6.1).

Table 6.1 Experiment 1. Results of a nested ANOVA of shoot length, shoot diameter and number of leaves of longest shoot and number of shoots, amongst and within ten *P. australis* populations grown in nutrient-stressed conditions.

Source of variance	d.f.	MS Shoot length	MS Shoot diameter	MS No. of leaves	MS No. of shoots
Block	4	4177^{ns}	0.2253^{ns}	3.503^{ns}	32.67^{ns}
Population	9	25392^{***}	1.0643^{**}	8.146^{***}	84.05^{***}
Family (Population)	83	3897^{ns}	0.3593^{ns}	2.161^{ns}	22.53^{ns}
Error	84	3209	0.3961	2.019	21.24

ns not significant, ** $P < 0.01$, *** $P < 0.001$

Table 6.2 Experiment 1. Mean values ± 1 SE of longest shoot length, shoot diameter, number of leaves of longest shoot and number of shoots of ten populations of *P. australis* grown in nutrient-stressed conditions. Populations are arranged in descending order of latitude with sample sizes in parentheses. Population abbreviations are given in Table 2.1.

Population (*n*)	Shoot Length (mm)	Shoot diameter (mm)	No. of leaves	No. of shoots
IM (3)	431 ± 44.5	1.43 ± 0.19	9.33 ± 1.20	10.7 ± 1.45
IT (18)	373 ± 13.5	1.72 ± 0.18	6.39 ± 0.34	15.7 ± 1.19
SM (20)	405 ± 14.2	1.54 ± 0.12	6.90 ± 0.24	15.4 ± 0.99
MH (22)	314 ± 10.4	1.83 ± 0.10	6.68 ± 0.21	13.4 ± 1.08
LM (20)	321 ± 11.8	1.42 ± 0.13	6.65 ± 0.25	9.05 ± 0.72
SF (20)	364 ± 15.1	1.55 ± 0.11	7.10 ± 0.25	13.6 ± 1.13
LH (20)	312 ± 15.1	1.84 ± 0.19	6.30 ± 0.36	10.9 ± 0.82
W (18)	337 ± 12.2	1.47 ± 0.16	6.50 ± 0.41	13.3 ± 1.20
MM (20)	285 ± 17.4	1.68 ± 0.10	6.20 ± 0.42	10.8 ± 1.46
NW (20)	353 ± 7.00	1.05 ± 0.15	8.30 ± 0.40	14.4 ± 0.96

None of the four characters showed high correlation within or among populations, although there was a negative weak and marginally significant correlation between shoot length and number of shoots (r = - 0.629, P = 0.051). There was also evidence of a latitudinal trend in shoot length, with southern populations producing shorter shoots when grown in nutrient stressed conditions (R^2 = 0.687, P < 0.01) (Figure 6.3).

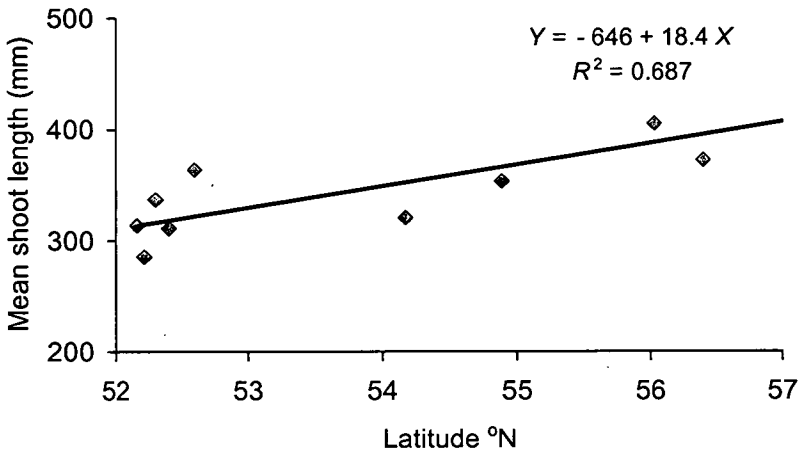
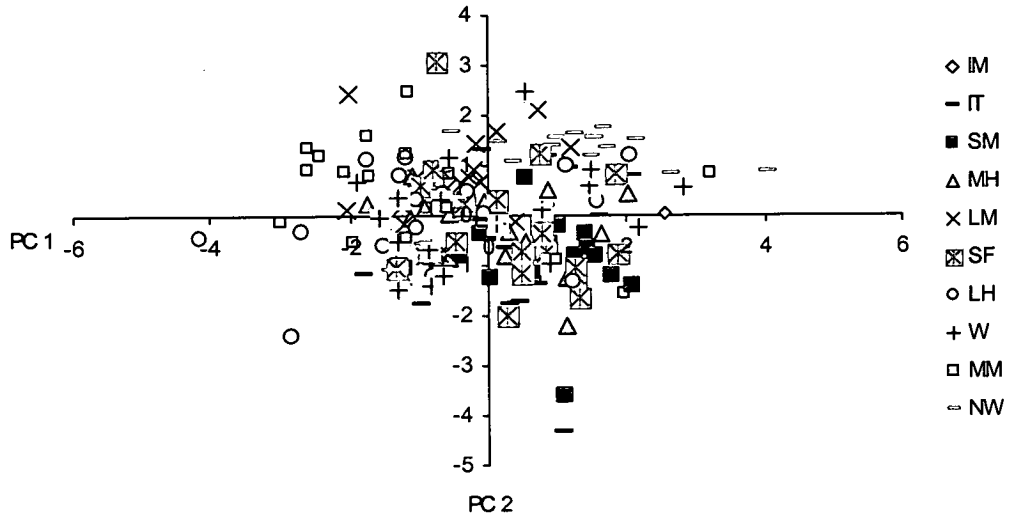


Figure 6.3 Experiment 1. Mean shoot length *vs* latitude for ten populations of *P. australis* (y axis starts at 200 mm).

Populations could not be separated into ecotypes, as an ordination plot of values of PC 2 against PC 1 revealed that there was considerable overlap between populations (Fig. 6.4a), even though a One-Way ANOVA showed there was a highly significant difference amongst populations for PC 1 and PC 2 values (both P < 0.001).

(a) Experiment 1, nutrient-stressed conditions



(b) Experiment 2, full-nutrient conditions

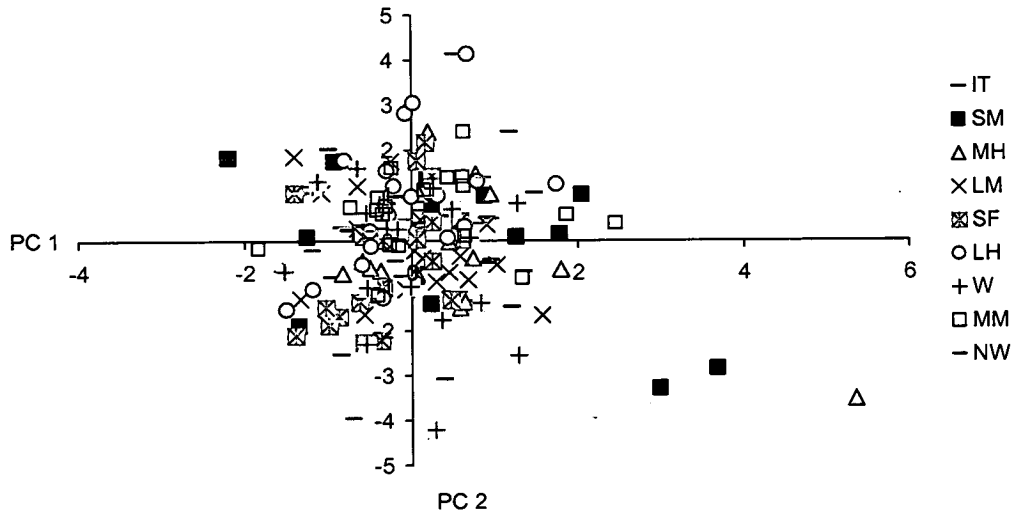


Figure 6.4 An ordination plot of values of principal components PC 1 and 2 for each population of *P. australis* for (a) Experiment 1 and (b) Experiment 2. Population abbreviations are given in Table 2.1.

6.3.2 Experiment 2: *P. australis* grown in a full-nutrient environment

6.3.2.1 Above ground measurements

Similar to Experiment 1, considerable differences were found in shoot length, shoot diameter and number of shoots among populations grown in full-nutrient conditions ($P < 0.001$) (Table 6.3). No significant differences were found within populations ($P > 0.05$). Once again, population differences in variables were substantial (Table 6.4). For instance, St. Margaret's Marsh had the shortest shoots (628 ± 28 mm) and they were only 0.74 the length, on average, of the Minsmere shoots (845 ± 20 mm) which were the longest. Lakenheath had the least number of shoots (23.6 ± 1.1) and there were 1.44 times more shoots, on average, at Strumpshaw (34.2 ± 2.2), which had the most shoots.

Similar to Experiment 1, there was no correlation between any of the variables either within or among populations ($P > 0.05$). There was evidence of a latitudinal trend in shoot length ($R^2 = 0.808$, $P < 0.01$), which was the opposite of that found in the nutrient-stressed conditions of Experiment 1: southern populations produced longer shoots than northern populations in full-nutrient conditions (Fig. 6.5a).

Table 6.3 Experiment 2. Results of a nested ANOVA of mean shoot length, mean shoot diameter and mean number of leaves of five longest shoot, total number of shoots and width/length ratio of longest leaf on longest shoot, amongst and within nine *P. australis* populations grown in a full-nutrient environment.

Source of variance	d.f	MS Mean shoot length	MS Mean shoot diameter	MS Mean no. of leaves	MS No. of shoots	MS Width/ Length Ratio
Block	4	35627^{ns}	0.191^{ns}	1.996^{ns}	66.760^{ns}	0.00025^{ns}
Population	8	46329**	0.594**	6.036^{ns}	225.560**	0.00022^{ns}
Family (Population)	80	16413^{ns}	0.161^{ns}	6.724^{ns}	76.810^{ns}	0.0002^{ns}
Error	83	19483	0.204	4.769	70.780	0.0002

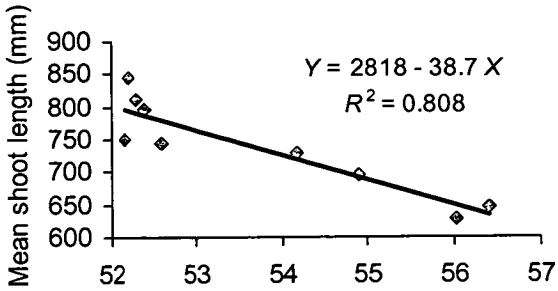
ns not significant, * $P < 0.05$, ** $P < 0.01$

Table 6.4 Experiment 2. Mean values of shoot length (SL), shoot diameter (SD) and number of leaves of five longest shoots (NoL), number of shoots (NoS), width/length ratio of longest leaf (W/L), total rhizome length (TRL), number of rhizome internodes (NoI), mean diameter of internodes (DoI), length between internodes (LbI), number of rhizome buds (NoB) of nine populations of *P. australis* grown in full-nutrient conditions. Errors in parentheses are 1 SE. Populations (Pop) are arranged in descending order of latitude, with sample size in parentheses. Population abbreviations are given in Table 2.1

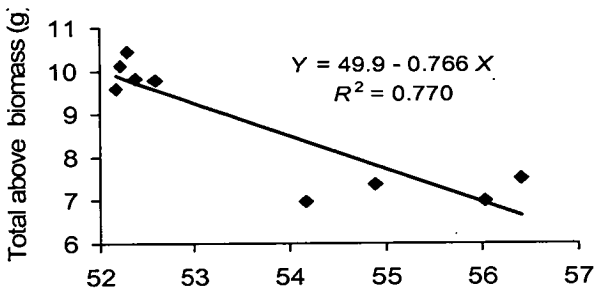
Pop (*n*)	SL (mm)	SD (mm)	NoL	NoS	W/L	TRL (cm)	NoI	DoI (mm)	LbI (mm)	NoB
IT (20)	646.43 (35.5)	1.31 (0.05)	12.58 (0.49)	24.95 (2.24)	0.049 (0.002)	119.30 (10.8)	49.20 (4.35)	4.74 (0.18)	39.45 (2.47)	9.70 (1.24)
SM (18)	628.16 (48.1)	1.56 (0.05)	13.00 (1.50)	24.88 (2.08)	0.051 (0.002)	106.00 (10.2)	49.00 (3.63)	4.72 (0.41)	37.61 (3.42)	11.44 (0.86)
MH (20)	694.78 (31.8)	1.65 (0.06)	13.25 (0.45)	25.20 (1.65)	0.043 (0.002)	129.85 (9.4)	50.05 (3.65)	4.68 (0.18)	35.10 (1.85)	7.25 (0.64)
LM (19)	728.43 (26.4)	1.82 (0.22)	14.59 (0.28)	28.35 (1.57)	0.042 (0.002)	144.42 (9.3)	63.32 (3.69)	4.49 (0.18)	36.16 (2.40)	7.47 (0.63)
SF (22)	742.93 (24.5)	1.53 (0.06)	13.34 (0.31)	34.24 (2.23)	0.046 (0.002)	149.68 (7.4)	59.45 (2.74)	4.60 (0.20)	34.95 (2.19)	9.23 (0.83)
LH (20)	794.74 (23.1)	1.93 (0.10)	13.57 (0.27)	23.60 (1.12)	0.049 (0.002)	159.60 (30.4)	51.05 (2.99)	4.88 (0.20)	35.25 (2.61)	7.21 (0.53)
W (20)	812.23 (30.0)	1.81 (0.07)	13.87 (0.42)	34.30 (2.17)	0.052 (0.007)	144.65 (12.5)	59.05 (5.54)	4.46 (0.16)	32.95 (1.74)	9.40 (0.86)
MM (20)	845.35 (19.8)	1.74 (0.06)	13.86 (0.32)	23.70 (1.62)	0.047 (0.002)	115.10 (9.30)	48.40 (3.42)	4.79 (0.16)	36.35 (2.44)	7.90 (0.87)
NW (18)	750.22 (31.4)	1.73 (0.07)	14.59 (0.29)	27.72 (2.62)	0.045 (0.002)	126.28 (9.20)	57.22 (3.97)	5.08 (0.24)	32.72 (1.55)	8.94 (1.00)

A Finlay-Wilkinson plot (Finlay and Wilkinson, 1963) of shoot height illustrates the genotype × environment interaction of each population grown in nutrient-stressed and then full-nutrient conditions (Figure 6.6). The southern populations (e.g. Minsmere, Lakenheath and Walberswick) generally grew less well and produced shorter shoots under nutrient-stressed conditions, but were the most responsive in a full-nutrient environment, producing the longest shoots.

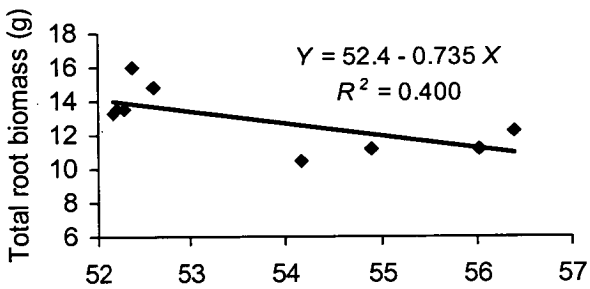
(a)



(b)



(c)



(d)

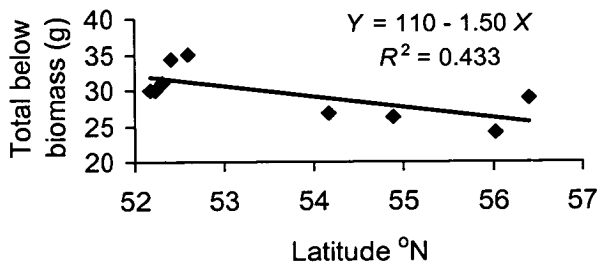


Figure 6.5 Experiment 2. Regression analysis of (a) mean shoot length (y axis starts at 600 mm), (b) total above ground biomass (y axis starts at 6 g), (c) total root biomass (y axis starts at 6 g), (d) total below ground biomass (y axis starts at 20 g) *vs* latitude.

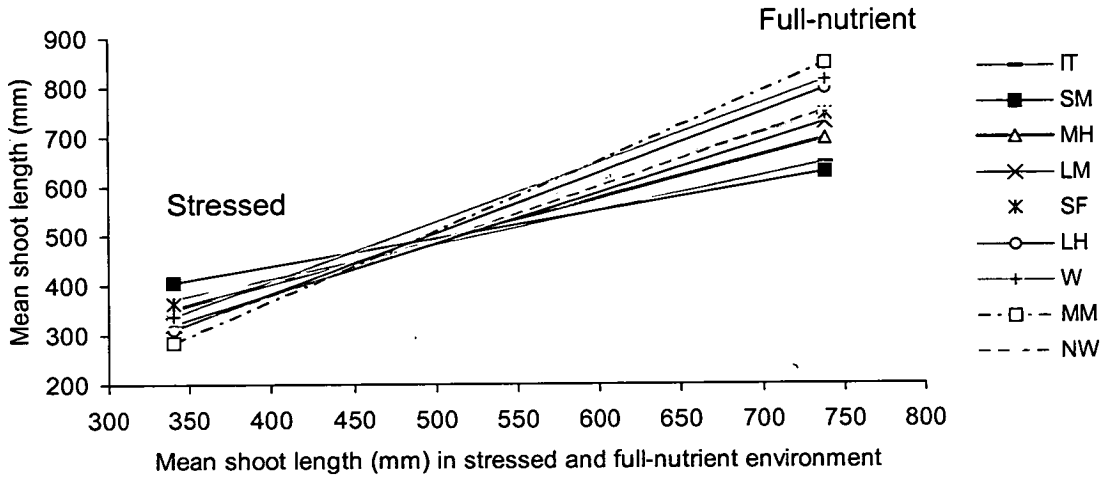


Figure 6.6 Mean shoot length of each population in each environment against mean shoot length of all populations in stressed and full-nutrient environment. Population abbreviations are given in Table 2.1.

6.3.2.2 Below ground measurements

Unlike the results for the above ground variables, no significant population differences were found for any of the five below ground characters measured (Table 6.5). Significant variability within populations was not found except for total number of buds, which showed a highly significant difference within populations (Table 6.5).

6.3.2.3 Above and below ground biomass

Although there were no differences amongst populations in the five below ground variables measured, there were significant differences amongst populations in above ground biomass, root biomass and total below ground biomass (Table 6.6).

Table 6.5 Experiment 2. Results of a nested ANOVA of total rhizome length, total number of internodes (Is), mean diameter of the two internodes (Is) of the thickest rhizome, length between the two internodes of the thickest rhizome and total number of buds, amongst and within populations.

Source of variance	d.f.	MS Total rhizome length	MS Total no. of Is	MS Mean diameter of two Is	MS Length between two Is	MS Total no. of buds
Block	4	0.019^{ns}	2420^{ns}	0.604^{ns}	12.79^{ns}	106.4^{ns}
Population	8	0.058^{ns}	24474^{ns}	0.610^{ns}	55.44^{ns}	1061.8^{ns}
Family (Population)	79	0.039^{ns}	33982^{ns}	1.090^{ns}	123.01^{ns}	839.7***
Error	82	0.041	34997	0.931	91.04	202.3

ns not significant, *** $P < 0.01$

No significant differences were found within populations except for above/below ground biomass ratio (Table 6.6). Once again where variability was found amongst populations, the differences were substantial. For instance, the Leighton Moss population had the smallest root biomass (10.4 ± 0.93 g) which was 0.63 on average, of Lakenheath which had the largest biomass (16.0 ± 0.91 g) (Table 6.7).

Table 6.6 Experiment 2. Results of a nested ANOVA of total biomass of root, rhizome, below ground, above ground, and above/below ground biomass, amongst and within populations, October 2004.

Source of variance	d.f.	MS Root	MS Rhizome	MS Below ground	MS Above ground	MS Above/ below ground
Block	4	18.08^{ns}	56.28^{ns}	86.81^{ns}	12.939^{ns}	0.00096^{ns}
Population	8	49.60**	60.06^{ns}	188.01*	24.151*	0.01246^{ns}
Family (Population)	80	15.71^{ns}	41.60^{ns}	83.21^{ns}	9.558^{ns}	0.01510**
Error	83	15.86	33.56	73.65	7.841	0.00850

ns not significant, *$P < 0.05$, ** $P < 0.01$

Similar to the finding that southern populations produced longer shoots compared to northern populations, there were also significant negative associations between latitude and above ground biomass ($R^2 = 0.77$, $P < 0.01$) (Fig. 6.5b), root biomass ($R^2 = 0.400$, $P < 0.05$) (Figure 6.5c) and total below ground biomass ($R^2 = 0.433$, $P < 0.05$) (Fig 6.5d). This implies that northern populations generally produced less above and below ground biomass as well as less root biomass and, when fully supplied with nutrients, the southern populations were the most productive.

Table 6.7 Experiment 2. Mean values of root, rhizome, total below ground, and above ground biomass and above/below ground biomass ratio of nine populations of *P. australis*. Errors in parentheses = 1 SE. Populations are arranged in descending order of latitude, with sample size in parentheses. Population abbreviations are given in Table 2.1.

Population (*n*)	Roots (g)	Rhizomes (g)	Total below ground (g)	Above ground (g)	Above/ below ground ratio
IT (20)	12.3 (0.87)	16.6 (1.28)	28.9 (1.94)	7.51 (0.65)	0.26 (0.02)
SM (18)	11.2 (1.29)	12.9 (1.79)	24.1 (2.77)	6.98 (0.87)	0.27 (0.04)
MH (20)	11.2 (1.02)	15.1 (1.34)	26.3 (2.03)	7.37 (0.66)	0.30 (0.02)
LM (19)	10.4 (0.93)	16.3 (1.82)	26.8 (2.53)	6.97 (0.81)	0.28 (0.03)
SF (22)	14.9 (0.95)	20.2 (1.13)	35.1 (1.73)	9.78 (0.74)	0.28 (0.02)
LH (20)	16.0 (0.91)	18.4 (1.24)	34.5 (1.93)	9.82 (0.52)	0.30 (0.02)
W (20)	13.6 (0.68)	17.5 (1.24)	31.1 (1.71)	10.45 (0.58)	0.35 (0.02)
MM (20)	13.5 (0.81)	16.5 (1.45)	30.0 (2.02)	10.10 (0.51)	0.36 (0.02)
NW (18)	13.3 (0.77)	16.7 (1.38)	30.0 (1.83)	9.60 (0.65)	0.33 (0.03)

Similar to populations grown in a nutrient-stressed environment, ecotypes were not found in full-nutrient conditions. An ordination plot of PC 2 against PC 1 values, for all the ten measured above and below ground variables (excluding biomass), showed that populations could not be easily separated (Fig. 6.4b).

6.3.3 Experiment 3. The effects of synthetic sewage on nine *P. australis* populations

A Two-Way ANOVA showed that there were significant differences among populations, regardless of treatment, for shoot length, number of shoots and total above ground biomass (Table 6.8).

Table 6.8 Experiment 3. Results of a Two-Way ANOVA testing the main effects of population and synthetic sewage treatment and a population x treatment interaction amongst nine populations of *P. australis* on mean shoot length and diameter of five longest shoots, total number of shoots, above ground and below ground biomass and above/below ground biomass ratio. Degrees of freedom given in parentheses.

Source of variance	MS Shoot length	MS shoot diameter	MS No. of shoots	MS Above ground biomass	MS Below ground biomass	MS Above/ below ground biomass
Block	50000^{ns} (4)	0.0070^{ns} (4)	267.8^{ns} (4)	37.57^{ns} (4)	117.0^{ns} (4)	0.0075^{ns} (4)
Population	103534* (8)	0.0110^{ns} (8)	607.5** (8)	102.14* (8)	698.1^{ns} (8)	0.0125^{ns} (8)
Synthetic sewage treatment	41095^{ns} (1)	0.0125^{ns} (1)	658.9† (1)	12.52^{ns} (1)	2027.1† (1)	0.0584* (1)
Population x treatment	44871^{ns} (8)	0.0124^{ns} (8)	116.1^{ns} (8)	43.50^{ns} (8)	435.0^{ns} (8)	0.0121^{ns} (8)
Error	51311 (149)	0.0075 (149)	208.2 (149)	44.80 (69)	531.7 (69)	0.0120 (69)

ns not significant, * † marginally significant $P < 0.08$, *$P < 0.05$, ** $P < 0.01$

The substantial differences between populations that were found in the previous experiments were also present here. For instance, Inner Tay had the shortest shoots (806 ± 54 mm) which were only 0.38 the length, on average, of the longest shoots from Minsmere (1112 ± 215 mm) (with sewage treatment) (Table 6.9). St. Margaret's Marsh had the lowest number of shoots (28.4 ± 3.5) (no treatment) and there were 1.82 times more shoots, on average, for Walberswick (51.7 ± 5.9), which had the most shoots (no treatment).

Sewage treatment had a marginal significant effect on the number of shoots produced ($P = 0.077$), total below ground biomass ($P = 0.055$) and above/below ground biomass ratio ($P < 0.05$), regardless of population (Table 6.8). Application of synthetic sewage resulted in a decrease in the number of shoots produced (mean over all populations receiving treatment = 35.41 ± 1.94; populations not receiving treatment = 39.46 ± 2.32), an increase in below ground biomass (55.36 ± 4.10 g for populations receiving treatment; 45.80 ± 3.22 g for populations that had no treatment) and a decrease in above/below ground biomass ratio (0.287 ± 0.015 for treatment populations compared to 0.334 ± 0.019 for untreated populations) (Tables 6.9 and 6.10). There was no evidence that populations responded differently to treatment as there were no significant population x treatment interaction for any of the variables (Table 6.8). Population responses to application of synthetic sewage are shown by the interaction plots for those variables that were significantly affected by treatment (Fig. 6.7). The interaction plots revealed that although many populations showed a similar direction of response to synthetic sewage, the experimental environment had a substantial effect in some populations.

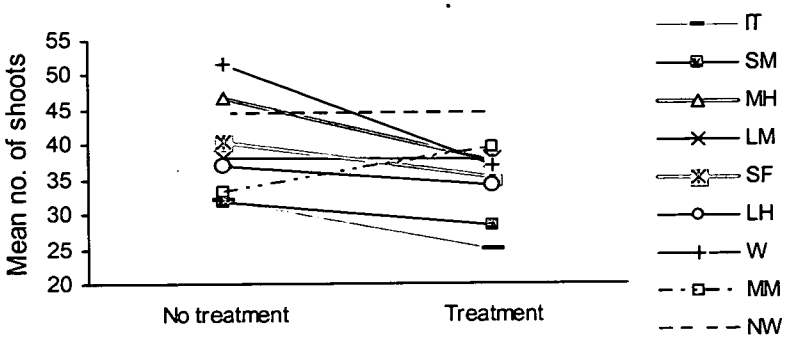
Table 6.9 Experiment 3. Population mean values of shoot length (mm), shoot diameter (mm) and number of shoots receiving no treatment (NT) or treatment with synthetic sewage (T). Errors in parentheses = 1 SE. Mean values combining all populations for each variable are also given with 1 SE shown in parentheses. Populations are arranged in descending order of latitude with sample sizes for populations receiving treatment and no treatment in parentheses. Population abbreviations are given in Table 2.1.

Population	Shoot length (mm)		Shoot diameter (mm)		No. of shoots	
	NT	*T*	*NT*	*T*	*NT*	*T*
IT (7,8)	849 (76.5)	806 (54.8)	4.38 (0. 32)	4.39 (0. 31)	32.1 (6.88)	24.9 (3.60)
SM (10,10)	8711 (84.9)	893 (92.7)	4.48 (0. 37)	4.55 (0. 34)	31.9 (6.16)	28.4 (3.51)
MH (9,10)	818 (56.8)	825 (48.9)	4.27 (0. 31)	3.98 (0.15)	46.7 (5.62)	37.6 (4.29)
LM (10,10)	854 (42.7)	932 (42.0)	4.47 (0.30)	4.43 (0.23)	38.1 (4.55)	37.8 (2.98)
SF (10,10)	1014 (35.4)	952 (36.4)	4.73 (0.21)	4.86 (0.20)	40.3 (3.11)	35.1 (6.53)
LH (10,10)	952 (50.0)	1031 (36.6)	4.77 (0.02)	5.22 (0.35)	36.8 (3.87)	34.2 (4.44)
W (10,10)	1031 (56.2)	852 (84.5)	4.97 (0.25)	3.68 (0.44)	51.7 (5.91)	36.9 (5.09)
MM (10,10)	1023 (47.0)	1112 (215)	4.78 (0.20)	4.26 (0.27)	33.1 (5.27)	39.4 (2.46)
NW (10,10)	8662 (41.1)	861 (38.8)	4.39 (0.21)	4.33 (0.12)	44.4 (3.72)	44.4 (3.24)
Mean	919.7 (28.4)	918.3 (33.6)	4.58 (0.08)	4.41 (0.15)	39.5 (2.32)	35.4 (1.94)

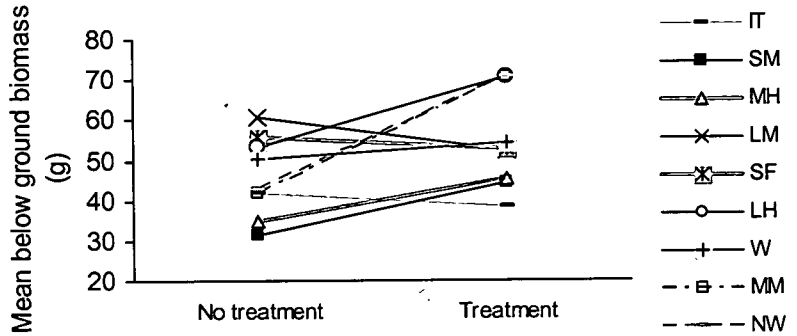
Table 6.10 Experiment 3.Population mean values of above ground biomass, below ground biomass and above/below ground biomass ratio receiving no treatment (NT) or treatment with synthetic sewage (T). Errors in parentheses = 1 SE. Mean values combining all populations for each variable are also given with 1 SE shown in parenthesis. Populations are arranged in descending order of latitude with sample sizes for populations receiving treatment and no treatment in parentheses. Population abbreviations are given in Table 2.1.

Population	Above ground biomass (g)		Below ground biomass (g)		Above/Below biomass ratio	
	NT	*T*	*NT*	*T*	*NT*	*T*
IT (5,5)	15.2 (4.44)	8.89 (1.62)	41.6 (9.24)	38.3 (5.45)	0.33 (0.05)	0.23 (0.03)
SM (5,5)	7.46 (1.63)	11.5 (3.02)	31.6 (6.33)	44.4 (10.88)	0.24 (0.04)	0.33 (0.09)
MH (5,5)	10.0 (1.48)	12.6 (1.98)	35.0 (7.59)	45.5 (6.58)	0.32 (0.05)	0.29 (0.04)
LM (5,5)	18.3 (2.79)	17.1 (3.63)	60.4 (6.10)	52.0 (6.50)	0.31 (0.05)	0.32 (0.04)
SF (5,5)	19.9 (3.32)	16.7 (2.24)	55.7 (4.85)	52.5 (12.0)	0.36 (0.05)	0.36 (0.06)
LH (5,5)	15.4 (2.45)	16.9 (4.69)	53.3 (5.48)	70.4 (10.1)	0.28 (0.02)	0.25 (0.05)
W (5,5)	18.6 (1.52)	11.4 (2.85)	50.1 (14.3)	54.3 (13.4)	0.33 (0.04)	0.23 (0.02)
MM (5,5)	16.7 (2.53)	19.1 (4.69)	41.7 (6.09)	70.4 (20.7)	0.41 (0.03)	0.29 (0.03)
NW (5,5)	16.1 (2.69)	19.1 (2.98)	42.9 (9.42)	70.6 (12.4)	0.42 (0.08)	0.29 (0.04)
Mean	15.3 (1.36)	14.3 (1.25)	45.8 (3.22)	55.4 (4.10)	0.33 (0.02)	0.29 (0.02)

(a)



(b)



(c)

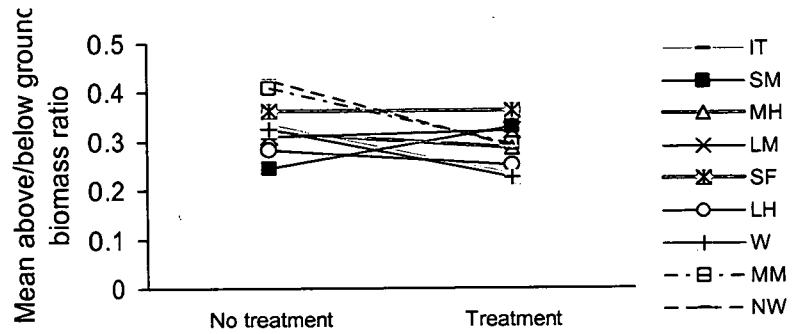


Figure 6.7 Experiment 3. Interaction plots showing the effect of synthetic sewage application (treatment) on (a) mean number of shoots, (b) mean below ground biomass (c) mean above/below ground biomass ratio, for each population. For above ground measurements: SM, LM, SF, LH, W, NW n = 20; MH, MM, n = 19; IT, n = 15. For above and below ground biomass: all populations, n = 5. Population abbreviations are given in Table 2.1.

6.4 DISCUSSION

In all three experiments significant and substantial morphological differences were found among British *P. australis* populations. The results were similar to the findings in earlier studies carried out in Sweden (Björk, 1967), the Netherlands (van der Toorn, 1972) Britain (Daniels, 1991), Denmark (Clevering, 2001) and Italy (Lambertini *et al.*, 2003). Because the experiments reported here used seeds obtained from many parents within each population that were grown under the same conditions for each experiment, it can be concluded that the population differences observed were of genetic rather than phenotypic origin (Daniels, 1991; Briggs and Walters, 1997). In each experiment where trait differences were found among populations, they were substantial.

For all experiments, *P. australis* seeds were collected in the field from each population rather than produced through propagation under controlled environmental conditions and as a consequence there may have been a maternal environment effect that could have preconditioned the seeds (Briggs and Walters, 1997; Clevering *et al.*, 2001). Thus the variation found between populations could have arisen due to maternal effects rather than as a result of the genotype (Roach and Wulff, 1987). However these effects are usually seen in early seedling growth rather than the late seedling stage when the genotype of the offspring begins to contribute to any observed variation (Roach and Wulff, 1987; Clevering, 1999). Therefore, in this study, the measurements that were made 18 weeks and > 52 weeks after germination are more likely to be determined by the genotype of the individual in the population rather than due to a maternal carry over effect.

Measurements of below ground morphology were made in the full-nutrient experiment and in contrast to the study of Clevering (2001), there was no significant difference amongst populations in rhizome diameter or total rhizome length. In fact none of the five below ground variables, apart from biomass, were significantly different amongst populations. This result is surprising considering the substantial differences found amongst populations in above ground variables and may have

arisen because of the confined growing space which resulted in some plants becoming pot bound. When the pots were removed at the end of October 2004 it was observed that the root and rhizome systems were distributed throughout the whole pot volume and in many cases were growing outside the pot. Significant differences were found in total root biomass, but not in rhizome biomass, perhaps due to the restrictions to rhizome growth imposed by the pots. This is quite probable since it has been estimated that annual rhizome growth can be 0.4 - 2 m per year (in this study, the mean rhizome length of populations was 1.06 - 1.60 m after 11 months, Experiment 2), with rhizomes living from 3 - 6 years (Haslam, 1972). In Experiment 2 populations also differed in below ground biomass, root biomass and above ground biomass whilst above ground biomass was also significantly different in Experiment 3, regardless of treatment.

The characters that showed consistent variation between populations when grown in the three environments were shoot length and number of shoots produced. Shoot diameter was variable in Experiments 1 and 2, but not 3. As no correlation was found between these characters either within or among populations, they appear to be independent.

Generally, for each experiment, variation amongst *P. australis* populations was greater than within populations. This may be explained by a combination of factors. Firstly, this study has found clone size within populations to be large in some of the populations sampled for this experiment (see Chapter 3 section 3.3.2.2). For instance, the Inner Tay population has one clone that may be as large as 5.1 ha. Therefore, within a population, families may be based on exactly the same female genotype. Secondly, if each *P. australis* population in this study was founded by only one seedling recruitment event, then the genotypic variability would be determined by the number of founding clones (founder effects) as well as the genetic diversity of the founding clones (Clevering, 1999). During reedbed establishment, inter-clonal competition would result in only those clones adapted to the hydrological, climatic and edaphic conditions spreading within the reedbed. Over time clonal diversity would be expected to decrease and only after disturbance such as drawdown, cutting

or burning sections of the reedbed would sexual recruitment occur (Koppitz *et al.*, 1997). Reed is cut commercially for thatching at Inner Tay and Walberswick, whilst at other sites the reedbed is managed by cutting and/or burning (Leighton Moss, Strumpshaw Fen, North Warren and Minsmere), so seedling recruitment after disturbance could be a regular occurrence in some of the populations studied. However these methods of management leave the rhizomes undisturbed and the existing reeds can probably outcompete young seedlings, establishing after a disturbance event for light (Haslam, 1970). Therefore intra-population diversity may be small compared to inter-population diversity and within population morphological variability observed within a reedbed may be the result of clonal plasticity (although the extent of this is under genetic control) rather than the presence of many different genotypes (Vretare *et al.*, 2001).

A geographic pattern of variability was found for certain characters. Shoot length of each population was dependent on nutrient conditions as well as geographic origin. The latitudinal trend in shoot length was positive under nutrient-stressed conditions and negative for full-nutrient conditions. Above and below ground and root biomasses were also negatively associated with latitude in Experiment 2. Generally, in nutrient-stressed conditions, northern populations produced longer shoots than southern ones (R^2 = 0.687), whilst the opposite occurred in full-nutrient conditions, with southern populations producing longer shoots (R^2 = 0.808) and greater above ground biomass (R^2 = 0.77) than the northern ones The positive association between shoot length and latitude found in Experiment 1 may be explained by the adaptation of the northern populations to growing in conditions with lower soil temperature. Under these conditions, nutrient cycling proceeds more slowly and nutrient availability is less than in southern soils, where temperatures are generally higher (Chapin and Chapin, 1981) and, as a consequence, northern populations are more tolerant of nutrient stress than southern ones (Clevering *et al.*, 2001). Therefore, under the nutrient stressed conditions of Experiment 1, the southern populations produced shorter shoots than would normally be expected, for example shoots from the Minsmere population were only two-thirds of the length of those from the Insh Marsh population.

The negative relationship between shoot length and latitude found in Experiment 2, has also been reported by Clevering *et al.*, (2001) and Karunaratne (2003) although the latitudinal effect in this experiment was over a smaller scale, as these studies investigated *P. australis* populations from different countries (Clevering *et al.*, 2001) and continents (Karunaratne, 2003). It has been suggested that for southern populations competition for light becomes more important with increasing length of the growing season (de Kroon and Kalliola, 1994) which introduces density dependent competition for light, such that southern populations produce taller but fewer shoots. In contrast northern populations are more subject to density independent selection by frost, which produces populations with more prostrate and abundant shoots. Whether this is a plausible explanation over the fine latitudinal scale examined here is not clear, and no latitudinal trend in shoot density was found in the experimental results.

Other studies have found that plants from northern climates have a higher allocation to below ground parts which is related to the lower nutrient availability in these environmental conditions and the increased carbohydrate reserve required to survive the winter (Chapin and Chapin, 1981; Clevering *et al.*, 2001; Karunaratne 2003). However such a north-south trend was not observed in this study (experiment 2) and in fact the opposite trend was observed, as there was a significant negative association between below ground biomass (R^2 = 0.433) and root biomass (R^2 = 0.400) with latitude. This is probably because the climatic gradients from north to south in this study were insufficient to reveal an adaptive response such as an increase in rhizome biomass in the northern populations, which would enable them to tolerate colder soil conditions. More probably the increase in below ground biomass found in the southern populations of this study arises due to comparatively greater and prolonged photosynthetic activity (due to a longer growing season) in the more abundant aerial parts which resulted in a greater number of assimilates transported to the rhizomes, roots and buds from late July onwards (Haslam, 1972; Hara *et al.*, 1993).

Although population differences in growth form were found in both the nutrient-stressed and full-nutrient environment, ecotypes such as those described by van der Toorn (1972), Dykyjová and Hradecká (1973) and Waisel and Rechav (1971), were not found. This was evident from the PCA ordination plots, which showed considerable overlap between populations. It was therefore concluded that populations exhibited clinal variation in this study and could not be separated into biotypes, which agrees with the findings of Clevering *et al.* (2001).

When the growth of populations in two different environments (nutrient-stressed and full-nutrient) was compared using a Finlay-Wilkinson plot, it was clear that the southern populations grew best (or at least taller) in full-nutrient soils, whilst northern populations were generally more tolerant of the nutrient-stressed conditions. Application of synthetic sewage resulted in a significant decrease in the number of shoots produced per population compared to those seedlings not receiving treatment, as well as a significant increase in below ground biomass. There was no significant difference amongst populations in number of shoots or below ground biomass and no significant genotype x environment interaction for these two variables. However the interaction plots (Fig. 6.7) revealed that although many populations showed a similar response to synthetic sewage, the environment had a substantial effect in some populations. For instance application of synthetic sewage to the Walberswick population resulted in a 47 % reduction in shoot production, whilst no change was recorded for North Warren. Minsmere produced 40 % more below ground biomass in response to synthetic sewage application whilst Strumpshaw Fen produced 5 % less.

These results contradict the findings of Hardej and Ozimek (2002), who found that application of sewage sludge increased shoot density, biomass and shoot diameter as well as shortening shoots, although their study compared a natural reed stand and a constructed wetland system which received a more concentrated form of sewage than in this experiment. In addition, their experiment was conducted over two years and no below ground measurements were made. It was hypothesised that the phenotypic response of *P. australis* in constructed wetlands receiving sewage effluent is to produce shorter and more abundant shoots to permit the rapid transportation of

oxygen to the rhizosphere and reduce the risk of tissue anaerobis. Although this plastic response was not found in this experiment, the increase in below ground biomass observed in some populations would ensure a large surface area for microbial activity and oxygen leakage in the rhizosphere, enabling the removal of organic matter, ammonium, nitrate and phosphate (a full explanation of the role of *P. australis* in CWS is given the next Chapter).

6.5 IMPLICATIONS FOR REEDBED RESTORATION FOR CONSERVATION AND USE IN CONSTRUCTED WETLAND SYSTEMS

The three experiments demonstrate that there are significant and substantial genetic differences amongst British populations of *P. australis* in morphology and biomass, which persist under different edaphic conditions. These findings are important for reedbed conservation for wildlife since a local reed source is not always available for planting and a decision must be made regarding the provenance of *P. australis* for reedbed establishment. This study has shown that seed source choice should account for the quantitative genetic differences that exist amongst British *P. australis* populations. Planting of a particular provenance could determine the presence or absence of certain faunal species. For instance phytophagous insect communities, which are monophagous for *P. australis*, have varying habitat requirements that are dependent on the thickness of *P. australis* shoots. (Tscharntke, 1999). The stem boring moth (*Archanara geminipuncta*) which is a key species in the reed ecosystem and greatly influences insect community structure has a preference for mean shoot diameter of 6.3 mm, whilst the gall inducing chloropid flies (*Lipara lucens* and *L. rufitarsis*) have a preference for much thinner shoots less than 4.5 mm in diameter (Tscharntke, 1990; Tscharntke, 1999). Variability in *P. australis* shoot diameter, density and height is important in the breeding habitat requirements of passerine

species (Poulin *et al.*, 2002). The great reed warbler (*Acrocephalus arundinaceus*) has a strong association with reedbeds having thick stems, whilst the reed warbler (*Acrocephalus scirpaceus*) has a preference for monospecific tall stems and the bearded tit (*Panurus biarmicus*) has a preference for dense, short and thin reed stems (Poulin *et al.*, 2002).

Consequently, reedbeds established or restored as nature reserves should contain a variety of *P. australis* shoot types for the development of species-rich faunal communities, whilst the attraction of particular species may lead to targeting of a certain *P. australis* population with a specific morphology. In this study, variability within populations was not found in either a nutrient-stressed or full-nutrient environment. If local provenances are not available for planting, a variety of reed sources should be used to promote genotypic diversity within the reedbed. In old reedbeds where clonal diversity may be low, the reedbed should be managed to promote sexual recruitment, rather than vegetative spread, thereby increasing genetic diversity and the variability in *P. australis* morphology.

While this study did not explore the plastic response of *P. australis* by transplant experiments (van der Toorn, 1972; Daniels, 1991), it was shown that southern populations grew best in full-nutrient conditions and should not be used as a seed source for more northern or nutrient poor habitats in Britain.

Although the sewage treatment experiment did not show a genotype x environment reaction, it was clear from the interaction plots that some populations responded more to sewage than others. For instance, Walberswick had a large decrease in shoot production when grown in synthetic sewage, whilst North Warren showed virtually no change. Likewise, Lakenheath had a substantial increase in below ground biomass, whilst the Inner Tay showed a slight decrease. In designing constructed wetland systems, the source of *P. australis* could influence the success of reed establishment, and it is probably good practice to closely match *P. australis* origin to the geographical location of CWS. This procedure is not presently carried out as

provenances from North Wales (53.08° N) are supplying CWSs in the Orkney Islands (58.99° N), which is located *c.* 661 km north (Reed from Seeds, pers comm. 2004).

In CWS, insect damage to *P. australis,* such as stem bore holes, may have a deleterious effect on CWS functioning, as the aeration of the rhizosphere may be significantly reduced. Armstrong *et al.* (1996) reported that stem boring insects can appreciably reduce and sometimes prevent convective flows, which aerate the rhizosphere, because of blockage (callus) formation or leakage from the shoots. Since experiment 3 showed that there was no difference between *P. australis* populations in shoot diameter (although substantial differences were found in full-nutrient and nutrient-stressed conditions) or an effect of synthetic sewage treatment, reed should be selected from populations with shoot thicknesses that would reduce the risk of insect attack, or have a variety of thicknesses, so that not all stems would be attacked.

Chapter 7 – Investigation of intraspecific differences in sewage treatment performance of *Phragmites australis* in CWSs

7.1 INTRODUCTION

Constructed wetland systems (CWSs) were originally developed in Europe and North America about 40 years ago, as natural alternatives to technical methods of wastewater treatment (Verhoeven and Meuleman, 1998; Shutes, 2001). The principal advantages of these systems over mechanised wastewater treatment are lower cost and maintenance, as well as improved aesthetics, particularly where the systems are landscaped (Gray and Biddlestone, 1995; Luederitz, *et al.*, 2001; Vymazal, 2002). Nutrients from wastewater, such as ammoniacal nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), phosphate (P) and biological oxygen demand (BOD), are removed below ground, either biologically by microbial degradation/transformations in aerobic and anaerobic zones, or by physicochemical processes (Cooper *et al.*, 1996; Stottmeister *et al.*, 2003).

7.1.1 NH_4-N and NO_3-N removal

Removal of NH_4-N in CWSs forms part of the complex nitrogen cycle in which ammonium is removed through nitrification and denitrification. Nitrification occurs in the aerobic zone and is a two step process in which ammonium is oxidised to nitrite (NO_2^-) by chemoautotrophic bacteria and then to nitrate (NO_3^-) by facultative bacteria (Kadlec *et al.*, 2001; Werker *et al.*, 2002). Removal of nitrate (denitrification) occurs under anoxic conditions and is carried out by chemoheterotrophs that use nitrates as the terminal electron acceptor in respiration resulting in the release of nitrous oxide or nitrogen gases during this irreversible process (Cooper *et al.*, 1996). The magnitude of ammonium and nitrate removal is dependent on temperature (growth of bacteria proceeds slowly below 5 °C), pH (optimum for nitrification 7.5 - 8.6; 7 - 8 for denitrification), C source (inorganic for

nitrification, organic for denitrification), and dissolved oxygen (> 1 mg l^{-1} for nitrification; inhibitory for denitrification) (Cooper *et al.*, 1996; Kadlec *et al.*, 2001). NH_4^+, NO_2^- and NO_3^- are also incorporated in plant biomass, but this removal route is of minor importance, especially in heavily loaded CWSs, since only a small portion of total nitrogen is removed in this way (5 - 10 %) (Stottmeister *et al.*, 2003). Furthermore, nitrogen is released from plant material by leaching and litter decomposition at the onset of senescence (Meuleman *et al.*, 2002).

7.1.2 P removal

In wastewater, phosphorus is usually present in one of three forms: orthophosphate, dehydrated orthophosphate or organic phosphorus (Cooper *et al.*, 1996). Once biological oxidation occurs, most phosphorus is converted to orthophosphate ($H_2PO_4^-$ HPO_4^{2-}, PO_4^{3-}). The latter forms are then removed by adsorption, complexation and precipitation with metals (e.g. Fe, Al, Mn), or complexation and precipitation with clay minerals in the substrate (Luederitz *et al.*, 2001). Adsorption is dependent upon the presence of oxidised iron (Fe^{3+}), aluminium or calcium which are found in clay minerals or bound to soil organic matter. In the oxidised state, Fe^{3+} binds phosphate to form stable complexes in a process where phosphate displaces water or hydroxyls from the surface of iron hydrous oxide (Cooper *et al.*, 1996). However under anaerobic, reducing conditions, Fe^{2+} has less adsorption capacity, resulting in P release. Adsorption to calcium only occurs under neutral to basic conditions, and both hydrous iron and calcium oxides are subject to P saturation due to limitation of adsorption sites. In addition, the process is also reversed if the substrate redox potential (Eh) or pH is lowered (Verhoeven and Meuleman, 1999). A more permanent P removal mechanism, which is less subject to saturation, is by precipitation with iron, aluminium and calcium compounds to form insoluble metal phosphates. The processes of P adsorption and precipitation are cyclical, so precipitation of P from previous adsorption sites, releases sites for further adsorption (Verhoeven and Meuleman, 1999).

In CWSs incorporation of P into plant biomass is insignificant compared to the other routes of removal. Plant biomass becomes P-saturated in a relatively short time and,

even when the plant is harvested, only 5 - 20 % of P is removed (Luederitz *et al.*, 2001; Meuleman *et al.*, 2002). Biotic P cycling occurs through growth, death and decomposition, and most of the P accumulated in plant biomass during the growing season is returned to the system. However a significant proportion is retained in residual organic matter that is resistant to decomposition, resulting in the permanent removal of P by accretion of newly formed sediments and soils (i.e. humic substances) (Verhoeven and Meuleman, 1999; Luederitz *et al.*, 2001).

7.1.3 BOD removal

BOD is the usual measure of soluble organic matter in polluted waters (Werker *et al.*, 2002). Two mechanisms are involved in the removal of organic matter in CWSs: physical removal by deposition and filtration and a biological removal in aerobic (by chemoheterotrophic and chemoautotrophic bacteria) and anaerobic zones (by heterotrophic bacteria (facultative or obligate)) (Kadlec *et al.*, 2001). The greatest removal is by chemoheterotrophs because they have a faster metabolic rate (Cooper *et al.*, 1996). Anaerobic degradation of organic compounds is much slower than aerobic degradation and is more sensitive to pH (which affects methane-forming bacteria) (Kadlec *et al.*, 2001).

7.1.4 CWS design

The three most common designs of CWSs are horizontal-surface (HSF) and subsurface-flow systems (SSF), in which the wastewater flows horizontally over/through the wetland substrate, and vertical-flow systems (VFS), in which the wastewater flows vertically through a highly permeable substrate and is collected in drains (Shutes, 2001; Verhoeven and Meuleman, 1998). CWSs have been used to treat urban run-off (Shutes, 2001) landfill leachate (Peverley *et al.*, 1995), mine water from abandoned coal/metal mines (Ye *et al.*, 2001), agricultural run-off (Kern and Idler, 1999) and secondary and tertiary treated municipal sewage effluent (Greenway and Woolley, 1999).

7.1.5 Role of *Phragmites australis* in CWSs

While a variety of aquatic plant species are used in CWSs, the common reed (*Phragmites australis)* is favoured in Europe since it is tolerant of a wide range of climatic conditions and exhibits high productivity in both above and below ground parts (Luederitz *et al.*, 2001; Shutes, 20001).

P. australis has physiological and anatomical attributes that enable it to grow and survive in waterlogged soils (Vartapetian and Jackson, 1997). Large internal airspaces (aerenchyma) afford oxygen transport to the root and rhizome system *via* passive molecular diffusion and three types of convective flow (thermal transpiration, humidity-induced pressurization and Venturi-induced convection) (Armstrong *et al.,* 1992; Brix, 1994). In this way the respiratory demands of the rhizosphere (root zone) are met, whilst leakage from the sub apical region of young white roots creates an oxygenated film < 1 mm thick surrounding the root hairs, which supports a large population of aerobic microorganisms, such as nitrifying bacteria (Armstrong, 1979; cited in Kadlec *et al.*, 2001; Brix and Schierup, 1990; Gray and Biddlestone, 1995).

Away from the roots, anoxic conditions prevail and anaerobic microbial processes such as denitrification dominate. In addition to rhizosphere aeration and provision of a large surface area for microbial attachment, *P. australis* serves other important functions in CWSs. The rhizosphere provides a large surface area for sediment attachment (adsorption) as well as aiding filtration and prevention of clogging, especially in VFS (Brix, 1994). The large aerial parts reduce surface wind velocity in the reedbed, enhancing conditions for sedimentation, as well as preventing algal growth through light attenuation (Brix H. pers. comm. 2004). In cold climates, winter vegetation cover insulates the soil and prevents freezing (Brix, 1994). The presence of plants also provides a carbon source which is essential for microbial denitrification. However, the role of *P. australis* in CWSs varies, depending on the design (Brix, 1994). For example, prevention of clogging by *P. australis* is more important in VFS than HSF and SSF systems, whilst light attenuation, oxygenation of the rhizosphere and reduction of water velocity are more important in SFS than VFS (Brix, 1994).

7.1.6 Intraspecific variability in *P. australis*

Whilst there have been numerous studies of the effects of interspecific variability (i.e. variability between plant species) on CWS performance (Ansola *et al.*, 1995; Rai *et al.*, 1995; Peterson and Teal, 1996; Greenway and Woolley, 1999; Karathanasis *et al.*, 2003), intraspecific variability (i.e. variability within a species) and its possible effects on water treatment in CWSs is a depauperate area of research. Although research has shown *P. australis* varies in morphology, biomass and phenology (timing of events such as flowering, start of growing season and onset of senescence) between populations (Björk, 1967; Haslam, 1972; van der Toorn, 1972), this has not been considered an important factor in design or performance of CWSs. Moreover, *P. australis* used in CWSs may be sourced from distant locations and may not be adapted to local conditions. For instance a CWS located in Northern Scotland (Orkney Islands, 58.99° N, 3.01° W) was planted with *P. australis* seeds originating from North Wales (53.08° N, 4.09° W), *c.* 661 km to the south (Reeds from Seeds pers. comm. 2002).

7.1.7 Aims

This study hypothesised that the genetic variability found within *P. australis* could affect removal of nutrients in CWSs. This was tested by creating mini-CWSs using rhizomes from five British *P. australis* populations and comparing their effectiveness in treating artificial sewage (measured by efficiency in removal of NH_4-N, NO_3-N, P and BOD) over a one-year period. In addition, amongst population differences during the growing season and dormant period and over three and nine day cycles were tested for each nutrient.

7.2 MATERIALS AND METHODS

7.2.1 Plant material

P. australis rhizome cuttings were used to establish the experimental wetlands rather than seed, as complete root-rhizome development from seed may take up to five

years. In addition, a litter layer which provides a carbon source for denitrification may not occur in a 'young' wetland (Cooper *et al.*, 1996). Rhizomes were collected from five *P. australis* populations (two in Scotland: Inner Tay, St. Margaret's Marsh; and three in England: Leighton Moss, Walberswick, Strumpshaw Fen) selected to represent a range of latitudes and site sizes (for location see Chapter 2, Fig. 2.1). At each reedbed in March 2003, three - five rhizomes *c.* 15 cm in length and possessing at least one apical bud and two nodes with lateral buds, were dug up from eight sampling points which were located along line transects at least 100 m apart to minimise the risk of resampling the same clone. Samples were placed in plastic bags and stored at 4 ° C in the dark until planting.

7.2.2 Experimental design and procedure

7.2.2.1 Mini-constructed wetland system (mini-CWS)

The design of each mini-CWS was loosely based on a vertical flow system (VFS) in which the influent is applied to the reedbed in a large batch and, after percolating through the substrate, is collected by a drainage network. The reedbed is drained completely and the substrate allowed to aerate before the next application, ensuring that nitrification is unimpeded (Cooper *et al.*, 1996). In these systems the drying out period and hydraulic residence time can vary from hours to days depending on the design and loading of the system (Ansola *et al.*, 1995; Cooper *et al.*, 1996; Tanner *et al.*, 1999; Verhoeven and Meuleman, 1999; Meuleman *et al.*, 2002). In VFSs, the substrate usually consists of layers of graded material (from fine sand to coarse gravel) arranged in fixed proportions (Cooper *et al.*, 1996; Luederitz, *et al.*, 2001).

In this experiment each mini-CWS comprised a 72 l plastic container, fitted at the base with a plastic tap to allow periodic drainage of effluent, and filled with a graded substrate (Fig. 7.1). The relative depths and nature of substrate were chosen to reflect design guidelines for VFSs (Cooper *et al.*, 1996). In April 2003, eight of the collected rhizomes were planted, one from each sampling point per population, into each mini-CWS according to the protocol of Hawke and José (1996) (i.e. planted at ≈ horizontal to 45 ° angle, so that at least one bud was buried to a depth of 4 cm). In total, there were 15 mini-CWSs consisting of five populations each replicated three

times, distributed randomly amongst five blocks (because each block did not contain a replicate of each population, the experiment was not a full randomised block design and was unbalanced) in an unheated greenhouse at the University of Edinburgh, (Table 7.1). To allow vegetation establishment (both aerial and root-rhizome) and litter development, the planted rhizomes were left to grow for a year before commencement of the experiment (Kadlec *et al.*, 2001) (Figure 7.2). During this period the plants were regularly watered and fertilized. Each month 3 l of Tomorite fertilizer (4 % N, 4.5 % P, 8 % K) and seaweed extract (Levington Horticulture Ltd., Ipswich, UK) were applied to each mini-CWS. Following an aphid infestation in the first year, the mini-CWSs were treated with an insecticide solution (Intercept; Scotts Company Ltd, UK) in March 2004, to prevent further attack. In April 2004, a month before the start of the experiment, each mini-CWS was dosed with 1 l of sewage effluent obtained from the Leitholm waste water treatment works, 60 km south of Edinburgh, to establish an appropriate microbial community.

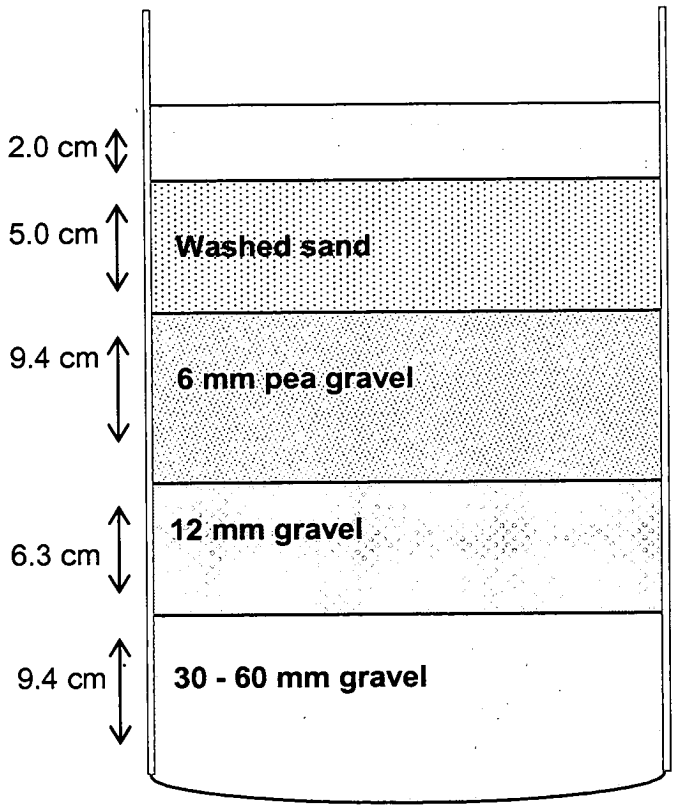


Figure 7.1 Cross section of a mini-CWS, composed of a 72 l plastic container fitted with a tap at the base and filled with a substrate of graded material (*P. australis* not shown).

Table 7.1 Layout of 15 mini-CWSs (5 populations x 3 replicates) in randomised block design.

Block 1	Block 2	Block 3	Block 4	Block 5
Leighton Moss	Inner Tay[a]	St. Margaret's	Walberswick	Strumpshaw Fen
Walberswick	Strumpshaw Fen	Leighton Moss	Leighton Moss	Walberswick
Strumpshaw Fen	Inner Tay	St. Margaret's	Inner Tay	St. Margaret's

[a], replicate was irreparably damaged, so no experimental results

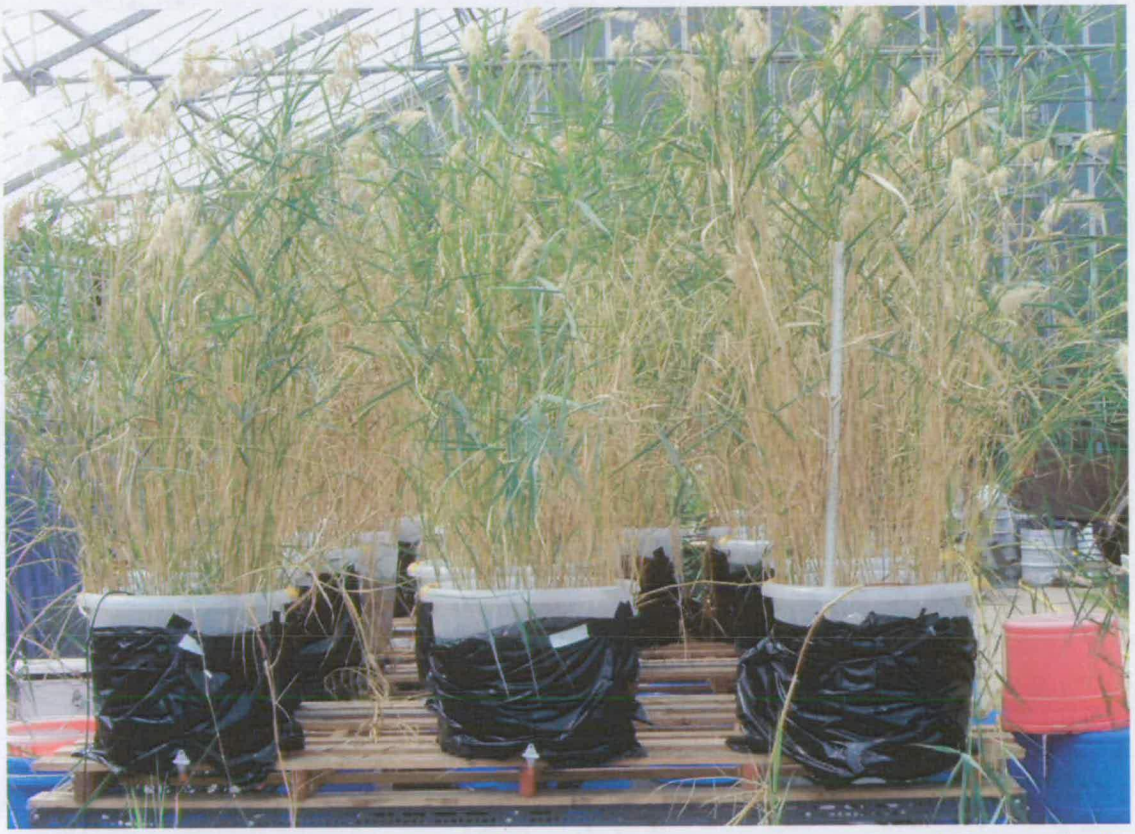


Figure 7.2 Experimental mini-CWSs after one year's growth of *P. australis*. Populations are arranged in a randomised block design in an unheated greenhouse. Height of *P. australis* indicated by metre rule in the right hand container.

The redox potential (Eh) of the mini-CWS rhizosphere was measured hourly, from May - November 2004, using OI11 combination platinum electrodes with an annular

ceramic junction and an AgCl reference system (Sentek) inserted into the substrate (*c.* 10 cm below the surface of the reedbed and in the centre) of a randomly chosen mini-CWS from each population, and connected to a Campbell CR10X data logger, To check the accuracy of the Eh measurements, the probes were placed in Zobell's solution (which should have an Eh of + 258 mV at 21 °C) (Langmuir, 1971) in the laboratory before insertion into the mini-CWSs. The deviation of these readings from the expected values was added to the Eh measurements in the mini-CWS to take account of any differences between the probes. The actual Eh was calculated by adding the standard potential of the AgCl reference (taking account of molarity of the KCl electrolyte and temperature) (Langmuir, 1971) to the adjusted Eh reading.

Prior to commencement of the experiment, the mini-CWSs were elevated 0.5 m above the ground, onto platforms, so that effluent could be drained into 40 l containers. One of the 72 l containers was irreparably damaged during the experimental set up so there were only two replicates of the Inner Tay population.

7.2.2.2 Rhizome nutrient analysis

To determine if there was a significant difference amongst populations in the nutrient content of the rhizomes, which could cause an environmental 'carry over effect', total nitrogen, phosphorus and potassium were determined in a 8 cm section of rhizome from each sampling point that had not been planted in the mini-CWSs. In total, there were 40 rhizome samples, one from each of the eight sampling points per population. The samples were oven dried at 80 °C for 48 hours, ground in a Glen Creston ball mill and then digested with 18M H_2SO_4 and 30 % w/v H_2O_2 in a heating block at 320 °C for 6 hours (Allen *et al.*, 1974). N and P concentrations and K concentration of the acid digests were determined colorimetrically using a continuous flow autoanalyser (Bran and Luebbe), and a flame atomic absorption spectrometer (Unicam M5) respectively. From statistical analysis of the results (One-way ANOVA), no significant difference amongst populations was found in total N ($F_{4,35} = 1.34$, $P = 0.276$), P ($F_{4,35} = 0.85$, $P = 0.505$) and K ($F_{4,35} = 0.17$, $P = 0.954$). The mean rhizome % (± 1 SE) nutrient content of N, P and K over all populations was 3.67 % ± 0.03, 0.18 % ± 0.01 and 1.64 % ± 0.04 respectively.

7.2.2.3 Synthetic sewage

For practical reasons it was decided to use synthetic rather than real sewage as the influent to the mini-CWS in the experiment as used by Drizo *et al.* (1997) and the Department of the Environment/National Water Council of Great Britain (DoE, 1981). Synthetic sewage was made up following the guidelines of the Department of the Environment (1981), and contains in 1 litre of tap water: 160 mg peptone, 110 mg meat extract, 30 mg urea, 7 mg sodium chloride, 4 mg calcium chloride dehydrate, 2 mg magnesium sulphate heptahydrate, and 8 mg dipotassium hydrogen phosphate. The synthetic sewage was made up in 1 l plastic bottles as concentrated solutions (200 times) which could be stored at 1 °C for up to a week before dilution.

7.2.2.4 Application of synthetic sewage

The experiment was conducted from 11 May 2004 to 14 of April 2005. During this period, each mini-CWS was periodically batch loaded with synthetic sewage (manual application) and drained. Full details of the volume and frequency of synthetic sewage application in each cycle and the total number of cycles are given in Table 7.2.

Table 7.2 Total number of applications of synthetic sewage to mini-CWSs and length of cycles from 11 May 2004 - 14 April 2005. Maximum volume of synthetic sewage applied in any one cycle and timing of application within a cycle are also shown.

Date	Cycle	Total number of cycles	Maximum volume added to each mini-CWS (l)	Days of application volume in cycle
11/05/04 - 17/08/04	14 days	6	40	Day 1, 15 l; Day 5, 10 - 15 l; Day 9, 5 - 10 l
17/08/04 - 2/11/04	9 days	3	35	Day 1, 15 l; Day 3, 5 l
	3 days	4	25	Day 1, 15 l; Day 2, 10 l
2/11/04 - 14/04/05	≥9 [a] days	9	15	Day 1, 15 l
	3 days	8	15	Day 1, 15 l

[a] = 7 x 9 day cycle; 2 x 18 day cycle

From 11 May - 2 November 2004 (during the growing period of *P. australis* and to the commencement of senescence), up to 40 l of synthetic sewage were applied over the course of each cycle, whilst from 2 November - 14 April 2005 (during the plants' dormant period) only 15 l of sewage was applied per cycle. For the first two months, a two week loading and draining cycle was followed then, from the middle of August 2004 until the end of the experiment, the synthetic sewage was applied in alternating nine and three days cycles. (It was decided to vary the length of cycles to determine if variability in hydraulic retention time (nine or three days) had an effect on nutrient removal (Headley, T. pers. comm. 2004).) The volume of synthetic sewage applied to each mini-CWS in each cycle varied during the experiment due to seasonal changes in evapotranspiration rates. At the end of each cycle, effluent from each mini-CWS was drained into the calibrated containers over night for 16 hours. The following morning, the height of the effluent in the collection containers and effluent temperature and pH, (H9023C meter, Hanna Instruments, calibrated with standards of pH 4.01 and 7.01), were measured. 25 ml of the effluent from each mini-CWS was stored in a vial at - 20 °C until analysis for ammonium, nitrate and orthophosphate and 750 ml of each effluent were analysed immediately for BOD_5. Initially, BOD_5 was measured in samples every two weeks and then usually at the end of the three day cycle. BOD_5 was not measured following a nine day cycle, due to time constraints.

In the afternoon of the same day, the next cycle started with the application of 15 l of synthetic sewage to each mini-CWS. Samples of the synthetic sewage were periodically analysed for BOD_5, NO_3-N, NH_4-N and orthophosphate (P) to determine the composition of influent.

7.2.2.5 Influent and effluent nutrient analysis

The concentrations of nitrate (NO_3-N), ammonium (NH_4-N) and phosphate (P), measured as orthophosphate, were determined colorimetrically using a continuous flow autoanalyser (Bran and Luebbe), as for the rhizome analysis. BOD_5 concentration was determined with the OxiTop® system (Wissenschaftlich-Technische Werkstätten (WTW) GmbH) in which piezoresistive electronic sensors detect changes in dissolved oxygen concentration in the sample, due to microbial

oxidation of organic compounds, as a change in pressure. Samples were first seeded with a nitrification inhibitor, N-allylthiourea (WTW), and then incubated in the dark at 20 ± 1 °C for five days.

Over the course of the experiment, 17 samples of influent were collected and the mean (± 1 SE) concentration of nutrients in the synthetic sewage applied was: BOD_5 = 194 ± 7.36 mg l^{-1}; NO_3-N = 1.31 ± 0.09 mg l^{-1}; NH_4-N = 5.59 ± 1.71 mg l^{-1}; P = 4.83 ± 0.29 mg l^{-1}. Initially, the influent ammonium concentrations were very variable (from 0.803 - 24.61 mg l^{-1}) due to use of an old batch of urea, but when new urea was used to make up the synthetic sewage the mean concentration became more constant (1.88 ± 0.30 mg l^{-1}). The variability in ammonium input was taken into account when calculating the mass applied to the mini-CWSs.

7.2.2.6 Mass balance calculations

The mass of each nutrient applied to and lost in effluent from each mini-CWS for each cycle were determined using equations 7.1 and 7.2 respectively. Percentage mass removal was determined using equation 7.3.

Influent mass of each nutrient (mg) = Volume of synthetic sewage added (l) × mean Influent nutrient concentration (mg l^{-1}) (Equation 7.1)

Effluent mass of each nutrient (mg) = Volume of Effluent drained (l) × Effluent concentration of nutrient (mg l^{-1}) (Equation 7.2)

$$\textit{Percentage mass removal of each nutrient} = \frac{\textit{Influent mass} - \textit{Effluent mass}}{\textit{Influent mass}} \times 100$$

(Equation 7.3)

In equation 7.1, the mean concentrations of NO-N, BOD_5 and P were used for all 32 cycles. As influent NH_4-N concentration varied, the measured influent concentrations were initially used for each cycle, but when the influent concentration stabilised from 12 December 2004 onwards, the mean value was used.

7.2.2.7 Evapotranspiration (E_T)

Percentage evapotranspiration for each mini-CWS per cycle was determined using equation 7.4.

$$E_T = \frac{(Volume\,of\,Influent - Volume\,of\,Effluent)}{Volume\,of\,Influent} \times 100 \qquad \text{(Equation 7.4)}$$

7.2.2.8 Above and below ground biomass

At the end of the experiment, each mini-CWS was drained and left to dry out for a week before a sub sample of above and below ground biomass was removed. Above ground biomass was harvested with scissors from a sector of approximately one eighth marked on the surface of each mini-CWS. The CWS plastic container was then removed and the below ground portion of the sector was obtained using a hack saw. Before washing the roots and rhizomes to remove substrate, the radius, height and arc length of the sector were measured, so the biomass of the whole mini-CWS could be calculated. The above and below ground biomass samples were dried in an oven at 80 °C for 72 hrs and then weighed. The total above and below ground biomass of each mini-CWS was estimated using equations 7.5 - 7.8:

$$Volume\,of\,mini\text{-}CWS = \pi \, x \, radius^2 \times height \qquad \text{(Equation 7.5)}$$

$$Volume\,of\,each\,sector = \frac{(Height \times arc\,length \times radius)}{2} \qquad \text{(Equation 7.6)}$$

$$Portion\,of\,each\,sector = \frac{Volume\,of\,each\,sector}{Volume\,of\,whole\,CWS} \qquad \text{(Equation 7.7)}$$

$$Biomass\ (above\ and\ below\ ground)\ of\ mini\text{-}CWS = Biomass\ x \frac{1}{Portion\,of\,each\,sector} \qquad \text{(Equation 7.8)}$$

7.2.3 Statistical analysis

All statistical analysis was carried out using MINITAB 14 for Windows. Each variable (% and mass NH_4-N, NO_3-N, P and BOD_5 removal, % evapotranspiration, above and below ground biomass, total biomass and above/below ground biomass ratio) was tested for normality and homoscedasticity among populations using Kolmogorov-Smirnov and Bartlett's tests, respectively (Sokal and Rohlf, 1995). As there was a significant departure from normality and homogeneity of variance for all variables except biomass, statistical analysis was conducted on the $\log_{10}$ transformed data.

To determine if there was a significant difference amongst populations in % and mass nutrient removal and % evapotranspiration, a three factor Analysis of Variance (ANOVA), incorporating Tukey's test, was performed, using the General Linear Model (GLM). The model was used to test for the main effects of block, population and date. The same model was used to test population differences for both the three and nine day cycles, and in the growth (11 May - 2 November 2004) and dormant period (2 November 2004 - 14 April 2005). Pearson's correlation was used to test for association between removal of different nutrients within populations. A One-Way ANOVA using GLM was used to test amongst population differences in biomass variables.

7.3 RESULTS

7.3.1 The system

The mean total volume of influent added to each mini-CWS population during the experimental period ranged from 669 ± 0.3 l (Inner Tay) to 633 ± 19.4 l (St. Margaret's Marsh) and the mean total volume of effluent removed varied from 328 l ± 15.6 (Strumpshaw Fen) to 281 ± 18.5 l (St. Margaret's Marsh). The mean concentrations of nutrients in the effluent for each population over time are shown in Fig. 7.3. Often the mean effluent concentrations were similar between populations for NH_4-N, NO_3-N, P and BOD_5. In general, for NH_4-N, NO_3-N and P, effluent concentrations were lower in the summer (growing period) and higher in winter (dormant period), although high effluent concentrations occurred on 14 September 2004, due to a high evapotranspiration rate (the mean % evapotranspiration of all mini-CWS for the cycle was 86 %, compared to the long term mean % rate of 40 %), coupled with an influent NH_4-N concentration of 9.9 mg l^{-1}. In winter there was more variability between populations in nutrient removal and BOD_5 concentration was generally lower than in the summer months. Within population differences in nutrient removal were variable, as illustrated in Fig. 7.4, for % NH_4-N removal of each replicate per population. For instance, one of the St. Margaret's Marsh replicates had consistently high % removal rates during the experiment (> 88 %), whilst the other two replicates have low removal rates predominantly from 10 December 2004 to 31 March 2005 (between - 203 and 7 %)

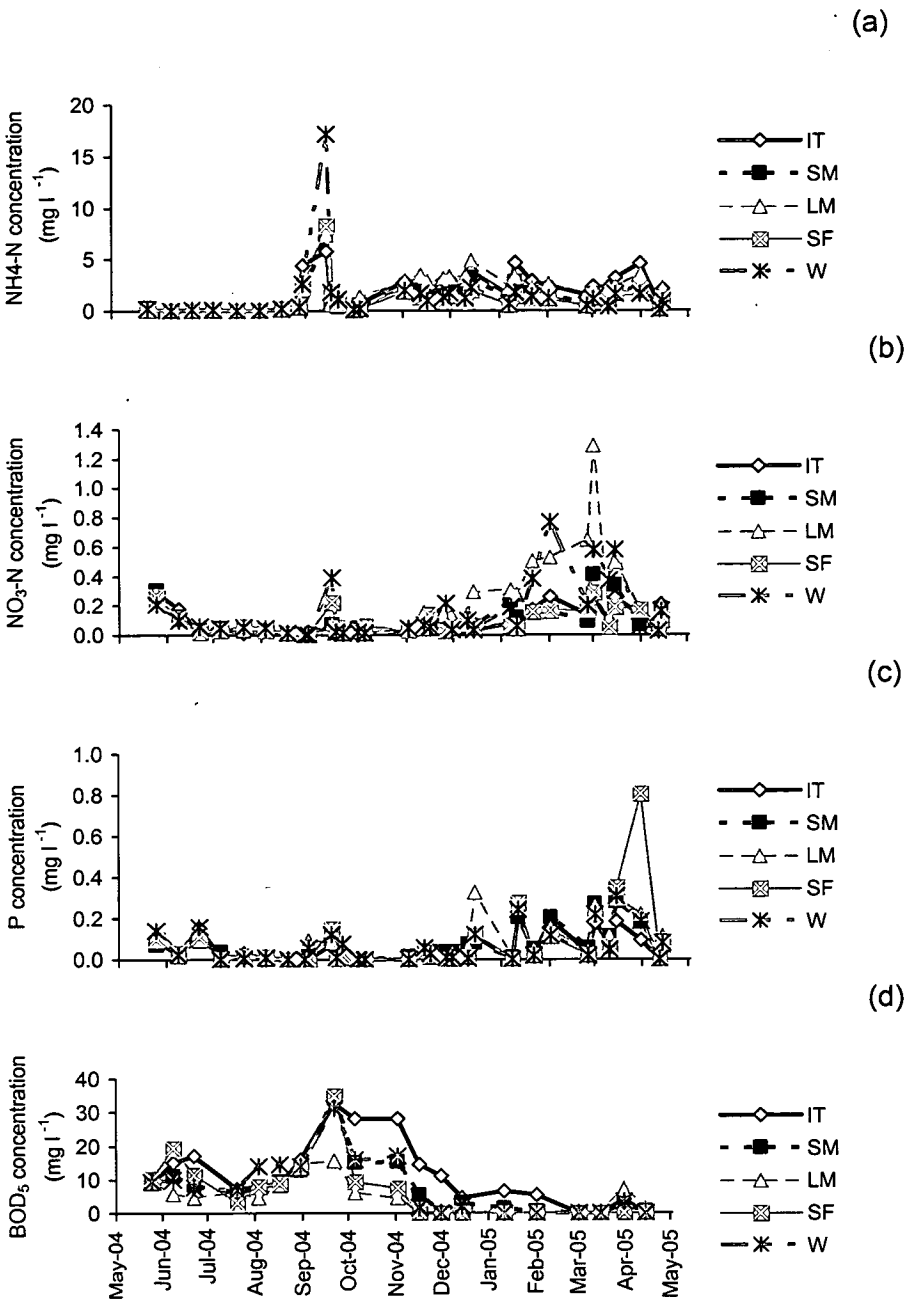


Figure 7.3 Mean nutrient concentrations in effluent from each population, sampled after every cycle (32 cycles for (a) NH_4-N, (b) NO_3-N and (c) P; and 19 cycles for (d) BOD_5), 11 May 2004 - 14 April 2005. Population abbreviations given in Table 2.1.

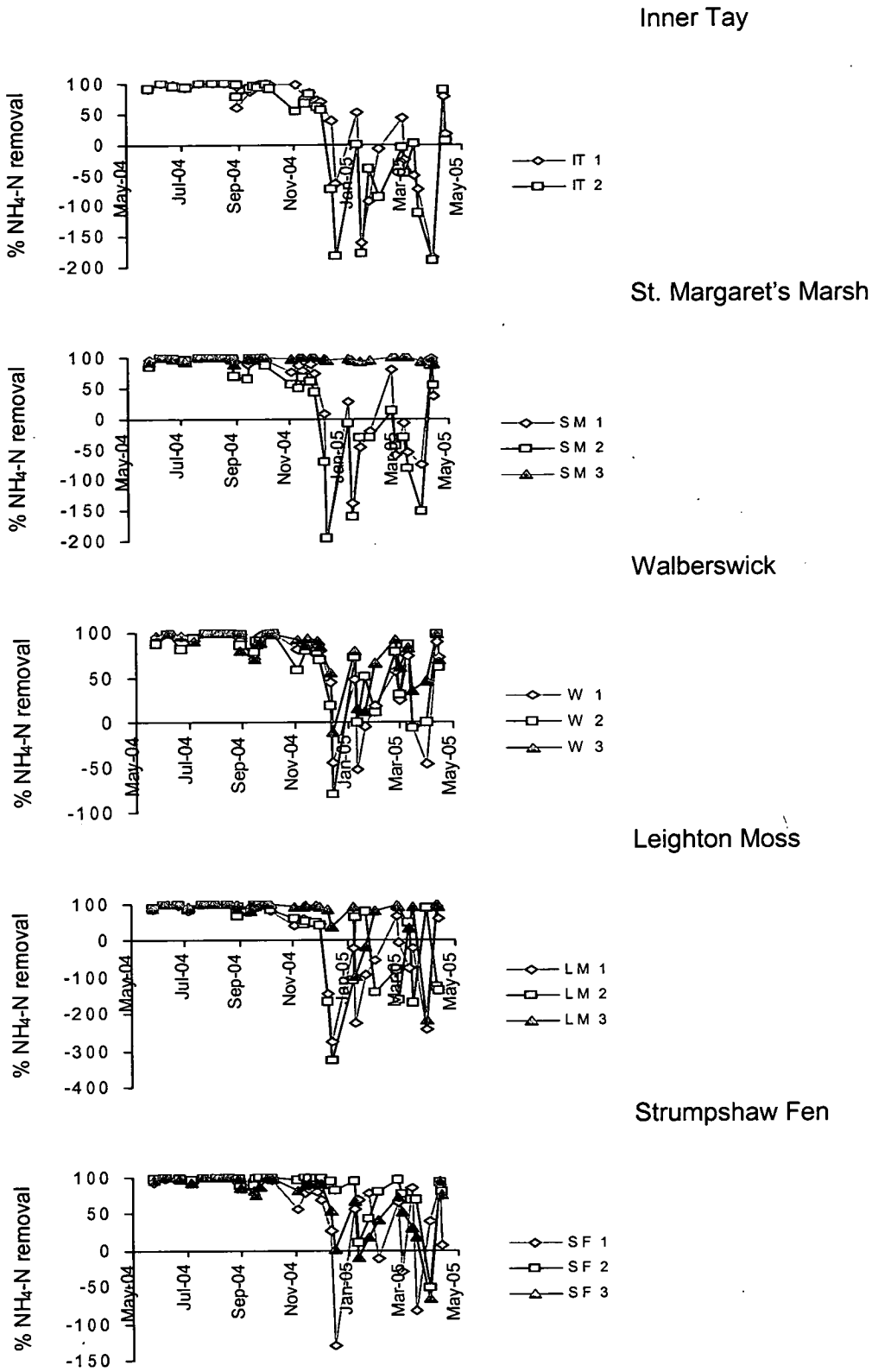


Figure 7.4 % NH_4-N removal for each replicate per population (11 May 2004 - 14 April 2005): Inner Tay, St. Margaret's Marsh, Walberswick, Leighton Moss and Strumpshaw Fen.

During each cycle, Eh in the substrate of the CWSs of the five populations was < - 100 mV and reducing conditions prevailed. Only when each mini-CWS was drained and refilled was a positive Eh recorded (up to + 680 mV). The Ehs measured hourly during a typical nine day cycle, which included drainage and refill, is shown in Fig. 7.5. After drainage and refill, reducing conditions returned within 12-20 hours.

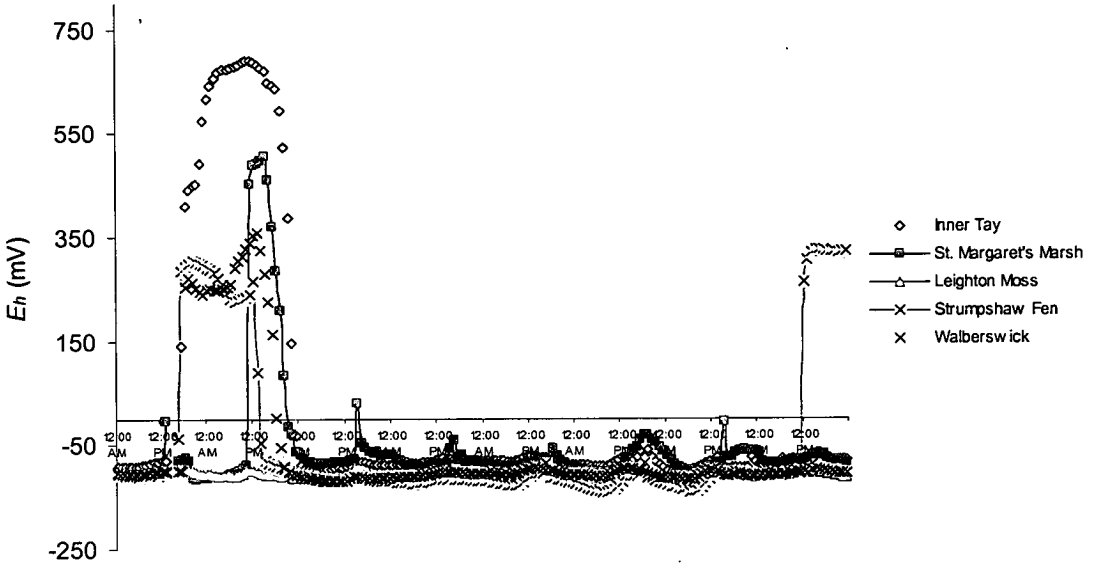


Figure 7.5 Redox potential measured every hour in each of the five populations in the mini-CWSs, 16 - 23 August 2004.

The constant negative Eh values (< - 90 mV) measured in the Leighton Moss CWS in this cycle, even after drainage/refill, were attributable to a shorter than usual drainage time (5 hours, rather than over night). Eh displayed a diurnal cycle in some populations (St. Margaret's Marsh and Strumpshaw Fen), with less negative Ehs occurring in the afternoon, possibly due to the peak of photosynthetic activity. During this time leaf stomata are open and oxygen enters the internal air spaces of the plant, through convective and diffusive flows, resulting in oxygen leakage from the roots. In addition, Eh is temperature dependent therefore the less negative readings recorded in the two populations may also have arisen due to high afternoon temperatures in the mini CWSs. For example on the 18 August at 3 pm the temperature in one of the mini CWSs was 26 °C.

Although air temperature was not measured during the experiment an indication of temperature variation over the year is given by the mean monthly temperature of the effluent after overnight drainage. The highest mean monthly temperature occurred in August 2004 (15 ± 0.2 °C) and the lowest in March 2005 (5.5 ± 0.05 °C).

The mean effluent pH varied between 6.8 ± 0.03 - 7.1 ± 0.05 among the five populations and there was no significant difference between populations ($F_{4,4,201}$ = 1.28; $P > 0.05$).

7.3.2 NH_4-N removal

During the total experimental period (11 May 2004 - 14 April 2005), there was a highly significant difference amongst populations in % NH_4-N removal ($P < 0.001$) and mass NH_4-N removal ($P < 0.001$) (Table 7.3).

Table 7.3 Three-way ANOVA of % and mass removal of NH_4-N and % NH_4-N removal over 3 and 9 day cycles and growing and dormant period, amongst populations. Degrees of freedom in parentheses.

Source of variance	MS % NH_4-N removal (Over all experiment)	MS Mass NH_4-N removal (Over all experiment)	MS % NH_4-N (3 day cycle)	MS % NH_4-N (9 day cycle)	MS % NH_4-N (growing)	MS % NH_4-N (dormant)
Block	0.0673*** (4)	0.0315*** (4)	0.0770*** (4)	0.0196** (4)	0.0001^{ns} (4)	0.1201*** (4)
Pop	0.0434*** (4)	0.0078*** (4)	0.0325* (4)	0.0237** (4)	0.0001^{ns} (4)	0.0784*** (4)
Date	0.0916*** (31)	0.5247*** (31)	0.1513*** (11)	0.0635*** (13)	0.0008*** (14)	0.1105*** (16)
Error	0.0074 (408)	0.0014 (408)	0.0130 (148)	0.0052 (174)	0.0093 (187)	0.0119 (213)

ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Percentage and mass removal of NH_4-N were significantly affected by date ($P <$ 0.001) (Fig. 7.6). There was also a block effect, which may have arisen due to the position of the mini-CWSs in the greenhouse since a Tukey's test showed that the outside blocks (one and five) had significantly higher removal rates than block three which was located in the centre.

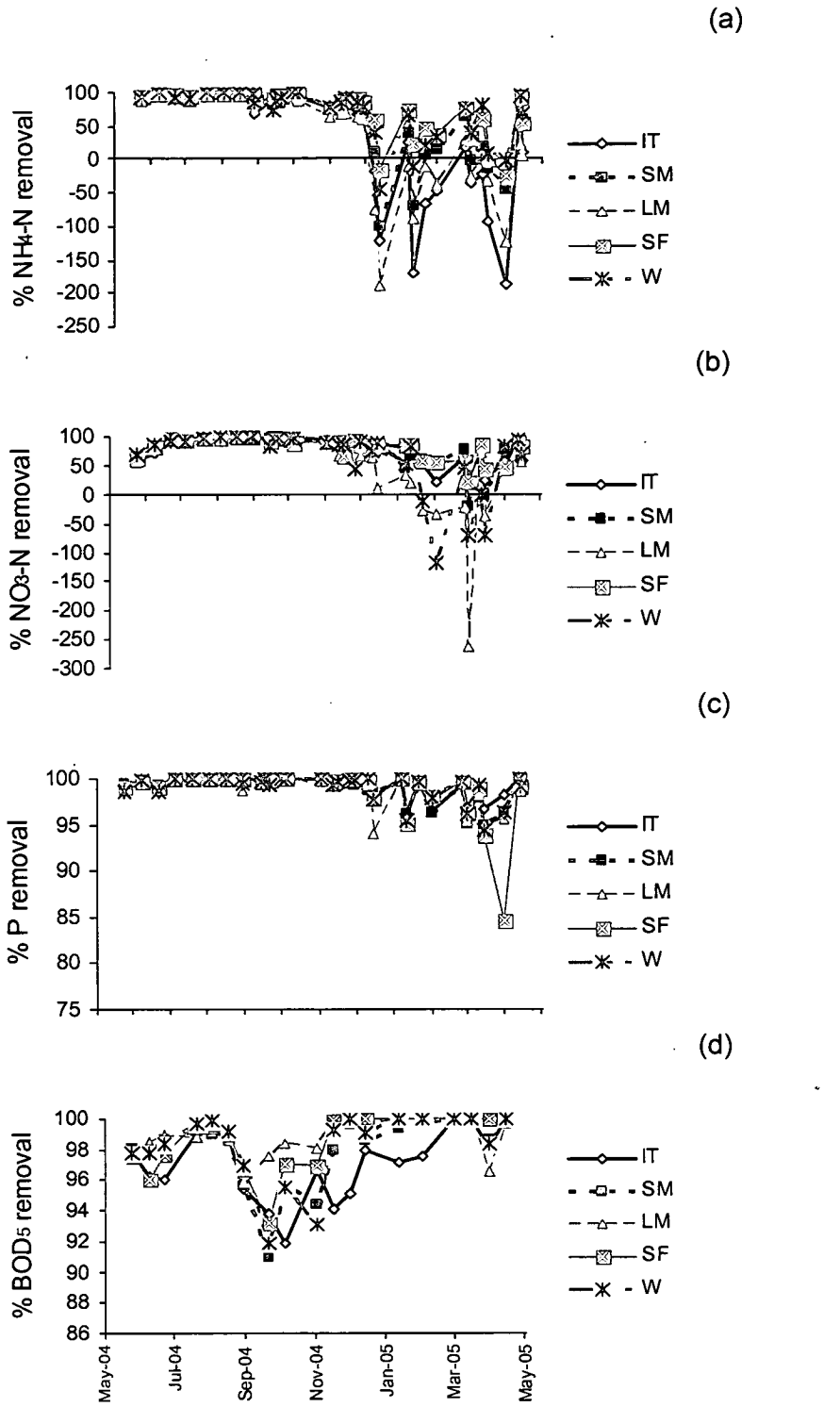


Figure 7.6 Mean % nutrient removal for each population over time 11 May 2004 - 14 April 2005: (a) NH_4-N; (b) NO_3-N; (c) P (y axis starts at 75 %); (d) BOD_5 (y axis starts at 86 %). Population abbreviations given in Table 2.1.

Removal rates amongst populations varied by up to *c.* 40 %, with Strumpshaw Fen recording the highest removal rate over the year (71.7 ± 4.50 %), whilst the Inner Tay had the lowest (33.1 ± 10.8 %). In general the northern populations had lower NH_4-N removal rates than the southern populations (Fig. 7.7). The lowest removal rates occurred during the dormant period from November 2004 - March 2005 (Fig. 7.6). During this time, the % NH_4-N removal was sometimes negative as the effluent concentration was greater than the influent.

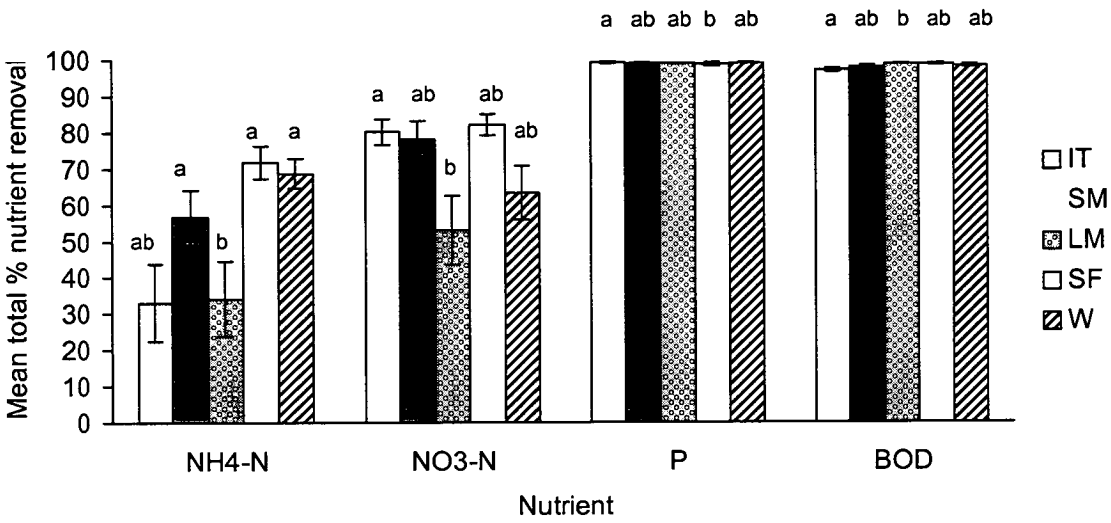


Figure 7.7 Mean total % nutrient removal by population over the experimental period 11 May 2004 - 14 April 2005. Populations arranged in order of decreasing latitude. Populations with the same letter are not significantly different for that particular nutrient. Error bars are ± 1 S. E. Population abbreviations given in Table 2.1.

In the nine and three day cycles from 17 August 2004 - 14 April 2005 there was a significant difference amongst populations in % NH_4-N removal over the nine day ($P < 0.01$), and three day cycles ($P < 0.05$) (Table 7.3). Once again there was a significant effect of date and block for both of these cycles. In the nine day cycle, % NH_4-N removal ranged from 78.6 ± 4.57 % for Strumpshaw Fen to 43.6 ± 11.7 % for Leighton Moss (Table 7.4). Removal rates in the three day cycle were lower, but the variability was similar to the nine day cycle. Strumpshaw Fen again had the highest removal rate (60.1 ± 9.03 %) whilst Inner Tay had the lowest (13.9 ± 19.5 %). A Tukey's test showed that St. Margaret's Marsh was significantly higher than Leighton

Moss during the three day cycle, whilst during the nine day cycle Leighton Moss was significantly lower than all populations except Inner Tay.

Table 7.4 Mean % NH_4-N removal for 9 and 3 day cycles and growing (11 May 2004 - 2 November 2004) and dormant periods (2 November 2004 - 14 April 2005). Errors are ± 1 S.E. Population abbreviations are given in Table 2.1

Population	% NH_4-N removal period			
	9 days	3 days	Growing period	Dormant period
IT	44.5 ± 12.5	13.9 ± 19.5	91.8 ± 2.10	- 25.7 ± 15.6
SM	66.6 ± 7.44	40.1 ± 15.3	93.5 ± 1.66	19.9 ± 12.8
LM	43.6 ± 11.7	18.4 ± 19.5	89.7 ± 2.03	- 21.4 ± 17.3
SF	78.6 ± 4.57	60.1 ± 9.03	94.5 ± 1.23	48.9 ± 7.62
W	76.4 ± 4.16	55.8 ± 8.05	92.2 ± 1.37	45.1 ± 6.49

There was no significant difference amongst populations in % NH_4-N removal over the growing period ($P > 0.05$), but there was a significant difference over the dormant period ($P < 0.001$) (Table 7.3). Once again, there was an effect of date for both periods, and an effect of block in the dormant period. All populations had high removal rates (90 - 95 %) in the growing period. During the dormant period % removal was much lower and ranged from 48.9 ± 7.62 % removal for Strumpshaw Fen to - 25.7 ± 15.6 % for Inner Tay (Table 7.4). A Tukey's test showed that St. Margaret's had significantly higher removal rates than Inner Tay and Leighton Moss during the dormant period.

7.3.3 NO_3-N removal

During the total experimental period there was a marginal significant difference amongst populations in % mass NO_3-N removal ($P = 0.081$) and mass NO_3-N removal ($P = 0.0581$) (Table 7.5). Percentage and mass removal of NO_3-N were significantly affected by date (both $P < 0.001$) (Table 7.5). There was also a block effect for mass removal of NO_3-N only, which may again have arisen due to position

of the mini-CWSs in the greenhouse. A Tukey's test showed that the inside blocks three and four, had significantly lower removal rates than the outside block five.

Table 7.5 Three-way ANOVA of % and mass removal of NO_3-N over 3 and 9 day cycles and growing and dormant period, amongst populations. Degrees of freedom in parentheses.

Source of variance	MS % NO_3-N removal (Over all experiment)	MS Mass NO_3-N removal (Over all experiment)	MS % NO_3-N (3 day cycle)	MS % NO_3-N (9 day cycle)	MS % NO_3-N (growing)	MS % NO_3-N (dormant)
Block	0.0104^{ns} (4)	0.0005^{**} (4)	0.0160^{ns} (4)	0.0026^{ns} (4)	0.000010^{ns} (4)	0.0191^{ns} (4)
Pop	0.0098† (4)	0.0002† (4)	0.0156^{ns} (4)	0.0035†	0.000005^{ns} (4)	0.0186† (4)
Date	0.0220^{***} (31)	0.0025^{***} (31)	0.04768^{***} (11)	0.0090^{***} (13)	0.000500^{***} (14)	0.0326^{***} (16)
Error	0.0047 (408)	0.0001 (408)	0.0105 (148)	0.0016 (174)	0.000011 (187)	0.0085 (213)

ns, not significant, † marginally significant ($P < 0.08$), $^{**}P < 0.01$, $^{***}P < 0.001$

Removal rates varied amongst populations with Strumpshaw Fen once again recording the highest NO_3-N removal rate over the year (82.0 ± 2.87 %), whilst Leighton Moss (which also recorded the second lowest % NH_4-N removal) recorded the lowest removal rate (53.0 ± 9.54 %) (Fig. 7.7). Unlike % NH_4-N removal, northern populations did not appear to have lower removal rates than the southern populations. As with % NH_4-N removal, lowest removal rates occurred during the dormant period from November 2004 - March 2005 (Fig.7.6). During this time the % NO_3-N removal was sometimes negative as the concentration in the effluent was greater than the influent.

There was no significant difference amongst populations in % NO_3-N removal over the three day cycle ($P = 0.239$), and a marginal population difference over the nine day cycle ($P = 0.076$) (Table 7.5). Once again NO_3-N removal was affected by date. The highest % removal during the nine day cycle was for Strumpshaw Fen (83.6 ± 3.28 %), and the lowest was recorded for Walberswick (63.5 ± 9.30 %) (Table 7.6).

There was no significant difference amongst populations in % NO_3-N removal over the growing period ($P > 0.05$), but there was a marginal significant difference over the dormant period ($P = 0.073$) (Table 7.4). Once again, there was an effect of date during both periods but no block effect. In the growing period mean removal rates were consistently high (> 90 %) for all populations, but during the dormant period removal rates were lower and more variable between populations, % removal varied from 71.0 ± 5.10 % for Strumpshaw Fen to 15.4 ± 17.4 % for Leighton Moss (Table 7.6). A Tukey's test showed that the removal rate from the Inner Tay was significantly higher than Leighton Moss during the dormant period.

Table 7.6 Mean % NO_3-N removal for 9 and 3 day cycles and growing (11 May 2004 - 2 November 2004) and dormant periods (2 November 2004 - 14 April 2005). Errors are ± 1 S.E. Population abbreviations are given in Table 2.1.

Population	% NO_3-N removal period			
	9 days	3 days	Growing period	Dormant period
IT	81.1 ± 3.96	78.5 ± 6.94	92.4 ± 2.05	67.8 ± 6.13
SM	83.2 ± 3.03	69.9 ± 11.9	92.6 ± 1.73	63.8 ± 9.13
LM	64.1 ± 7.38	34.6 ± 22.2	90.7 ± 2.15	15.4 ± 17.4
SF	83.6 ± 3.28	79.3 ± 5.38	93.1 ± 1.43	71.0 ± 5.10
W	63.5 ± 9.30	63.1 ± 12.3	92.8 ± 1.32	34.0 ± 13.5

7.3.4 P removal

During the total experimental period there was a marginal significant difference amongst populations in % P removal ($P = 0.057$) and a highly significant difference in mass P removal ($P < 0.001$) (Table 7.7). Percentage and mass removal of P were significantly affected by date ($P < 0.001$) (Fig.7.6). There was also a block effect, which may have arisen due to position of the mini-CWSs in the greenhouse.

Although there was a marginally significant difference in % removal amongst populations, all populations performed well with each having a mean % P removal over the experimental period of > 98 %. Although % P removal was lower during the dormant period, all populations maintained > 90% removal rates during this period,

apart from Strumpshaw Fen in March 2005 (Fig. 7.6). A Tukey's test showed that Inner Tay had significantly higher removal rates than Strumpshaw Fen and Walberswick during the dormant period.

Although there was a significant difference amongst populations in % P removal over the three day ($P < 0.01$) and the nine day cycles ($P < 0.05$) (Table 7.7), removal rates only varied from 98.0 ± 0.48 % at Leighton Moss to 98.8 ± 0.39 % for Inner Tay (Table 7.8). Once again P removal was affected by date and there was also a random block effect for the three day cycle.

Table 7.7 Three-way ANOVA of % and mass removal of P and % P removal over 3 and 9 day cycles and growing and dormant periods, amongst populations. Degrees of freedom in parentheses.

Source of variance	MS % P removal (Over all experiment)	MS Mass P removal (Over all experiment)	MS % P (3 day cycle)	MS % P (9 day cycle)	MS % P (growing)	MS % P (dormant)
Block	0.0010*** (4)	0.0059*** (4)	0.00056*** (4)	0.0007† (4)	0.000006* (4)	0.0018*** (4)
Pop	0.0003† (4)	0.0039*** (4)	0.00106** (4)	0.0003* (4)	0.000002^{ns} (4)	0.0006† (4)
Date	0.0008*** (31)	0.3065*** (31)	0.00018*** (11)	0.0010*** (13)	0.000030*** (14)	0.0013*** (16)
Error	0.0001 (408)	0.0007 (408)	0.00005 (148)	0.0003 (174)	0.000002 (187)	0.0003 (213)

ns not significant, † marginally significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

There was no significant difference amongst populations in % P removal during the growing period ($P = 0.385$) but there was a marginally significant difference amongst populations during the dormant period ($P = 0.06$) (Table 7.7), although during the latter period % P removal rates only varied from 97.7 ± 0.95 % for Strumpshaw Fen to 98.6 ± 0.30 % for Inner Tay. Once again % P removal was affected by date and block.

Table 7.8 Mean % P removal for 9 and 3 day cycles and growing (11 May 2004 - 2 November 2004) and dormant period (2 November 2004 - 14 April 2005). Errors are ± 1 S.E. Population abbreviations are given in Table 2.1

Population	% P removal period			
	9 days	3 days	Growing period	Dormant period
IT	99.4 ± 0.18	98.8 ± 0.43	99.8 ± 0.07	98.6 ± 0.35
SM	99.0 ± 0.24	98.5 ± 0.39	99.8 ± 0.05	98.1 ± 0.30
LM	99.2 ± 0.24	98.0 ± 0.48	99.7 ± 0.07	97.9 ± 0.39
SF	98.6 ± 1.04	98.5 ± 0.46	99.8 ± 0.05	97.7 ± 0.95
W	99.4 ± 0.19	98.4 ± 0.36	99.7 ± 0.08	98.4 ± 0.30

7.3.5 BOD removal

There was a highly significant difference amongst populations in % BOD_5 removal and mass BOD_5 removal (both $P < 0.01$) (Table 7.9). Similar to the removal of the other nutrients, % and mass removal of BOD_5 were significantly affected by date ($P < 0.001$).

Table 7.9 Three-way ANOVA of % and mass removal of BOD_5 and % BOD_5 removal over growing and dormant period, amongst populations.

Source of variance	MS % BOD_5 removal (Over all experiment)	MS Mass BOD_5 removal (Over all experiment)	MS % BOD_5 (growing)	MS % BOD_5 (dormant)
Block	0.0002* (4)	0.0010^{ns} (4)	0.0001^{ns} (4)	0.00014* (4)
Population	0.0003** (4)	0.0025** (4)	0.0001^{ns} (4)	0.00024*** (4)
Date	0.0009*** (18)	0.3125*** (18)	0.0011*** (8)	0.00010* (9)
Error	0.0001 (239)	0.0006 (239)	0.0001 (109)	0.00004 (122)

ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Although the difference in % removal amongst populations was significant, all populations performed well, with each having mean % BOD_5 removal rates > 97 % over the whole experiment (Fig. 7.7). The difference between the highest removal rate (Leighton Moss, 98.8 ± 0.21 %) and the lowest (Inner Tay, 97.1 ± 0.51 %) was negligible. Unlike the other nutrients, the highest BOD_5 removal rates in each population occurred during the dormant period although the Inner Tay had lower removal rates than the other populations (Table 7.10). A Tukey's test showed that removal rates for the Inner Tay was significantly lower than for Leighton Moss.

Table 7.10 Mean % BOD_5 removal during growing (11 May 2004 - 2 November 2004) and dormant period (2 November 2004 - 14 April 2005). Errors are ± 1 S.E. Population abbreviations are given in Table 2.1.

Population	% BOD_5 removal period	
	Growing period	Dormant period
IT	96.6 ± 0.69	97.8 ± 0.76
SM	96.9 ± 0.55	99.4 ± 0.23
LM	98.2 ± 0.27	99.5 ± 0.27
SF	97.5 ± 0.40	99.9 ± 0.01
W	97.1 ± 0.66	99.6 ± 0.21

There was no significant difference amongst populations in % BOD_5 removal during the growing period ($P > 0.05$) but a highly significant difference occurred during the dormant period ($P < 0.001$). In the latter period mean % removal rate ranged from 100 ± 0.01 for Strumpshaw Fen to 97.8 ± 0.76 % for Inner Tay (Table 7.10), with a Tukey's test showing that the removal rate for the Inner Tay population was significantly lower than for Strumpshaw Fen. BOD_5 removal rate was affected by date for both periods and there was a block effect in the dormant period.

7.3.6 Correlation among nutrient removal, within populations

Correlation coefficients between nutrients were calculated to see if the timing of removal of different nutrients was similar. There was a significant positive correlation between % NH_4-N *vs* NO_3-N removal amongst four out of five populations (Table 7.11). There was also a highly significant positive association for % P *vs* % NH_4-N and NO_3-N removal for all populations. Only one out of five populations had a significant negative correlation for % BOD_5 *vs* % NH_4-N, NO_3 and P removal.

Table 7.11 Correlation coefficient (*r*) of % nutrient removal (NH_4-N *vs* NO_3-N; NH_4-N *vs* P; NH_4-N *vs* BOD_5; NO_3-N *vs* P; NO_3 *vs* BOD_5; P *vs* BOD_5) within populations during experimental period 11 May 2004 - 14 April 2004. Population abbreviations are given in Table 2.1.

Population	NH_4-N *vs* NO_3-N	NH_4-N *vs* P	NH_4-N *vs* BOD_5	NO_3-N *vs* P	NO_3-N *vs* BOD_5	P *vs* BOD_5
IT	0.499**	0.749***	- 0.316^{ns}	0.772***	- 0.506*	- 0.408^{ns}
SM	0.397*	0.688***	- 0.315^{ns}	0.702***	- 0.424^{ns}	- 0.477*
LM	0.261^{ns}	0.815***	- 0.176	0.524**	- 0.328^{ns}	- 0.247^{ns}
SF	0.607***	0.736***	- 0.480*	0.536**	- 0.405^{ns}	- 0.314^{ns}
W	0.375*	0.732***	- 0.301^{ns}	0.505**	- 0.327^{ns}	- 0.343^{ns}

ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

7.3.7 Evapotranspiration (E_T) from mini-CWSs

There was no significant difference amongst populations in % E_T from the mini-CWSs ($P > 0.05$) (Table 7.12). E_T was affected by date (Fig 7.8) and block (position in greenhouse). Mean % E_T removal during the whole experiment ranged from 43.3 ± 2.58 % (Strumpshaw Fen) to 38.3 ± 2.55 % (St. Margaret's Marsh). The highest E_T rates occurred on 17 August 2004 and 14 September 2004, whilst the lowest occurred from November 2004 - March 2005 (Fig. 7.8).

Table 7.12 Three-way ANOVA of % evapotranspiration (E_T) during total experiment and over growing (11 May 2004 - 2 November 2004) and dormant periods (2 November 2004 - 14 April 2005) amongst populations. Degrees of freedom shown in parentheses

Source of variance	MS % E_T (Over all experiment)	MS % E_T (growing)	MS % E_T (dormant)
Block	0.094*** (4)	0.021† (4)	0.103** (4)
Population	0.017^{ns} (4)	0.012^{ns} (4)	0.054† (4)
Date	1.340*** (31)	0.307*** (14)	0.845*** (16)
Error	0.018 (408)	0.009 (187)	0.024 (213)

ns not significant, † marginally significant $P < 0.08$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

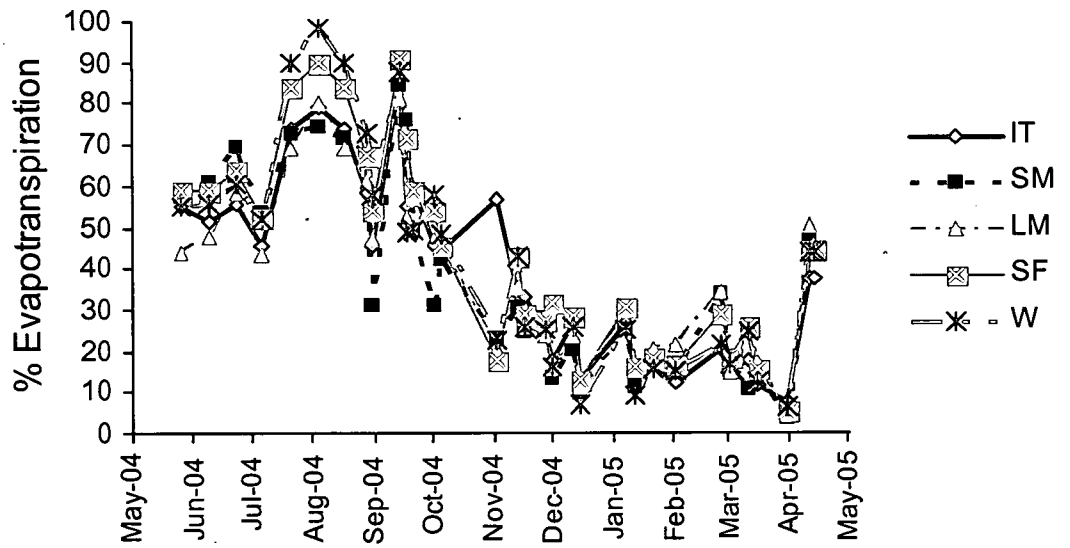


Figure 7.8 Mean % evapotranspiration for each population, from 11 May 2004 - 14 April 2005. Population abbreviations given in Table 2.1

There was no significant difference amongst populations in % E_T during the growing period ($P > 0.05$) with populations having a mean % E_T between 54 – 63 %, but there was a marginally significant difference amongst populations during the dormant period ($P = 0.067$) (Table 7.12). The highest % E_T in the dormant period was again recorded for Strumpshaw Fen (25.52 ± 1.68 %) whilst the lowest was for St. Margaret's Marsh (21.47 ± 1.73 %). A Tukey's test showed no significant difference between the populations. E_T was affected by date for both periods and there was a positional effect of block. A Tukey's test showed that the central block had a lower evapotranspiration rate than the other blocks.

7.3.8 *P. australis* biomass differences amongst populations

There was no significant among population differences in above ground biomass, below ground biomass, total biomass, and above/below ground biomass ratio (all cases, $P > 0.05$) (Table 7.13). There was great variability of biomass measurements within populations which was reflected in the high standard error values (Table 7.14).

Table 7.13 One way ANOVA of above ground biomass, below ground biomass, total biomass and above/below ground biomass ratio amongst populations

Source of variance	d.f.	MS Above ground biomass	MS Below ground biomass	MS Total biomass	MS Above/below ratio
Block	4	57350^{ns}	63586^{ns}	117218^{ns}	0.06663^{ns}
Population	4	60675^{ns}	22200^{ns}	92939^{ns}	0.04316^{ns}
Error	13	61395	91811	205929	0.05327

ns not significant

Table 7.14 Mean population above and below ground biomass m^{-2}, total biomass m^{-2} and above/below ground biomass ratio. Errors are ± 1 SE. Population abbreviations given in Table 2.1.

Population	Mean above ground biomass (g m^{-2})	Mean below ground biomass (g m^{-2})	Total biomass (g m^{-2})	Above/below ratio
IT	2153 ± 376	2830 ± 249	4984 ± 625	0.75 ± 0.07
SM	2091 ± 169	2490 ± 598	4582 ± 588	0.93 ± 0.20
LM	1939 ± 429	2310 ± 351	4249 ± 761	0.82 ± 0.09
SF	1994 ± 315	2869 ± 71	4863 ± 261	0.70 ± 0.13
W	2494 ± 213	2570 ± 179	5064 ± 73	0.99 ± 0.15

7.4 DISCUSSION

7.4.1 NH_4-N removal

In this study the mean percentage mass removal of NH_4-N from the five mini-CWS populations ranged from 33 - 72 % and was in accordance with the literature (Table 7.15)

The substantial and significant differences in percentage mass removal of NH_4-N between populations for the whole experiment and also for the three and nine day cycles and the dormant period could have arisen from population differences in morphology, phenology and/or physiology. For instance, differences in magnitude of oxygen release into the rhizosphere caused by plant structure variability may affect nitrification and hence NH_4-N removal. Many authors acknowledge that oxygen leakage from the roots creates aerobic zones in CWSs (Armstrong *et al.*, 1992; Sorrell and Armstrong, 1994; Münch *et al.*, 2005) which are essential for nitrification.

Table 7.15 Mean % NH_4-N and NO_3-N removal reported in this study (in bold) and other studies, calculated from mass or concentration, in horizontal surface (HSF), horizontal subsurface (HSS) or vertical flow (VFS) wetlands planted with *P. australis* or other species.

Nutrient and location	Mean % removal	Type of wastewater	CWS design	Planted with	Area of bed (m^2)	Reference
NH_4-N						
Unheated greenhouse	**33 - 72 (Mass)**	Synthetic sewage	VFS	*P. australis*	0.17	This study
Czech Republic	84.3 (Conc)	Domestic or municipal	HSFs	*P. australis*	18 - 4493	Vymazal, 2002
Laboratory	50 (Conc)	Primary treated sewage	SSF Microcosms	*Pennisetum purpureum*	0.24	Yang *et al.*, 2001
Unheated Greenhouse	100 (Mass)	Synthetic sewage	HSF Laboratory - scale	*P. australis*	0.49	Drizo *et al.*, 1997
Queensland, Australia	73 (Mass) 33 (Mass)	Secondary sewage effluent	Constructed free water surface wetlands	*P. australis* and other native plant species	- -	Greenway and Woolley, 1999
Austria	82 (Conc)	Domestic sewage.	VFS 4 beds, operated in parallel or as two pairs	*P. australis*	400 (each bed)	Kadlec *et al.*, 2001
NO_3-N						
Unheated greenhouse	**53 - 82 (Mass)**	Synthetic sewage	VFS	*P. australis*	0.17	This study
Florida, USA	94 (Mass)	Animal	Constructed free water surface wetlands	*Panicum hemitomon, Pontederia cordata and Nymphaea odorata* dominant spp.	750	Carr and Rushton; 1995; cited in Kadlec, 2001
Germany	96 (Conc) 67 (Conc)	Domestic and agricultural	HSF (2 beds) VFS (2 beds)	*P. australis*	64 70	Kern and Idler, 1999
Austria	0 - 80 (Conc) (mean 25)	Domestic sewage	VFS (4 beds)	*P. australis*	149 (each bed)	Kadlec *et al.*, 2001

Weißner *et al.* (2002) found a positive correlation between oxygen release rates and shoot size and above ground biomass in *Juncus effusus* and *Typhus latifolia* but no correlation with total root biomass, presumably because only the youngest white roots, just behind the apical tip, are involved in oxygen release (Sorrell B. pers. comm. 2004).

However some authors have expressed doubts concerning the importance of oxygen root leakage, since the quantities recorded in some experiments have been extremely low (Bedford *et al.*, 1991; Gries *et al.*, 1990), although Sorrell and Armstrong (1994) have suggested that the methodology employed (i.e. bathing the roots in oxygen depleted solutions) may underestimate the oxygen transport potential of aquatic plants. In this experiment differences in above ground biomass between populations were found, with Leighton Moss having the lowest and Walberswick the largest above ground biomass. Although the differences were not significant, this may be attributed to small sample size ($n = 3$ for all populations, apart from Inner Tay $n = 2$) and the fact that the roots were 'pot bound' after two seasons' growth in the 72 l containers. The CGE (Chapter 6), which included the five populations here, found significant differences in above ground biomass between ten British populations of *P. australis,* with the lowest occurring for Leighton Moss when grown in full-nutrient conditions. Therefore, in this study, the low NH_4-N removal rate for Leighton Moss may be explained by low above ground productivity, which had a quantitative effect on root oxygen leakage.

In VFSs (the wetland design used in this experiment) the substrate is aerated during the draining and refilling cycle, so the plant's role in supplying oxygen may be less significant (Brix and Schierup, 1990). However, the hydraulic retention time in the mini-CWSs in this study was longer than in most VFSs (Gray and Biddlestone, 1995; García *et al.*, 2003; Meuleman *et al.*, 2002) and ranged mostly from three to nine days (there were also eight cycles ≥two weeks). Stottmeister *et al.* (2003) stated that the hydraulic retention time, including the length of time that the water is in contact with the plant roots, affects the extent to which the plant plays a significant role in the removal or breakdown of pollutants. In this experiment highly significant differences between populations were found for % NH_4-N removal for both the nine and three

day cycles and more NH_4-N was removed during the nine day (54 ± 8 % removal over all populations) compared to the three day cycle (38 ± 10 % removal over all populations). This suggests that the plants played a significant role in NH_4-N removal and that the variability in removal was attributable to population differences.

Population differences in below ground biomass may have affected % NH_4-N removal in the mini-CWSs because the size of the microbial community is dependent on the surface area available for attachment as well as the amount of oxygen released (for aerobic bacteria). Leighton Moss had the lowest below ground biomass, so there would be a smaller surface area available for microbial attachment (and temporary adsorption of NH_4^+) compared to the other mini-CWSs, although population differences in below ground biomass were not significant. However, the CGE (Chapter 6) found significant differences in below ground biomass between ten populations of *P. australis* with the second lowest below ground biomass occurring in Leighton Moss. On the other hand Leighton Moss may have performed poorly in the mini-CWSs because this population was unable to adapt as successfully as the other populations to the hostile rhizosphere environment created in the CWS, as reflected in its total biomass that was the lowest of all populations at the end of the experiment.

During the growing season no significant differences were found between populations in mass % NH_4-N removal, with all populations having high removal rates between 90 % and 95 %. Low effluent concentrations of NH_4-N occurred during this period due to a combination of factors. Warm temperatures ensured that microbial nitrification proceeded unimpeded. Coupled with this, aeration of the rhizosphere would be enhanced by day time humidity induced-diffusion *via* the leaf stomata (Armstrong and Armstrong, 1990). This was shown by the diurnal fluctuation in Eh in some populations (Fig. 7.5), attributable to photosynthetic activity of the plant. Furthermore, NH_4-N was incorporated into plant biomass (temporary storage).

During the dormant period there were significant population differences in mass % NH_4-N removal with the northern populations (Inner Tay, St. Margaret's Marsh and Leighton Moss) having mean NH_4-N removals of < 20 % compared to the southern

populations (Strumpshaw Fen and Walberswick) which maintained removal rates of > 45 %. Lower NH_4-N removal would be expected in the dormant period compared to the growing season, due to lower temperature, plant dormancy and/or reduced oxygen transport (Werker *et al.*, 2002). Differences between populations in NH_4-N removal during the dormant period may be attributed to phenological variation such as different lengths of growing season. For instance, Clevering *et al.* (2001) found that populations from lower latitudes had genetically determined longer growing seasons than northern ones. Therefore, in this experiment the onset of senescence and translocation of nutrients from the leaves to the rhizomes may have occurred earlier in the northern populations than southern populations (the timing of senescence was not recorded in the experiment), resulting in greater losses of NH_4-N from the plants through leaching and litter decomposition (Meuleman *et al.*, 2002). In addition to nutrient losses, leaf senescence causes cessation of humidity-induced diffusion (Armstrong *et al.*, 1996), reducing aeration of the rhizosphere and impeding nitrification. Coupled with this, reduction in temperature during the dormant period would result in decreased microbial activity in all mini-CWSs.

7.4.2 NO_3-N removal

In this study mean % mass removal of NO_3-N ranged from 53 - 82 %, which agreed with previous research (Table 7.15). Unlike NH_4-N removal, population differences in percentage mass removal were only marginally significant. There was a marginally significant difference between populations for the nine day cycle and dormant period, but no significant difference in the growing season or the three day cycle.

As NO_3-N removal involves denitrification in anaerobic zones, the plant's effect on the process may be minimal. On the other hand, denitrification by heterotrophic bacteria is dependent on an organic carbon source which may be supplied to the rhizosphere by plants (dead plant material and root exudates) (Stottmeister *et al.*, 2003; Münch *et al.*, 2005). This carbon source is not trivial and has been estimated at 10 - 40 % of the net photosynthetic production of agricultural crops (Stottmeister *et al.*, 2003). However, rhizodeposition may only be of importance in CWSs that have a low carbon load (e.g., those treating mine drainage) (Stottmeister *et al.*, 2003).

Denitrification could also be inhibited by root oxygen release, but Münch *et al.* (2005) suggest that there is strong heterogeneity in the rhizosphere, so that nitrification and denitrification occur side by side in aerobic and anaerobic microzones. If radial oxygen release decreased denitrification then, in this experiment, populations with the highest NH_4-N removal should have the lowest NO_3-N removal (i.e. the southern populations). Although the northern populations of St. Margaret's Marsh and Inner Tay had higher removal rates than Walberswick, Leighton Moss had the lowest % NO_3-N removal, whilst Strumpshaw Fen had the highest % NO_3-N and % NH_4-N removal compared to all other populations. Correlation analysis also confirmed that there is no negative association between % NH_4-N and NO_3-N removal in the mini-CWSs (Table 7.11). These observations suggest that intraspecific plant variability in radial oxygen release may have only a small effect on % NO_3-N removal.

There was a marginally significant difference in % NO_3-N removal between populations for the nine day, but not for the three day cycle. In the nine day cycle more NO_3-N was removed (75 ± 4 % over all populations) compared to the three-day cycle (65 ± 7 %), probably due to the longer duration of anaerobic conditions in the substrate, rather than to a plant effect. The limited effect of plants on NO_3-N removal is also evident during the dormant period, when three of the five populations maintained removal rates of > 60 %. The marginally significant difference between populations during the dormant period was caused by the low mean removal rates of Leighton Moss and Walberswick (15 % and 34 % respectively). During this period it would be expected that additional input of NO_3-N to the CWSs would occur because of senescence and litter decomposition, but this does not appear to have affected removal to the same extent as for NH_4-N, as negative % removal values occurred for shorter periods of time (Fig. 7.6).

7.4.3 P removal

Removal of P from the mini-CWSs was extremely high and ranged from 98.7 - 99.9 % which was in accordance with the results of other studies, but much higher than Greenway and Woolley (1999) (11 and 42 %) (Table 7.16).

Since P removal occurs mainly through substrate adsorption and precipitation, population differences in percentage removal in the mini-CWSs were not expected. Indeed, Drizo *et al.* (1997) observed that planting with *P. australis* in shale HFS improved the % P removal by only one percent. Nevertheless, in this study, there was a marginally significant difference between populations in % mass removal rates, as well as a significant difference between populations in the three and nine day cycles and a marginally significant difference in the dormant period. However, all populations maintained > 97 % removal rates in both the growing and dormant period and in the nine and three day cycles and the range of % removal between populations was negligible. Furthermore, unlike NH_4-N and NO_3-N that can be permanently removed from the CWS as nitrogen gas or nitrous oxide, P is stored in the CWS by adsorption and precipitation until saturation is reached when the wetland is no longer effective for P removal (Vymazal, 2002). The high removal rates of the mini-CWSs in this study therefore reflect the 'young age' of the system.

There are two possible processes by which the plants could affect P removal. Firstly oxygenation of the rhizosphere would promote Fe^{3+} precipitation in the form of iron plaques on the root and substrate surface, which act as sites for P adsorption (Peverley *et al.*, 1995). In this experiment iron plaques were observed, but not quantified, on many of the root and rhizome surfaces during root washing. Therefore variability in rhizosphere oxygenation between populations could result in disparate plaque formation and P adsorption.

Table 7.16 Mean % P and BOD_5 removal reported in this study (in bold) and other studies, calculated from mass or concentration, in horizontal surface (HSF), horizontal subsurface (HSS) or vertical flow(VFS) wetlands planted with *P. australis* or other species.

Nutrient and location	Mean % removal	Type of wastewater	CWS design	Planted with	Area of bed (m^2)	Reference
P						
Unheated greenhouse	**98 (Mass)**	Synthetic sewage	VFS	*P. australis*	0.17	This study
Unheated Greenhouse	> 98 (Mass)	Synthetic sewage	HSF Laboratory - scale	*P. australis*	0.49	Drizo *et al.*, 1997
Czech Republic	84.3 (Conc)	Domestic or municipal	HSFs	*P. australis*	18 - 4493	Vymazal, 2002
Queensland, Australia	42 (Mass) 11 (Mass)	Secondary sewage effluent	Constructed free water surface wetlands	*P. australis* and other native plant species	- -	Greenway and Woolley, 1999
Austria	0-80 (Conc) (mean 25)	Domestic sewage	VFS (4 beds)	*P. australis*	149 (each bed)	Kadlec *et al.*, 2001
BOD_5						
Unheated greenhouse	**97 - 98 (Mass)**	Synthetic sewage	VFS	*P. australis*	0.17	This study
Austria	97 (Conc)	Domestic sewage	VFS	*P. australis*	42.25	Kadlec *et al.*, 2001
Czech Republic	83.5 (Mass)	Domestic or municipal	HSFs	*P. australis*	18 - 4493	Vymazal, 2002
Kentucky, USA	73 (Mass)	Domestic	SSF	*Typha latifolia* L. or *Festuca arundinacea* Schreb.).	44[a]	Karathanasis *et al.*, 2003
Queensland, Australia	54 (Mass) 66 (Mass)	Secondary sewage effluent	Constructed free water surface wetlands	*P. australis* and other native plant species	- -	Greenway and Woolley, 1999

[a] average size of 12 wetland systems

Whether such potential variations contributed to population differences or differences observed in the three day cycle and dormant period are uncertain. The only other way that *P. australis* could affect P removal is through the formation of humics (accretion)

that adsorb P. However this is a slow process compared to the other forms of P removal, and is unlikely to have caused population differences (Kadlec, 1997).

Unlike NH_4-N and NO_3-N removal, biological influences on P removal are relatively unimportant. Therefore, P removal is not temperature dependent as the main removal processes are chemical precipitation and physicochemical adsorption, neither of which are influenced by temperature (Vymazal, 2002). Consequently seasonality was not expected to have a significant effect on % P removal. However this was not the case, as there was a highly significant effect of date on removal for all cycles and periods. This significant result may have been caused by a temperature or seasonal effect on *P. australis*, such as increased root oxygenation and temporary uptake of P during the growing period or release of P from leaching and litter decomposition during senescence and in the dormant period.

7.4.4 BOD removal

BOD removal from the mini-CWSs was extremely high and ranged from 97.1 - 98.8 % which was in accordance with other studies, although Greenway and Woolley (1999) had lower removal rates (54 % and 64 %) (Table 7.16). Although the percentage mass removal from all populations was > 97 % there were still significant differences between populations during the whole experiment and the dormant period.

Since microbial growth and attachment are affected by rhizosphere surface area and root zone oxygenation (for aerobic bacteria), population differences in total below ground biomass (i.e. surface area variability) and total above ground biomass (i.e. oxygenation variability) may account for the significant differences in removal rates. However, the Inner Tay had the lowest % BOD removal, but also the second highest total biomass amongst populations. Plant material decomposition from this population may have increased BOD in the mini-CWSs, although if this effect occurred it accounted for only a *c.* 1 % difference between populations. In contrast to NH_4-N, NO_3-N and P, the highest BOD removal rates occurred in the dormant period, although the removal rates were only about 1 % higher than in the growing season. Although Karathanasis *et al.* (2003) reported the opposite trend in their study, lower

BOD during the dormant period may have arisen from reduced solubility of some organic compounds in lower temperatures and the removal of colloidal organic matter by filtration (Werker *et al.*, 2002).

7.5 SUMMARY

In conclusion, this study is the first to show that there are significant and substantial intraspecific differences in nutrient removal in CWSs by *P. australis*. It is probable that the genetic variability manifest as morphological differences among British *P. australis* populations affects performance, particularly for NH_4-N removal. Generally, southern populations showed a higher NH_4-N removal rate than those from the north. These findings not only have implications for the sourcing of seeds for establishing CWSs (e.g. CWSs designed to maximise NH_4-N removal should not be planted with seeds sourced from Leighton Moss), but also suggest that other polymorphic plant species that are used as alternatives to *P. australis* may show similar results.

Chapter 8 – General Discussion

8.1 INTRODUCTION

The successful utilization and domestication of wild plants requires a thorough understanding of the species' biology and ecology. The present study investigated natural populations of *P. australis* in Britain because the conservation and restoration of reedbeds, in which this species dominates, has become a priority for statutory and non-governmental organisations. In addition *P australis* is the plant of choice for CWSs in Britain which have increased in prominence over the last 20 years, since their introduction in the early 1980s (Cooper *et al.*, 1996).

Although *P. australis* is one of the most extensively studied wetland species in the world (Brix, 1999), there remain extensive gaps in scientific knowledge, particularly regarding the resource in Britain. This formed the basis for the investigations undertaken in this study. A number of key questions were presented in Chapter One. The results of the research aimed at answering these questions will first be summarised. The information will then be used to make recommendations for the management of the *P. australis* resource for reedbed establishment and conservation as well as for use in CWSs. The final section discusses future areas of research which have arisen from this study.

8.2 KEY RESEARCH QUESTIONS

8.2.1 What are the chromosomal races of British populations of *P. australis?*

The ten populations of *P. australis* examined in this study by cytological investigations were all tetraploids ($2n = 4x = 48$). The chromosomal race of the Loch Leven population could not be determined due to lack of plant material. However, it

is likely that this population is also tetraploid, as the 'gigantism' associated with higher ploidy (Paucã-Comãnescu *et al.*, 1999) was not observed in the natural stand. *P. australis* is considered to be an allo-polyploid of ancient origin (Gorenflot *et al.*, 1979; cited in Clevering and Lissner, 1999) that has arisen through interspecific hybridisation and chromosome doubling (Soltis and Soltis, 2000). Allo-tetraploids are characterised by bivalent formation of homologous chromosomes at meiosis and disomic inheritance at each locus (Jenkins and Rees, 1991). Since only one chromosomal race is found in Britain, all crosses among *P. australis* populations within Britain should be fully fertile. It is also important to note that morphological and ecological variability among British populations of *P. australis* is not caused by differences in ploidy levels.

8.2.2 What is the clonal structure of *P. australis* populations in Britain?

There was a wide range of variability in clonal diversity amongst the British populations of *P. australis* detected using four polymorphic microsatellite loci. Whilst nine of the 11 populations maintained high levels of diversity, such that all sampled ramets displayed different genotypes, two populations, Loch Leven and Inner Tay, exhibited impoverished clonal diversity. In fact, Loch Leven was monoclonal, and the clone was $\approx$1.9 km long. Clonal diversity may decrease over time within a population (Koppitz *et al.*, 1997) unless sexual recruitment occurs to introduce genotypes that differ from the maternal parents. Therefore the lack of management of the Loch Leven population, as well as site homogeneity, probably contributed to the presence of only one clone. In the Inner Tay population, three large clones were found, the largest at the eastern end of the reedbed being 5.1 ha in size. As clonal diversity increased in this reedbed from east to west, which was also associated with a decrease in salinity, it was hypothesised that only those clones adapted to highly brackish conditions colonised the eastern part of the reedbed.

The clones found in this research were the largest that have been recorded in any study of *P. australis* using molecular markers. Furthermore the likelihood that the repeatedly sampled individuals of each clone arose independently (i.e. through

separate sexual events) was very small. The maintenance of clonal diversity within the other populations investigated in this study probably arose through a combination of factors, including active management, such as clearing/burning/commercial harvesting of vegetation and water level manipulation, site heterogeneity and recent establishment.

This study suggests that lack of management intervention, to encourage sexual recruitment in a population, may lead to a decrease in clonal diversity. Over time a monoclonal population, such as was found at Loch Leven, could develop. Low clonal diversity within a population may not only affect the ability of a population to adapt to future environmental change but will also affect seed production This is because *P. australis* possesses a self-incompatibility system (Ishii and Kadono, 2002) Compatible pollen limitation may thus occur where clonal diversity is low and probably explains why no seed was found in the monoclonal reedbed of Loch Leven over the two year study period.

Low clonal diversity was also shown to be associated with a high incidence of *Claviceps purpurea* infection. The latter is a systemic infection that spreads through all parts of the clone; so the presence of large clones, which are susceptible to infection, will result in a high prevalence of *C. purpurea* throughout the reedbed. Low seed production and high *C. purpurea* infection may have a deleterious effect on the wildlife communities that depend on the seed of *P. australis* within British reedbeds. A final point is that the clonal structure of a population has implications for effective sampling strategies for the collection of germplasm within a population. For example, the large clones found in this study emphasise the need to collect plant material at widely-spaced locations to ensure that the same clone is not repeatedly sampled.

8.2.3 What is the population genetic structure of British populations?

High levels of genetic diversity as measured by the expected heterozygosity, H_e, detected by microsatellite markers, were found within most populations of *P. australis* and over all populations studied the mean expected heterozygosity was

high. These findings add to the increasing number of studies that have found that clonal plant species maintain the same levels of genetic diversity for molecular markers as non-clonal species (Ellstrand and Roose, 1987; Jonsson *et al.*, 1996; Reusch *et al.*, 2000; Ainsworth *et al.*, 2003).

There was a moderate degree of genetic differentiation (calculated as θ) amongst populations based on the microsatellite data, implying that there is restricted gene flow between populations. In addition, populations did fit the isolation by distance model; populations that were separated by the greatest geographic distance had the least gene flow. However, pairwise comparisons of genetic distances (F_{ST}) gave some surprising results. Whilst some pairs of populations that were separated by only a few kilometres had low estimates of F_{ST} (e.g. Walberswick and Minsmere) as expected in the isolation by distance model, others that were separated by large distances (e.g. Inner Tay and Mersehead) also showed low values, indicating that there is long distance gene flow between them. It may be that a small amount of gene flow (in the form of seed transport) has been maintained by humans through activities such as commercial thatching and planting of non-local provenances into reedbeds.

8.2.4 What is the breeding system of *P. australis*?

This study was the first to determine the outcrossing rate of *P. australis* using genetic analysis. The multilocus outcrossing rate of *P. australis*, found by using microsatellite markers in one population, the Inner Tay, was close to one and the selfing rate (in a natural population) was less than 4 %. This result complements the findings of Ishii and Kadono (2002), who found that *P. australis* was mainly self-incompatible. However, the outcrossing rate in this study was most likely over estimated, because it was determined using only those progeny that showed successful germination and growth. Self fertilised individuals may have failed to germinate and grow to a stage where they could be analysed. The mating system analysis also gave evidence of biparental inbreeding, most likely caused by clone structure. Indeed, evidence of genetic substructure (θ = 0.038) was detected within populations using samples grouped by geographic location. In addition, it is likely

that the presence of large clones accounted for the observation that 20 % of siblings shared the same father (i.e. same pollen donor).

This study also found a remarkable difference in genetic diversity between the sexually produced progeny (40 alleles detected) and the adult population (16 alleles detected) indicating that the potential genetic diversity of this population is high, although it may never be realised unless there is successful seedling recruitment into the reedbed. It was concluded that removal of competition through disturbance (clearing of existing vegetation) is probably the only way to increase the genetic diversity of an established reedbed and, to encourage genetic diversity in a new reedbed, seeds must be planted rather than vegetative propagules.

8.2.5 Are there differences in seedset and germination rates amongst British populations of *P. australis*?

Seedset, germination rates and *C. purpurea* infection were highly variable amongst British populations of *P. australis*. The highest seedset was obtained from large reedbeds that had a low incidence of *C. purpurea* infection and high mean maximum October temperatures (when seed maturation occurs). It was hypothesised that site size was a surrogate measure for clonal diversity (i.e. larger sites contained more clones). The latter is an important factor for cross-pollination in a highly outcrossing species. The most northerly population (Insh Marsh) had low seedset, probably because it was at the edge of the species' range. In addition it is likely that compatible pollen limitation further contributed to the high infertility of this population and also to the total lack of seed production in the monoclonal Loch Leven population.

Germination rates in all populations, except Insh Marsh, were > 50 %. Since there was a positive association between seedset and germination rate, populations with a high seedset should be favoured for harvesting of seed as the germination rate should also be high.

C. purpurea infection was more prevalent in the more northerly populations which recorded lower temperatures in August and October compared to southern

populations. It was hypothesised that low pollination of flowers in the northern populations was a contributory factor. In addition, more infection was found where clonal diversity was low.

8.2.6 Are there quantitative genetic differences in morphology within and among populations of *P. australis* in Britain?

In all three common garden experiments (nutrient-stressed, full-nutrient and synthetic sewage), substantial genetic differences in morphological characters were found amongst populations. The characters that showed consistent variation in all three experiments were shoot height and number of shoots produced. Shoot diameter was variable in the first two experiments, but not in the synthetic sewage treatment. Generally northern populations produced smaller shoots than southern populations in full-nutrient conditions, whilst the reverse was found in nutrient-stressed conditions. It was concluded that plant material from southern populations would be unsuitable for planting into northern sites, because it is not adapted to growing in nutrient poor soils.

As there were no significant morphological differences within populations grown in the three environments, it was hypothesised that the morphological differences that are observed within a reedbed are the result of phenotypic plasticity (which is itself under genetic control). However another explanation for a low estimate of within population genetic diversity is that certain maternal genotypes were repeatedly sampled due to their extensive clonal growth (e.g. at the Inner Tay population).

The findings of this research have implications for provenance choice for reedbed establishment. Currently sites for panicle harvesting are selected on the basis of high seedset and the morphology of the chosen provenance has not been considered. However it is known that phytophagous insect communities and certain avian species have specific preferences in *P. australis* growth form (Tscharntke, 1999; Poulin *et al.*, 2002). Because this study has shown that the genetic differences in characters may be substantial, this factor should be accounted for in provenance selection, particularly if reedbeds are established for certain targeted wildlife species. This should also be considered for the selection of reed stems for thatching purposes. For

example, if a reedbed is to be established for commercial purposes (as well as wildlife) then shoot density becomes an important factor (thatchers prefer stem densities > 200 stems m^{-2} (Hawke and José, 1996)). In this case it would not be prudent to collect seed from Lakenheath or Minsmere, as they recorded the lowest number of shoots.

Application of synthetic sewage resulted in a decrease in the number of shoots produced and a significant increase in below ground biomass for all populations (compared to populations that had not received synthetic sewage). Although there was no significant GE interaction, some populations were more affected than others by the application of sewage. Therefore seed source for reedbed establishment in more nutrient-rich conditions should be from populations more tolerant to high nutrients.

8.2.7 Are there genetic differences in ability to remove nutrients among populations?

Substantial intraspecific variability in nutrient removal from synthetic sewage was found in mini-CWSs planted with *P. australis* from five different British populations. Of the four nutrients examined, % NH_4-N removal from synthetic sewage was most affected by genetic differences among populations. The disparity in % removal rate among populations was not trivial (ranging from 33 to 72 % NH_4-N removal) and generally the southern populations showed a higher % removal of NH_4-N than the northern populations.

It was hypothesised that population variability in morphology could have accounted for the results, although no significant differences in above or below ground biomass were found in the mini-CWSs this may be attributable to small sample size, as the investigation of population variability in quantitative traits found substantial and significant genetic differences among populations (section 8.2.6).

This study is the first to show that certain plant genotypes are more efficient in nutrient removal in CWSs than others, and, this should be a consideration in CWS establishment, particularly for systems designed to maximise NH_4-N removal.

8.3 MANAGEMENT RECOMMENDATIONS

This section synthesises the findings of this study to make recommendations for the future management of *P. australis* in Britain under categories: management of the resource for (i) reedbed establishment; (ii) conservation of existing reedbeds; (iii) use in CWSs.

8.3.1 Reedbed Establishment

8.3.1.1 Choice of plant material

This study has shown that the clonal diversity (and hence genetic diversity) of a population profoundly affects the seed fertility of a reedbed (which in turn affects future seedling recruitment). In addition, variability in quantitative traits and incidence of *C. purpurea* infection (which also affects seedset) are influenced by the inherent genetic diversity of the reedbed. Moreover a reedbed will only be sustainable if it is sufficiently genetically variable to withstand future environmental change. Since this study found that sexual progeny had remarkably higher levels of genetic diversity compared to the adult population, it is imperative that a reedbed is created from seed rather than vegetative propagules.

8.3.1.2 Source of seed

The first priority in creating a reedbed is to define the management objectives, as this will influence decision making regarding seed source. New reedbeds are frequently created to provide a habitat for a variety of wildlife, or are carefully designed to target a particular species (such as the bittern). In addition, it is often the case that the reedbed may be considered for commercial harvesting, because it is accepted that some of the reedbed will have to be cut annually, to prevent litter build up and successional change. More rarely a reedbed may be specifically established for commercial harvesting for thatching. Recommendations are given below for seed source for different reedbed establishment objectives.

8.3.1.2.1 Creating a habitat for wildlife

If the management objective is the creation of a species-rich faunal community, then a variety of *P. australis* growth forms (i.e. different genotypes) should be planted. This would create a reedbed that is sufficiently diverse to support the multitude of invertebrate and bird species that are dependent on reedbeds for food. Diversity is required by phytophagous insect communities that have a variety of preferences for shoot thickness (Tscharntke 1990; Tscharntke, 1999). In addition, breeding bird species (e.g. reed warbler, bearded tit) (Poulin *et al.*, 2002) show a preference for reedbed habitats containing various growth forms (such as shoot height, density or thickness) of *P. australis*.

8.3.1.2.2 Creating a habitat for a specific faunal species

The habitat requirements of the targeted species should be established before choosing the seed source. If this is not possible, seed should be sourced from reedbeds that are already colonised by the targeted species, as this increases the likelihood of recreating the essential (but possibly unknown) *P. australis* genotype(s) required by the species in the new reedbed. Since this study has shown that expression of growth form is under genetic control and is dependent on environmental conditions, consideration should be given to reproduce similar edaphic conditions (e.g. nutrient and hydrological status) in the new reedbed. Furthermore, if the seed source is local, the genotypes will most likely be adapted to the climatic conditions.

8.3.1.2.3 Creating a habitat for thatching

The creation of a reedbed for commercial harvesting must produce reed stems of a sufficient quality that is suitable for thatching. For instance a reedbed should be dense (> 200 stems m^{-2}) and contain reed that is 1 - 2 m tall and has a shoot thickness between 2 – 7 mm (Hawke and José, 1996). These characteristics are induced by single wale cutting (i.e. cutting reed every year), although the influence of genotype is unknown. Therefore, similar to the recommendations above for targeting a particular species, it would be prudent to collect seed from populations that are already used for commercial purposes.

8.3.1.3 Selection of the source population (s)

Ideally the source population should fulfil the following criteria:

1. *Have a high seedset*

This study has found that *P. australis* is mainly outcrossing. Therefore prolific seedset may be indicative of high clonal diversity within a population, because the fertility of *P. australis* is dependent on cross-pollination. This relationship was demonstrated in the monoclonal population of Loch Leven which produced no seed in two consecutive years. In addition, as seedset increases so does the germination rate, which implies a high reproductive potential. The latter is an important consideration if large scale planting is required. (Other factors associated with high seedset, which are discussed below, include size of reedbed (large sites produce more seed), low incidence of *C. purpurea* infection and high October temperatures).

2. *Be local*

Seeds should be harvested from locally sourced populations. This would ensure that the genetic processes which have been found in this study (i.e. very limited gene flow between distant populations), is replicated in managed populations. In addition the progeny of locally sourced seed are more likely to be adapted to the climatic conditions of the newly created reedbed. Moreover, southern populations should not be used as a seed source for establishing reedbeds in northern Britain (and *vice versa*).

3. *Have similar edaphic conditions*

Since the CGE showed that populations may exhibit different growth forms (e.g. shoot height) depending on the environmental conditions in which they are grown, seed should be collected from a reedbed with characteristics similar to the one to be established (e.g. have a similar water regime and edaphic conditions). In addition, the source population should be thoroughly examined to ensure that the morphology of the reed is sufficiently variable and fulfils the management objectives.

4. *Have no incidence of Claviceps purpurea infection*

Infection of a reedbed by *C. purpurea* decreases *P. australis* seedset and is also associated with low clonal diversity. Both of these traits are undesirable for seed collection. In addition, the presence of *C. purpurea* infection may afford the reedbed some defence from herbivory, through alkaloid production by the pathogen (Clay,

1990b), which may be detrimental to phytophagous insect communities and thus not meet the management objectives (e.g. creating a species-rich community). The source reedbed should be investigated for signs of *C. purpurea* infection, which only requires a simple inspection of the panicle (from September onwards) to detect the dark brown cigar shaped ergots which replace the seed in the floret. Since it is unknown whether *C. purpurea* is transmitted through seed, the precautionary principle should be applied and seed should not be collected from populations with a high incidence of infection. This predominantly applies to collection of seed from northern populations as they carry more infection than southern ones.

5. *Be from a large reedbed*

This study has shown that large reedbeds produce more seed compared to small sites, which is not only desirable for efficient panicle harvesting, but may also be a surrogate measure of clonal diversity (i.e. large reedbeds, with high seedset are likely to contain many clones).

8.3.1.4 Collection of seed from the source population

Once the source population has been selected, seeds should be collected for distantly located parts of the reedbed (e.g. at least 250 m apart in large reedbeds) as this study has shown that individual clones may be large. This ensures that the progeny do not possess the same maternal genotype and decreases the chance of biparental inbreeding (i.e. sharing the same maternal and paternal genotype), both of which would decrease the genetic diversity of the progeny.

8.3.1.5 Examining panicles for seed

In the field, an examination of five spikelets per panicle is a quick and reliable indicator of seedset. If no seeds are found, it is highly likely that the whole panicle is devoid of seed. More seed will be collected in years that have had high October temperatures (when seed maturation occurs) but if frost occurred during this month, the germination rate may be less than 50 %.

8.3.2 Conservation of an existing reedbed

The goal of conserving an existing reedbed is often to manage it to provide a sustainable and diverse habitat for wildlife. However, the sustainability of the reedbed will only be assured if it is sufficiently genetically variable to adapt to future environmental change.

Therefore the successful conservation of a reedbed is greatly dependent on the genetic diversity of *P. australis* within the population and, as clonal diversity of a reedbed is thought to decrease over time (Koppitz *et al.*, 1997) because of inter-clonal competition and limited seedling recruitment, it is imperative to adopt management that encourage seedling recruitment.

8.3.2.1 Assessing the genetic diversity of a reedbed

Before undertaking management practices for enhancing the genetic diversity of the reedbed, the reedbed should be investigated for indicators of genetic diversity or 'health' so that monoclonal areas within the reedbed can be identified and prioritised for management. Addressing the following questions should enable the genetic diversity of the reedbed to be assessed without costly, specialised genetic analysis.

1. *Does the reedbed have a high seedset?*

The seedset of the reedbed should be monitored over at least two years to take account of inter annual variability. Abundant seedset is suggestive of high clonal diversity. Seedset may be heterogeneous within the population and areas where seed production is low may be caused by the presence of large clones. High seedset is also necessary for increasing the likelihood of sexual recruitment. The promotion of sexual recruitment through disturbance will only be achieved if the population is fertile. For instance, clearing patches of the existing Loch Leven reedbed would not result in seedling recruitment because the population is infertile. Therefore in this population, externally sourced seedlings would have to be planted. It is also essential that any planted seedlings differ in the self-incompatibility genotype (this could be tested, by controlled pollination from the existing population), otherwise the population will remain devoid of seed.

2. *Is the reedbed infected with C. purpurea infection?*

High incidence of infection may indicate low clonal diversity. Since the infection spreads throughout the whole clone, large areas of vegetation (including the rhizomes) may need to be cleared. As it is unclear if the seed transmits infection, seedlings may need to be planted from a genotype within the reedbed that is resistant to infection. Furthermore a fungicide treatment (of the soil/sediment) may be needed to inhibit germination of *C. purpurea* sclerotia (e.g. production of spores) which may be present in the soil, and could infect establishing seedlings ((Dabkevičius and Mikaliūnaitė, 2005). Periodic monitoring (approximately every 5 years) of the reedbed for *C. purpurea* will be needed in the future because infection builds up over time (Bradshaw, 1958).

3. *Does the reedbed hold many invertebrate species?*

A variable habitat containing many *P. australis* genotypes will provide a multitude of niches for invertebrate communities. Therefore an inventory of the invertebrate species present may give an indication of the diversity of growth forms of *P. australis*. Although invertebrate preferences have been found (Tscharntke, 1999) a correlation between clonal diversity and invertebrate diversity has not been tested. Lack of invertebrate species may also indicate the presence of *C. purpurea* infection.

4. *Does the reedbed hold many bird species?*

The absence of a resident bearded tit population may signify poor seedset. A low number of species may imply that habitat preferences are not met, or food is unavailable. The latter is most important during the breeding season when a diverse diet of invertebrates is necessary for rearing young birds.

5. *Is the reedbed expanding or contracting in size?*

Shrinkage of a reedbed in size may occur because of deleterious environmental effects (e.g. eutrophication, increase in salinity or climate change) which may be exacerbated by low clonal diversity as there may be insufficient genotypes present within the population that are capable of adaptation to the changed environment.

6. *Is the habitat mainly homogeneous or heterogeneous?*

It is likely that in a heterogeneous environment more genotypes will be present. Although not tested in this study, it was hypothesised that in the homogeneous reedbed of Loch Leven one clone, that was adapted to growth in deep water,

outcompeted all others which over time led to the monoclonal population that is present today.

By addressing the above questions, the extent of genetic diversity in a reedbed should be ascertained, without the need for genetic analysis. The next section discusses the methods to increase the genotypic diversity of an established reedbed.

8.3.2.2 Recommended management practices to increase the genetic diversity of an old reedbed

Old reedbeds should be managed to promote genotypic diversity. This is best achieved by creating areas of disturbance which may involve extensive clearing/burning of existing vegetation and removal of rhizomes (if *C. purpurea* infection is present). To encourage seedling survival, water levels in the disturbed site may have to be lowered because seedlings are intolerant of flooded conditions (Haslam, 1972). However it is important that the newly disturbed area does not dry out as establishing seedlings are killed by desiccation (Haslam, 1970).

Where seed production is inherently low, seedlings may have to be planted from seeds collected elsewhere in the reedbed (with similar environmental conditions) or from another population. Seedlings, planted or recruited from the existing population, should be monitored over a long period of time to make sure they endure and are not outcompeted by existing clones.

By carrying out these practices, the genetic diversity of the reedbed should be substantially increased. Coupled with this, a dramatic increase in seedset should be experienced which may have profound ecological benefits, so long as the clonal diversity can be maintained over time. Periodically the reedbed should be monitored for signs of changes in clonal diversity, using the above questions as a guide.

8.3.3 Use of *P. australis* in CWSs

Because this study has shown that there are substantial genetic differences in nutrient removal (especially for NH_4-N) among *P. australis* populations, consideration must be given to the source of seed/seedlings to be planted in a CWS. In addition, the

CGE showed that the expression of growth form is under genetic control and that northern populations grew best in nutrient poor conditions compared to southern populations.

As this is the first investigation of intraspecific differences in efficacy of nutrient removal, many questions remain to be answered (which will be discussed below) before recommendations can be made for the most suitable source of reed to be used in CWSs. However it is probably good practice to plant CWSs with locally sourced seed, as some of the genotypes will already be adapted to the climatic conditions. In addition, seed should be collected from populations occurring in nutrient-rich conditions. These could be obtained from natural populations and existing CWS (if seed is available), or more likely from a commercial grower that may select for populations which show abundant growth under nutrient-rich conditions.

As it was generally found that populations from southern Britain removed the most nutrients (at least for NH_4-N) these should be considered superior to northern sources of seed. However if there is a large difference in climate between the source population and the proposed CWS site, the source population may not be adapted to the changed conditions. For this reason, seeds or seedlings should be planted and not vegetative propagules because this ensures the initial presence of many different genotypes, some of which will be adapted to the edaphic and climatic conditions present in the CWS. It is probable that over time, inter-clonal competition will lead to a reduction in the number of genotypes, although the clonal diversity of a CWS has never been investigated.

8.4 FUTURE RESEARCH

This section outlines areas of future research and is divided into two sections. The first part comprises questions that remain concerning reedbed conservation, whilst the second part outlines the numerous questions that have arisen out of the CWS experiment, which was the first study to show that there are intraspecific genetic differences in efficacy of nutrient removal.

8.4.1 Reedbed conservation

8.4.1.1 Does the genetic diversity of a reedbed decline over time?

Throughout this research it has often been stated that the genetic diversity of a clonal population is believed to decline over time (Ellstand and Roose, 1987; Widén *et al.*, 1994; Koppitz *et al.*, 1997). The creation of a new reedbed by the RSPB at Lakenheath provides an opportunity to test this hypothesis and would involve an extensive investigation of the *P. australis* clonal structure (i.e. number and size of clones present) including the 20 clones that were detected in this study, by microsatellite markers, in material collected in 2003. To determine the extent of inter-clonal competition, the reedbed should be re sampled over time using handheld GPS to relocate previously sampled genets or ramets. A comparison of trends in genetic diversity over time between sites could also be carried out by sampling from other newly created reedbeds, such as Mersehead or Ham Wall in south west England (Hawk and José, 1996).

8.4.1.2 Do sexually recruited seedlings survive in an established *P. australis* population?

This research has emphasised the need to create areas of disturbance to encourage seedling recruitment (either from a natural seed source within the reedbed or planted from an external source) and thus increase the genetic diversity of a reedbed. However the fate of the seedlings over time is unknown and it is possible that surrounding clones that are already adapted to the environmental conditions outcompete and replace the introduced genotypes.

This could be examined using the monoclonal Loch Leven population and would involve clearing a portion of the reedbed and planting it with seedlings from an external source (derived from a similar habitat). Successful genotype establishment in the long term could be monitored by examining the population for the presence of seeds. At present compatible pollen limitation causes infertility, therefore the introduction of new genotypes should ensure that cross-pollination occurs.

8.4.1.3 Do reedbeds with a high incidence of *C. purpurea* infection contain less invertebrate species?

It is thought that *C. purpurea* imparts a degree of herbivory defence to infected *P. australis* clones, through the production of alkaloids (Clay, 1990b). It was hypothesised in this study that a high incidence of infection would be associated with low invertebrate diversity. This hypothesis should be investigated because lack of invertebrate species would also be detrimental to avian communities inhabiting reedbeds. A comparison of invertebrate species in reedbeds containing high *C. purpurea* infection and low/no infection would be needed to test the hypothesis. This investigation could also determine if seeds transmit infection and the best methods for permanently removing infected clones from a reedbed.

8.4.2 Constructed wetland systems

8.4.2.1 Does the genetic diversity of a reedbed in a CWS decrease over time?

Similar to a natural reedbed, the genetic diversity of *P. australis* in a CWS may decline over time and small systems (as well as large) could become monoclonal, or possess a limited number of clones. This would affect the ability of a reedbed to respond to environmental change (e.g. changes in climate or nutrient application, or pest and pathogen attack) which may be dependent on the number of genotypes present in the population. Genetic diversity could also be studied over time between CWSs, differing in design and size.

8.4.2.2 Do CWSs containing southern populations of *P. australis* remove more NH_4-N than northern populations when planted in northern locations?

This study found that, in general, southern populations of *P. australis* had a higher removal of NH_4-N compared to northern populations. However it is not known if southern populations would exhibit a genotype x environment response when transplanted into northern locations, such that the efficacy in nutrient removal would diminish because the southern genotypes are not adapted to the local climate. This is

an important consideration since in Britain seed collected from southern populations is used for planting in CWSs located many hundreds of kilometres to the north (Reeds from Seeds pers. comm. 2002). Therefore a transplant experiment should be conducted in CWSs of the same design and nutrient application, planted with northern and southern populations of *P. australis* and a comparison in nutrient removal should be made when the populations are grown in northern and southern climates.

8.4.2.3 What are the genetic differences responsible for variability in nutrient removal among populations?

Although this study found substantial genetic differences among populations in effectiveness in nutrient removal, particularly for NH_4-N, the morphological differences (among populations) that were responsible could not be fully explained. Furthermore disparity in physiology and phenology (e.g. ability to oxygenate rhizosphere and length of growing season) between populations was not examined. Therefore future studies should investigate and determine the genetic differences in *P. australis* responsible for the variability in nutrient removal in CWSs so that superior genotypes of *P. australis* can be identified and used to increase nutrient removal efficiency.

8.4.2.4 Does ploidy level of *P. australis* affect nutrient removal?

In this study, the CWS experiment was conducted using the same chromosomal races (i.e. tetraploids). However it is known that morphological and ecological differences exist between different chromosomal races (Paucã-Comãnescu *et al.*, 1999). Therefore it would be instructive to learn whether octoploids ($2n = 8x = 96$) are superior in nutrient removal compared to hexaploids ($2n = 6x = 72$) or tetraploids.

8.4.2.5 Do other polymorphic species used in CWS show intraspecific genetic differences in nutrient removal?

Although, *P. australis* is the plant of choice in European CWS, it is not the only aquatic macrophyte used in CWS. For instance, in North America CWSs are rarely planted with *P. australis*, because it is considered an invasive species (Keller, 2000) and instead are planted with species such as yellow flag (*Iris pseudacorus*), cattail

(*Typha domingensis*) and bulrushes (*Typha latifolia*) (Kadlec *et al.*, 2001). Where other macrophytes are used in CWS, the inherent polymorphism in the species should be investigated because intraspecific genetic differences may affect the efficacy of nutrient removal in similar ways to those found in the present study.

References

Adams, W.T., Allard, R.W., 1977. Effects of polyploidy on phosphoglucose isomerase diversity in *Festuca microstachys*. Proceeding of the National Academy of Sciences of the United States. 74 (4), 1652-1656.

Ainsworth, E.A., Tranel, P.J., Drake, B.G., Long, S.P., 2003. The clonal structure of *Quercus geminata* revealed by conserved microsatellite loci. Molecular Ecology. 12, 527-532.

Allen, S.E., Grimshaw, H.M., Parkinson J.A., Quarmby, C., 1974. Chemical analysis of ecological methods. Blackwell Scientific Publications, Oxford, UK.

Amsberry, L., Baker, M.A., Ewanchuk, P.J., Bertness, M.D., 2000. Clonal integration and the expansion of *Phragmites australis*. Ecological Applications. 10 (4), 1110-1118.

Ansola, G., Fernández, C., de Luis, E., 1995. Removal of organic matter and nutrients from urban wastewater by using an experimental emergent aquatic macrophyte system. Ecological Engineering. 5, 13-19.

Armstrong, J., Armstrong, W., 1990. Light-enhanced convective throughflow increases oxygenation in rhizomes and rhizosphere of *Phragmites australis* (Cav.) Trin. ex Steud. New Phytologist. 114, 121-128.

Armstrong, J., Armstrong, W., Beckett, P.M., 1990. Measurement and modelling of oxygen release from roots of *Phragmites australis*. In: P.F. Cooper and B.C. Findlater (Eds.), Constructed wetlands in water pollution control, proceedings of the international conference on the use of constructed wetlands in water pollution control. Pergamon Press, Oxford, UK, pp. 41-51.

Armstrong, J., Armstrong, W., Beckett, P.M., 1992. *Phragmites australis*: Venturi- and humidity-induced pressure flows enhance rhizome aeration and rhizosphere oxidation. New Phytologist. 120, 197-207.

Armstrong, J., Armstrong, W., Armstrong, I.B., Pittaway, G.R., 1996. Senescence, and phytotoxin, insect, fungal and mechanical damage: factors reducing convective gas-flows in *Phragmites australis*. Aquatic Botany. 54, 211-226.

Aspinwall, N., Christian, T., 1992. Clonal structure, genotypic diversity, and seed production in populations of *Filipendula rubra* (Rosaceae) from the northcentral United States. American Journal of Botany. 79 (3), 294-299.

Barrett, S.C.H., Eckert, C.G., Husband, B.C., 1993. Evolutionary process in aquatic plant populations. Aquatic Botany. 44. 105-145.

Bedford, B.L., Bouldin, D.R., Beliveau, B.D., 1991. Net oxygen and carbon-dioxide balances in solutions bathing roots of wetland plants. Journal of Ecology. 79, 943-959.

Bibby, C.J., Lunn, J., 1982. Conservation of reed beds and their avifauna in England and Wales. Biological Conservation. 23, 167-186.

Björk, S., 1967. Ecological investigations of *Phragmites communis*. Studies in theoretical and applied limnology. Folia Limnologica Scandinavica. 14, 1-248.

Bradshaw, A. D., 1958. Population differentiation in *Agrostis tenuis* Sibth. II. The incidence and significance of infection by *Epichloe typhina*. New Phytologist. 58, 310-315.

Brennan, A.C., Harris, S.A., Hiscock, S.J., 2005. Modes of selfing and associated inbreeding depression in the self-incompatible plant *Senecio squalidus* (Asteraceae): a successful colonizing species in the British Isles. New Phytologist. 168, 475-486.

Briggs, D., Walters, S.M., 1997. Plant variation and evolution. (3rd Edition). Cambridge University Press, UK.

Brix, H., 1994. Functions of macrophytes in constructed wetlands. Water Science Technology. 29, 71-78.

Brix, H., 1999. Genetic diversity, ecophysiology and growth dynamics of reed (*Phragmites australis*). Aquatic Botany. 64, 179-184.

Brix, H., Schierup, H.H., 1990. Soil oxygenation in constructed reed beds: the role of macrophytes and soil-atmosphere interface oxygen transport. In: P.F. Cooper and B.C. Findlater (Eds.), constructed wetlands in water pollution control, proceedings of the international conference on the use of constructed wetlands in water pollution control. Pergamon Press, Oxford, UK, pp. 53-66.

Callaghan, T.V., Carlsson, B.Å., Jónsdóttir, I.S., Svenssson, B.M., Jonasson, S., 1992. Clonal plants and environmental change: introduction to the proceedings and summary. Oikos. 63, 341-347.

Chapin III, F.S., Chapin, M.C., 1981. Ecotypic differentiation of growth processes in *Carex aquatilis* along latitudinal and local gradients. Ecology. 62, 1000-1009.

Charlesworth, D., Charlesworth, B., 1987. Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics. 18, 237-268.

Cheliak, W.M., Morgan, K., Strobeck, C., Yeh, F.C.H., Dancik, B.P., 1983. Estimation of mating system parameters in plant populations using the EM algorithm. Theoretical and Applied Genetics. 65, 157-161.

Clarke, E., Baldwin, A.H., 2002. Responses of wetland plants to ammonia and water level. Ecological Engineering. 18, 257-264.

Clay, K., 1990a. Fungal endophytes of grasses. Annual Review of Ecology and Systematics. 21, 275-297.

Clay, K., 1990b. Insects, endophytic fungi and plants. In: J. J. Burdon, and S.R. Leather (Eds.), Pests pathogens and plant communities. Blackwell Scientific Publications, Oxford, UK, pp. 111-130.

Clevering, O.A., 1999. Between- and within- population differences in *Phragmites australis* 1. The effects of nutrients on seedling growth. Oecologia. 121, 447-457.

Clevering, O.A., Lissner, J., 1999. Taxonomy, chromosomes number, clonal diversity and population dynamics of *Phragmites australis*. Aquatic Botany. 64, 185-208.

Clevering, O.A., Brix, H., Lukavská, J., 2001. Geographic variation in growth responses in *Phragmites australis*. Aquatic Botany. 69, 89-108.

Cooper, P.F., Job, G.D., Green, M.B., Shutes, R.B.E., 1996. Reed beds and constructed wetlands for wastewater treatment. WRc Swindon, Wiltshire, UK.

Coops, H., Geilen, N., Verheij, H.J., Boeters, R., van der Velde, G., 1996. Interaction between waves, bank erosion and emergent vegetation: an experimental study in a wave tank. Aquatic Botany. 53, 187-198.

Curran, P.L., 1969. Fertility of *Phragmites communis* Trin. Irish Naturalist. 16, 242.

Dabkevičius, Z., Mikaliūnaitė, R., 2005. The effect of fungicidal seed treaters on germination of rye ergot (*Claviceps purpurea* (Fr.) Tul.) sclerotia and on ascocarps formation. Crop Protection. (Article in Press).

Daniels, R.E., 1991. Variation in performance of *Phragmites australis* in experimental culture. Aquatic Botany. 42, 41-48.

Department of Environment (DoE), 1981. Assessment of biodegradability. Methods for the examination of waters and associated materials. HMSO, London, UK.

Dole, J., Ritland, K., 1993. Inbreeding depression in two *Mimulus* taxa measured by multigenerational changes in the inbreeding coefficient. Evolution.47 (2), 361-373.

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus. 12, 13-15.

Drizo, A., Frost, C.A., Smith, K.A., Grace, J., 1997. Phosphate and ammonium removal by constructed wetlands with horizontal subsurface flow, using shale as a substrate. Water Science Technology. 35, 95-102.

Duran, S., Pascual, M., Estoup, A., Turon, X., 2004. Strong population structure in the marine sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers. Molecular Ecology. 13, 511-522.

Durand, J., Garnier, L., Dajoz, I., Mousset, S., Veuille, M., 2000. Gene flow in a facultative apomictic Poacea, the savannah grass *Hyparrhenia diplandra.* Genetics. 156, 823-831.

Dykyjová, D., Hradecká, D., 1973. Productivity of reed-bed stands in relation to the ecotype, microclimate and trophic conditions of the habitat. Polskie Archiwum Hydrobiologii. 20, 111-119.

Ellstrand, N.C., Roose, M.L., 1987. Patterns of genetic diversity in clonal plant species. American Journal of Botany. 74 (1), 123-131.

EN (English Nature), 2003. Definition of Reedbeds. http://www.english-nature.org.uk/livingwiththesea/projects_details/good_practice_guide/habitatcrr/ENRestore/Habitats/Reedbed/Definition.htm (accessed January 2006).

Ennos, R.A., Worrell, R., Malcolm, D.C., 1998. The genetic management of native species in Scotland. Forestry. 71, 1-23.

Eriksson, O., 1997. Clonal life histories and the evolution of seed recruitment. In: H. de Kroon and J. van Groenedael (Eds.), The ecology and evolution of clonal plants. Backhuys Publications, Netherlands, pp. 211-226.

Estoup, A., Jarne, P., Cornuet, J.M., 2000. Homoplasy and mutation at microsatellite loci and their consequences for population genetic analysis. Molecular Ecology. 11, 1591-1604.

Europa, 2003. Bird species listed in Annex I of Council Directive 79/409/EEC http://europa.eu.int/comm/environment/nature/bird-an.htm (accessed September 2003).

Fairlamb, D., 2000. Mersehead management plan, 2000-2005. RSPB, Sandy, Bedfordshire, UK.

Finlay, K.W., Wilkinson, G.N., 1963. The analysis of adaptation in a plant-breeding programme. Australian Journal of Agricultural Research. 14, 742-754.

García, J., Vivar, J., Aromir, M., Mujeriego, R., 2003. Role of hydraulic retention time and granular medium in microbial removal in tertiary treatment beds. Water Research. 37, 2645-2653.

Gibbons, D., Avery, M., Baillie, S., Gregory, R., Kirby, J., Porter, R., Tucker, G., Williams, G., 1996. 1. Bird Species of Conservation Concern in the United Kingdom, Channel Islands and Isle of Man: revising the Red Data List. RSPB Conservation Review. 10, 7-18.

Gilbert, G., Painter, M., Smith, K.W., 1996. An inventory of British Reedbeds in 1993. RSPB Conservation Review. 10, 39-44.

Goudet, J., 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). available from: http://www.unil.ch/izea/softwares/fstat.html. Updated from Goudet (1995).

Gray, A.J., Drury, M., Raybould A.F., 1990. *Spartina* and the ergot fungus *Claviceps purpurea* - a singular contest? In: J.J. Burdon, and S.R. Leather (Eds.), Pests pathogens and plant communities. Blackwell Scientific Publications, Oxford, UK, pp. 63 -79.

Gray, K.R., Biddlestone, A.J., 1995. Engineered reedbed systems for wastewater treatment. Trends in Biotechnology. 13, 248-252.

Green, S., Hawkins, I., Howe, A., Kemp, M., Needle, A., Parfitt, A., Patrick, L., Rose, T., Welch, G., 2003. Minsmere Reserve plan, 2003 - 2008. RSPB, Sandy, Bedfordshire, UK.

Greenway, M., Woolley, A., 1999. Constructed wetlands in Queensland: Performance efficiency and nutrient bioaccumulation. Ecological Engineering. 12, 39-55.

Gries, C., Kappen, L., Lösch, R., 1990. Mechanisms of flood tolerance in reed *Phragmites australis* (Cav.) Trin. ex Stuedel. New Phytologist. 114, 589-593.

Guo, W., Wang, R., Zhou, S., Zhang, S., Zhang, Z., 2003. Genetic diversity and clonal structure of *Phragmites australis* in the yellow River delta of China. Biochemical systematics and ecology. 31 (10), 1073-1220.

Gustafsson, Å. and Simak, M., 1963. X-ray photography and seed sterility in *Phragmites communis* Trin. Hereditas. 49, 442-450.

Hanganu, J., Mihail, G., Coops, H., 1999. Responses of ecotypes of *Phragmites australis* to increased seawater influence: a field study in the Danube Delta, Romania. Aquatic Botany. 64, 351-358.

Hara, T., van der Toorn, J., Mook, J.H., 1993. Growth dynamics and size structure of shoots of *Phragmites australis*, a clonal plant. The Journal of Ecology. 81, 47-60.

Hardej, M., Ozimek, T., 2002. The effect of sewage sludge flooding on growth and morphometric parameters of *Phragmites australis* (Cav.) Trin. ex Stuedel. Ecological Engineering. 18, 343-350.

Haslam, S., 1969. Stem types of *Phragmites communis* Trin. Annals of Botany. 33, 127-131.

Haslam, S., 1970a. The development and establishment of young plants of *Phragmites communis* Trin. Annals of Botany. 35, 1059-1072.

Haslam, S., 1970b. The performance of *Phragmites communis* Trin. in relation to water-supply. Annals of Botany. 34, 867-877.

Haslam, S., 1972. *Phragmites communis* Trin. (*Arundo Phragmites*.L.,? *Phragmites australis* (Cav.) Trin. ex Steudel). Journal of Ecology. 60, 585-610.

Hauber, D.P., White, D.A., Powers, S.P., DeFrancesch, F.R., 1991. Isozyme variation and correspondence with unusual reflectance patterns in *Phragmites australis* (Poaceae). Plant Systematics and Evolution. 178, 1-8.

Hawke, C.J., José, P.V., 1996. Reedbed management for commercial and wildlife interests. The Royal Society for the Protection of Birds, Sandy, Bedfordshire, UK.

ter Heerdt, G.N.J., Drost, H.J., 1994. Potential for the development of marsh vegetation from the seed bank after a drawdown. Biological Conservation. 67, 1-11.

Hellings, S.E., Gallagher, J.L., 1992. The effects of salinity and flooding on *Phragmites australis*. Journal of Applied Ecology. 29, 41-49.

HMSO, 1994. Biodiversity: The UK steering group report. Volume 2: Action Plans. HMSO Publications Centre, London, UK.

Hubbard, C.E., 1992. Grasses. (3rd Edition). Penguin Books Ltd., London.

Ishii, J., Kadono, Y., 2002. Factors influencing seed production of *Phragmites australis*. Aquatic Botany. 72, 129-141.

Jarne, P., Charlesworth, D., 1993. The evolution of the selfing rate in functionally hermaphrodite plants and animals. Annual Review of Ecological Systematics. 24, 441-466.

Jarne, P., Lagoda, P.J.L., 1996. Microsatellites, from molecules to populations and back. Trends in Evolution and Ecology. 11 (10), 424-429.

Jenkins, G., Rees, H., 1991. Strategies of bivalent formation in allopolyploid plants. Proceedings of the Royal Society London B series. 243, 209-214.

Jenkinson, J.G., 1958. Ergot infection of grasses in the south-west of England. Plant Pathology. 7, 81-85.

JNCC, 2001. Information sheet on Rasmar wetlands-Loch Leven. http://www.jncc.gov.uk/pdf/RIS/7UK007.pdf (accessed January 2006).

Jones, N., Ougham, H., Thomas, H., 1997. Markers and mapping: we are all geneticists now. New Phytologist. 137,165-177.

Jong, K., Möller, M., 2000. New chromosome counts in *Streptocarpus* (Gesneriaceae) from Madagascar and the Comoro Islands and their taxonomic significance. Plant Systematics and Evolution. 124, 173-182.

Jonsson, L., 1999. Birds of Europe. Christopher Helm Publishers Ltd, London, UK.

Jonsson, B.O., Jonsdottir I.S., Cronberg, N., 1996. Clonal diversity and allozyme variation in populations of the arctic sedge *Carex bigelowii* (Cyperaceae). The Journal of Ecology. 84 (3), 449-459.

Kadlec, R.H., 1997. An autobiotic wetland phosphorous model. Ecological Engineering. 8, 145-172.

Kadlec, R.H., Knight, R.L., Vymazal, J., Brix, H., Cooper, P., Haberl, R., 2001. Constructed wetlands for pollution control. Scientific and Technical Report No. 8. IWA Publishing, London, UK.

Karathanasis, A.D., Potter, C.L., Coyne, M.S., 2003. Vegetation effects on faecal bacteria, BOD, and suspended solid removal in constructed wetlands treating domestic wastewater. Ecological Engineering. 20, 157-169.

Karunaratne, S., Asaeda, T., Yutani, K., 2003. Growth performance of *Phragmites australis* in Japan: influence of geographic gradient. Environmental and Experimental Botany. 50, 51-66.

Keller, B.E.M., 2000. Genetic variation among and within populations of *Phragmites australis* in the Charles River watershed. Aquatic Botany. 66, 195-208.

Kern, J., Idler, C., 1999. Treatment of domestic and agricultural wastewater by reed bed systems. Ecological Engineering. 12, 13-25.

Klekowski, E.J.J., 1997. Somatic mutation theory of clonality. In: H. de Kroon and J. van Groenendael, (Eds.), Ecology and Evolution of Clonal Plants. Backhuys Publications, The Netherlands, pp. 227-242.

Koppitz, H., 1999. Analysis of genetic diversity among selected populations of *Phragmites australis* world-wide. Aquatic Botany. 64, 209-221.

Koppitz, H., Kühl, H., Hesse, K., Kohl, J.G., 1997. Some aspects of the importance of genetic diversity in *Phragmites australis* (Cav.) Trin. ex Stuedel for the Development of Reed Stands. Botanica Acta 110, 217-223.

de Kroon, H., Kalliola, R., 1994. Shoot dynamics of the giant grass *Gynerium sagittatum* in Peruvian Amazon floodplains, a clonal plant that does show self-thinning. Oecologia. 101, 124-131.

Lambertini, C., Brix, H., Speranza, M., 2003. Clonal variability of the common reed, *Phragmites australis* (Cav.) Trin. ex Steudel, in the Po Plain. Agroindustria. 2 (2-3), 121-126.

Langmuir, D., 1971. Eh-pH determination. In: R.E. Carver (Ed.), Procedures in Sedimentary Petrology. John Wiley, Chichester, UK, pp. 597-634.

Leitch, I.J., Bennett, M.D., 1997. Polyploidy in angiosperms. Trends in Plant Science. 2 (12), 470-476.

Les, D.H., Philbrick, C.T., 1993. Studies in hybridisation and chromosome number variation in aquatic angiosperms: evolutionary implications. Aquatic Botany. 44 (2-3), 181-228.

Lin, Y.F., Jing, S.R., Lee, D.Y., Wang, T.W., 2002. Nutrient removal from aquaculture wastewater using a constructed wetland system. Aquaculture. 209. 169-184.

Loo-Dinkins, J., 1992. Field design test. In: L. Fins, S.T. Friedman, J.V. Brotschol (Eds.). Handbook of quantitative forest genetics. Kluwer Academic Publications, The Netherlands, pp. 96-139.

Lovett Doust, L., 1981. Population dynamics and local specialization in a clonal perennial (*Ranunculus repens*). I The dynamics of ramets in contrasting habitats. Journal of Ecology. 69, 743-755.

Lowe, A., Harris, S., Ashton, P., 2004. Ecological genetics. Blackwell Science Ltd. Oxford, UK.

Luederitz, V., Eckert, E., Lange-Weber, M., Lange, A., Gersberg, R.M., 2001. Nutrient removal efficiency and resource economics of vertical flow and horizontal flow constructed wetlands. Ecological Engineering. 18, 157-171.

Mahy, G., Vekemans, X., Jaquemart A.L., 1999. Patterns of allozymic variation within *Calluna vulgaris* populations at seed bank and adult stages. Heredity 82. 432-440.

Martin, E., Hine, R.S., 2000. Oxford dictionary of biology. Oxford University Press, Oxford, UK.

Mayr, E., 1942. Systematics and the origin of species. Columbia University Press, New York.

McKee, J., Richards, A.J., 1996. Variation in seed production and germinability in common reed (*Phragmites australis*) in Britain and France with respect to climate. New Phytologist. 133, 233-243.

McKone, M.J., Thom, A.L., Kelly, D., 1997. Self-compatibility in *Chionochloa pallens* and *C. macra* (Poaceae) confirmed by hand pollination of excised styles. New Zealand Journal of Botany. 35, 259-262.

McLellan, A.J., Prati, D., Kaltz, O., Schmid, B., 1997. Structure and analysis of phenotypic and genetic variation in clonal plants. In: H. de Kroon and J. van Groenedael (Eds.), The ecology and evolution of clonal plants. Backhuys Publications, The Netherlands, pp. 211-226.

Meuleman, A.F.M., Beekman, J.H.P., Verhoeven, J.T.A., 2002. Nutrient retention and nutrient -use efficiency in *Phragmites australis* stands after wastewater application. Wetlands. 22 (4), 712-721.

Mueller, U.G., Wolfenbarger, L., 1999. AFLP genotyping and fingerprinting. Trends in Ecology and Evolution. 14, 389-394.

Münch, C., Kuschk, P., Roske, I., 2005. Root stimulated nitrogen removal: only a local effect or important for wastewater treatment. Water Science and Technology. 51 (9), 185-192.

Nei, M., 1987. Molecular evolutionary genetics. Columbia University Press, New York

Neuhaus, D., Kühl, H., Kohl, J.G., Dörfel, P., Börner, T., 1993. Investigation on the genetic diversity of *Phragmites* stands using genomic fingerprinting. Aquatic Botany. 45, 357-364.

Nikolajevskij, V.G., 1971. Research into the biology of the common reed (*Phragmites communis* Trin.) in the U.S.S.R. Folia Geobotanica Phytotaxonomica. 6, 221-230.

Parker, M.A., 1988. Genetic uniformity and disease resistance in a clonal plant. The American Naturalist. 132 (4), 538-549.

Parks, J.C., Werth, C.R., 1993. A study of spatial features of clones in a population of Bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). American Journal of Botany. 80 (5), 537-544.

Paucã-Comãnescu, M., Clevering, O.A., Hanganu, J., Gridin, M., 1999. Phenotypic differences among ploidy levels of *Phragmites australis* growing in Romania. Aquatic Botany. 64, 223-234.

Pemberton, J.M., Slate, J., Bancroft, D.R., Barrett, J.A., 1995. Non-amplifying alleles at microsatellite loci: a caution for parentage and population studies. Molecular Ecology. 4, 249-252.

Peterson, S.B., Teal, J.M., 1996. The role of plants in ecologically engineered wastewater treatment systems. Ecological Engineering. 6, 137-148.

Peverley, J.H., Surface, J.M., Wang, T., 1995. Growth and trace metal absorption by *Phragmites australis* in wetlands constructed for landfill leachate treatment. Ecological Engineering. 5, 21-35.

Poulin, B., Lefebvre, A., Mauchamp, A., 2002. Habitat requirements of passerines and reedbed management in southern France. Biological Conservation. 107, 315-325.

Prescott, T., 2000. Insh Marshes management plan 2000 - 2005. RSPB, Sandy, Bedfordshire, UK.

Preston, C.D., Pearman, D.A., Dines, T.D., 2002. New atlas of the British and Irish flora. Oxford University Press, Oxford, UK.

Proctor, M., Yeo, P., Lack, A., 1996. The natural history of pollination. Harper Collins Publishers, UK.

Rai, U.N., Sinha, S., Tripathi, R.D., Chandra, P., 1995. Wastewater treatability potential of some aquatic macrophytes: Removal of heavy metals. Ecological Engineering. 5, 5-12.

Reynolds, T., 2004. An Investigation into the effects of soil properties on seedset and germination in *Phragmites australis*. Unpublished BSc. dissertation, School of GeoSciences, University of Edinburgh.

Reusch, T.B.H., Stam, W.T., Olsen, J.L., 1998. Size and estimated age of genets in eelgrass, *Zostera marina*, assessed with microsatellite markers. Marine biology. 133, 519-525.

Reusch, T.B.H., Stam, W.T., Olsen, L., 2000. A microsatellite-based estimation of clonal diversity and population subdivision min *Zostera marina*, a marine flowering plant. Molecular Ecology. 9, 127-140.

Ricklefs, R.E., 1990. Ecology. (3rd Edition). W.H. Freeman and Company, New York.

Ritland, K., 2002. Extensions of models for the estimation of mating systems using *n* independent loci. Heredity. 88, 221-228.

Ritland, K., Jain, S., 1981. A model for the estimation of outcrossing rate and gene frequencies using *n* independent loci. Heredity. 47, 35 -52.

Roach, D.A., Wulff, R.D., 1987. Maternal effects in plants. Annual Review of Ecological Systematics. 18, 209-235.

Rodwell, J.S., 1995. British plant communities. Volume 4. Press Syndicate of the University of Cambridge, Cambridge, UK.

Rousset, F., 1997. Genetic differentiation and estimation of gene flow from *F*-Statistics under isolation by distance. Genetics. 145, 1219-1228.

Ruggiero, M.V., Capone, S., Pirozzi, P., Reusch, T.B.H., Procaccini, G., 2005. Mating system and clonal architecture: a competitive study in two marine angiosperms. Evolutionary Ecology. 19, 487-499.

Saltonstall, K., 2003. Microsatellite variation within and among North American lineages of *Phragmites australis*. Molecular Ecology. 12, 1689-1702.

Schluter, D., 1998. Ecological Causes of Speciation. In: D.J. Howard and S.H. Berlocher (Eds.), Endless Forms. Oxford University Press, New York, pp. 114-129.

Self, M., 1999. Effective establishment of reedbeds in created wetlands for wildlife. Cranfield University, Silsoe College, School of Agriculture, Food and Environment. Ph.D.

Self, M., Hawke, C.J., José, P.V., 1996. Reedbed Enhancement and Creation at RSPB Nature Reserves. RSPB Conservation Review. 10, 45-53.

Shutes, R.B.E., 2001. Artificial wetlands and water quality improvement. Environment International. 26, 441-447.

Silverton, J. W., Charlesworth, D., 2001. Introduction to plant population biology. (4th Edition). Blackwell Science Ltd., Oxford, UK.

Sokal, R.R., Rohlf, F.J., 1995. Biometry. (3rd Edition). W.H. Freeman and Co., New York.

Soltis, P.S., Soltis, D.E., 2000. The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Science. 97 (13), 7051-7057.

Sorrell, B.K., Armstrong, W., 1994. On the difficulties of measuring oxygen release by root systems of wetland plants. Journal of Ecology. 82, 177-183.

Stottmeister, U., Wießner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R.A., Moorman, H., 2003. Effects of plants and microorganisms in constructed wetlands for wastewater treatment. Biotechnology Advances. 22, 93-117.

Sun, M., Ritland K., 1998. Mating system of yellow starthistle (*Centaurea solstitialis*), a successful colonizer in North America. Heredity. 80, 225-232.

Tanner, C.C., D'Eugenio, J., McBride, G.B., Sukias, J.F.S., Thompson, K., 1999. Effect of water level fluctuation on nitrogen removal from constructed wetland mesocosms. Ecological Engineering. 12, 67-92.

Tscharntke, T., 1990. Fluctuations in abundance of a stem-boring moth damaging shoots of *Phragmites australis*: Causes and effects of overexploitation of food in a late-successional grass monoculture. The Journal of Applied Ecology. 27, 679-692.

Tscharntke, T., 1999. Insects on common reed (*Phragmites australis*): community structure and the impact of herbivory on shoot growth. Aquatic Botany. 64, 399-410.

Tuley, G., 1995. Small native pinewoods - possible ways of saving them. Scottish Forestry. 49, 22-27.

United Nations, 1997. U. N. Conference on Environment and Development (1992)

http://www.un.org/geninfo/bp/enviro.html (accessed September 2003).

van der Toorn, J., 1972. Variability of *Phragmites australis* (Cav.) Trin. ex Stuedel in relation to the environment. Van Zee Tot Land. 48, 1-122.

Vartapetian, B.B., Jackson, M.B., 1997. Plant adaptations to anaerobic stress. Annals of Botany. 79 (Supplement A.), 3-20.

Véber, K., 1981. Evaluation of introduced forms in experimental plantations of common reed (*Phragmites communis* Trin.). Polskie Archiwum Hydrobiologii. 28, 33-42.

Verhoeven, J.T.A., Meuleman, A.F.M., 1999. Wetlands for wastewater treatment: opportunities and limitations. Ecological Engineering. 2, 5-12.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research. 23, 4407-4414.

Vretare, V., Weisner, S.E.B., Strand, J.A., Granéli, W., 2001 Phenotypic plasticity in *Phragmites australis* as a functional response to water depth. Aquatic Botany. 69, 127-145.

Vymazal, J., 2002. The use of sub-surface constructed wetlands for wastewater treatment in the Czech Republic: 10 years experience. Ecological Engineering. 18, 633-646.

Wang, Z.Y., Ge, Y., Scott, M., Spangenberg, G., 2004. Viability and longevity of pollen from transgenic and nontransgenic tall fescue *(Festuca arundinacea)* (Poaceae) plants. American Journal of Botany. 91, 523-530.

Waisel, Y., Rechav, Y., 1971. Ecotypic differentiation in *Phragmites communis* Trin. Hydrobiologia. 12, 259-266.

Weir, B.S., Cockerham, C.C., 1984. Estimating *F*-statistics for the analysis of population structure. Evolution. 38, 1358-1370.

Weißner, A., Kuschk, P., Kästner, M., Stottmeister, U., 2002. Abilities of helophyte species to release oxygen into rhizosperes with varying redox conditions in laboratory-scale hydroponic Systems. International Journal of Phytoremediation. 4 (1), 1-14.

Werker, A.G., Dougherty, J.M., McHenry, J.L., Van Loon, W.A., 2002. Treatment variability for wetland wastewater treatment design in cold climates. Ecological Engineering. 19, 1-11.

Widén, B., Cronberg, N., Widén, M., 1994. Genotypic diversity, molecular markers, molecular markers and spatial distribution of genets in clonal plants: a literature survey. Folia Geobotanica Phytotaxa. 29, 245-263.

Workneh, F., Rush, C.M., 2002. Evaluation of the relationship between weather patterns and prevalence of sorghum ergot in the Texas Panhandle. Phytopathology. 92 (6), 659-666.

Wright, S., 1943. Isolation by distance. Genetics. 28, 114-138.

Wright, S., 1951. The genetical structure of populations. Annals of Eugenics. 15. 323-354.

Wynne, G., Avery, M., Campbell, L., Gubbay, S., Hawkswell, S., Juniper, T., King, M., Newberry, P., Smart, J., Steel, C., Stones, T., Stubbs, A., Taylor, J., Tydeman, C., Wynde, R., 1995. Biodiversity Challenge (2nd Edition). RSPB, Sandy. Bedfordshire, UK.

Yang, L., Chang, H.T., Lo Huang, M.N., 2001. Nutrient removal in gravel- and soil-based wetland microcosms with and without vegetation. Ecological Engineering. 18, 91-105.

Ye, Z.H., Whiting, S.N., Lin, Z.Q., Qian, J.H., Terry, N., 2001. Removal and distribution of iron, manganese, cobalt and nickel within a Pennsylvania constructed wetland treating coal combustion by-product leachate. Journal of Environmental Quality. 30 (4), 1464-1473.

Zeidler, A., Schneider, S., Jung, C., Melchinger, A.E., Dittrich, P., 1994. The Use of DNA fingerprinting in ecological studies of *Phragmites australis* (Cav.) Trin. ex Steudel. Botanica Acta. 107, 237-242.