Appendices

Appendix 1: Chapter 2

Figure 1: The distribution of ploidy levels across the British and Irish angiosperms in the four families with the highest number of species. Shown are Rosaceae, Poaceae, Asteraceae and Fabaceae. Each family has distinct distributions of ploidy levels.

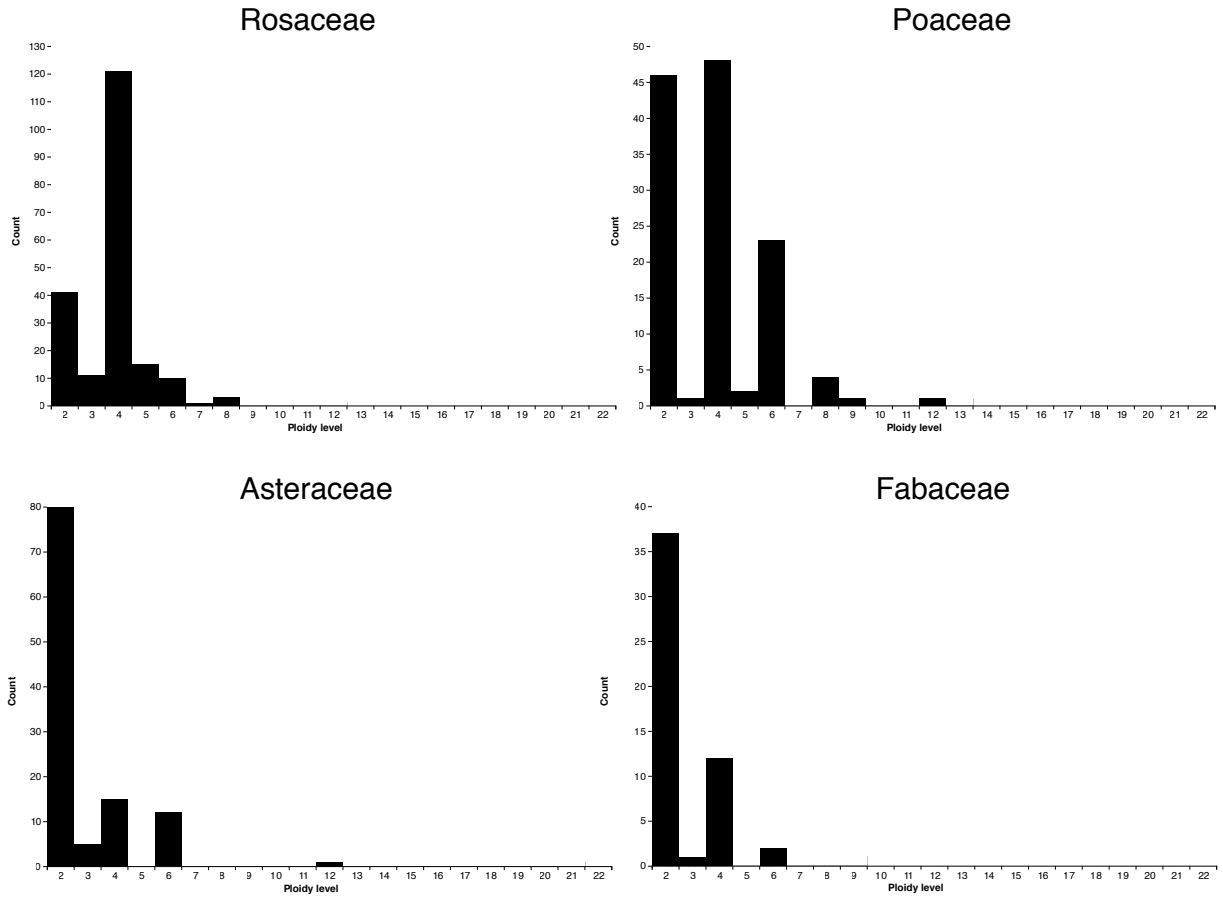


Table 1: Search strings for Google Scholar searches used to generate the list of examples of cross-ploidy hybrids in Chapter 2. Note that other examples were added if they were deemed to be important and/or well known.

Journal	Search string
Molecular Ecology	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:"Molecular Ecology"
Evolution	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid site:onlinelibrary.wiley.com source:"Evolution" -source:"and Evolution" -source:"Organic Evolution"
Heredity	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:"Heredity"
Annals of Botany	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:"Annals of Botany"
American Journal of Botany	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" American Journal of Botany"
New Phytologist	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" New Phytologist"
PNAS	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" PNAS"
Biological Journal of the Linnean Society	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" Biological Journal of the Linnean Society"
Botanical Journal of the Linnean Society	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" Botanical Journal of the Linnean Society"
Journal of Evolutionary Biology	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" Journal of Evolutionary Biology"
PLoS One	Ploidy hybrid genetic introgression diploid OR tetraploid OR hexaploidy OR octoploid source:" PLoS One"

Appendix 2: Chapter 3

Figure 2: Trees with root nodes containing the highest and lowest posterior mean probability of hybridisation from Model 1 (BLUP's of nodes in the phylogeny). A is the top tree (subset of Orchidaceae) whilst B is the tree with lowest probability of hybridisation (Fabaceae and Polygalaceae).

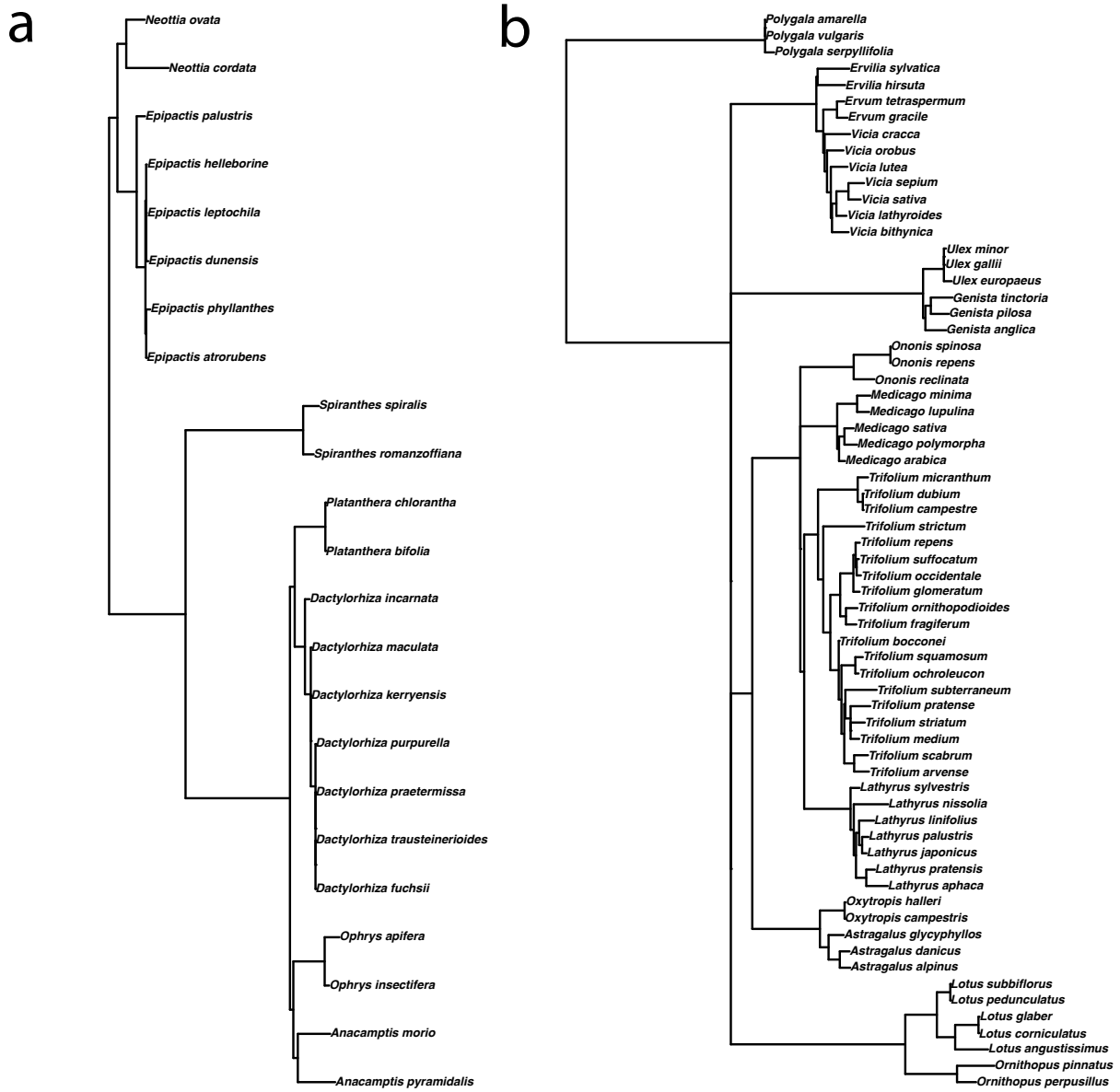


Figure 3: The joint probability of hybridisation between two parental species give both branch length between species (tree based genetic distance) and geographical overlap between parental species (measured as overlap in occupancy of 10x10km grid squares in the UK). The degree of shading in the scale bar and tiles represent the posterior probability of hybridisation from Model 1 given parameter values for each variable. Estimates are visualised at mean genus size, for annual-perennial parental combinations and accounting for phylogenetic relationships between species.

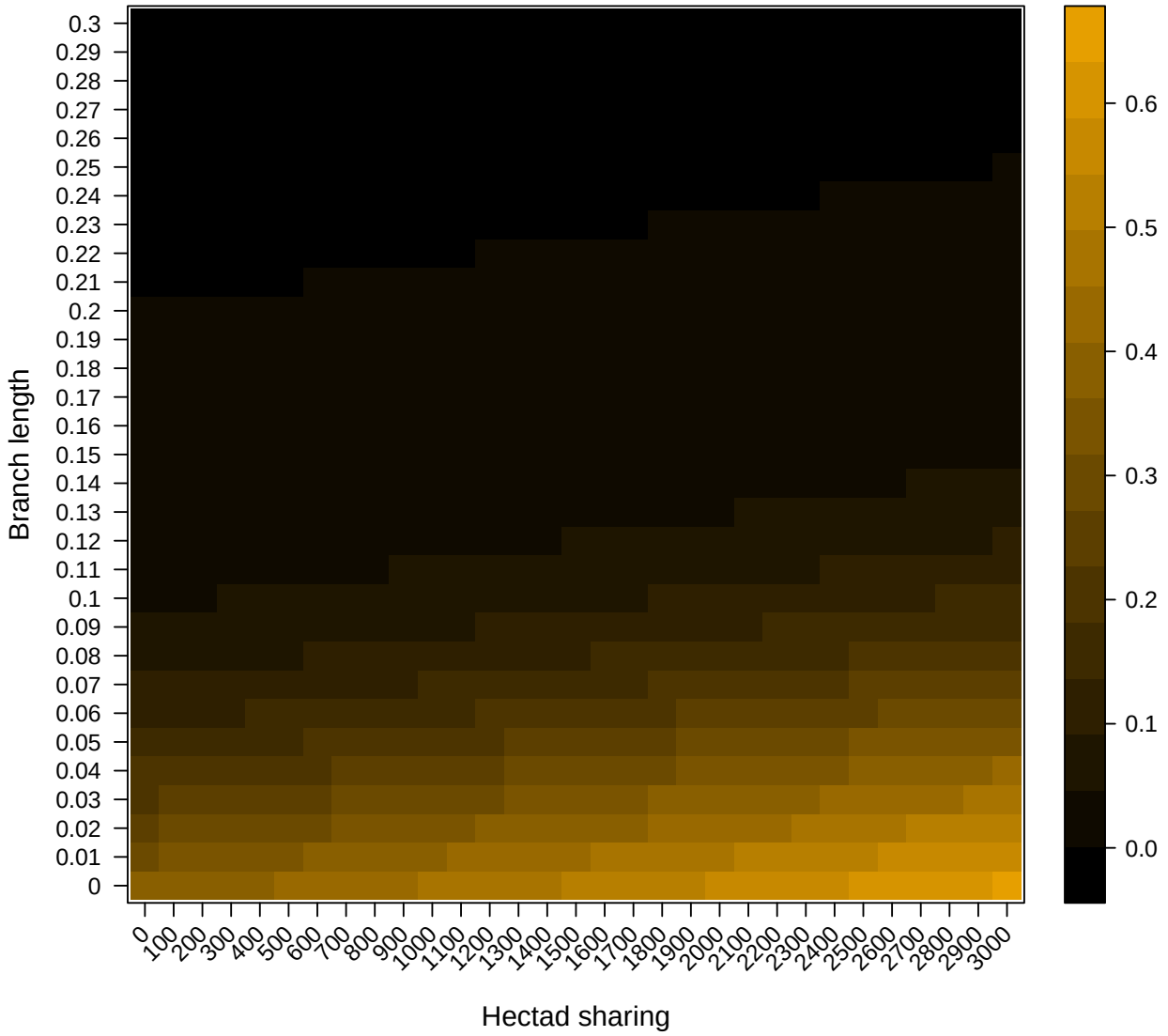


Figure 4: Predicted fit of probability of hybridisation given hectad sharing and ploidy difference of parental species from Model 2. Dashed lines indicate the 95% Credible Intervals, and the bold lines represent the posterior mode of the coefficients of congeneric pairs of species hybridising as a function of pairwise overlap in distribution, conditional on parental ploidy status. The effect is visualised at mean genetic distance for annual-perennial parent combinations and accounting for phylogenetic effects. The bold red dashed line indicates mean pairwise overlap in distribution (10x10km^2).

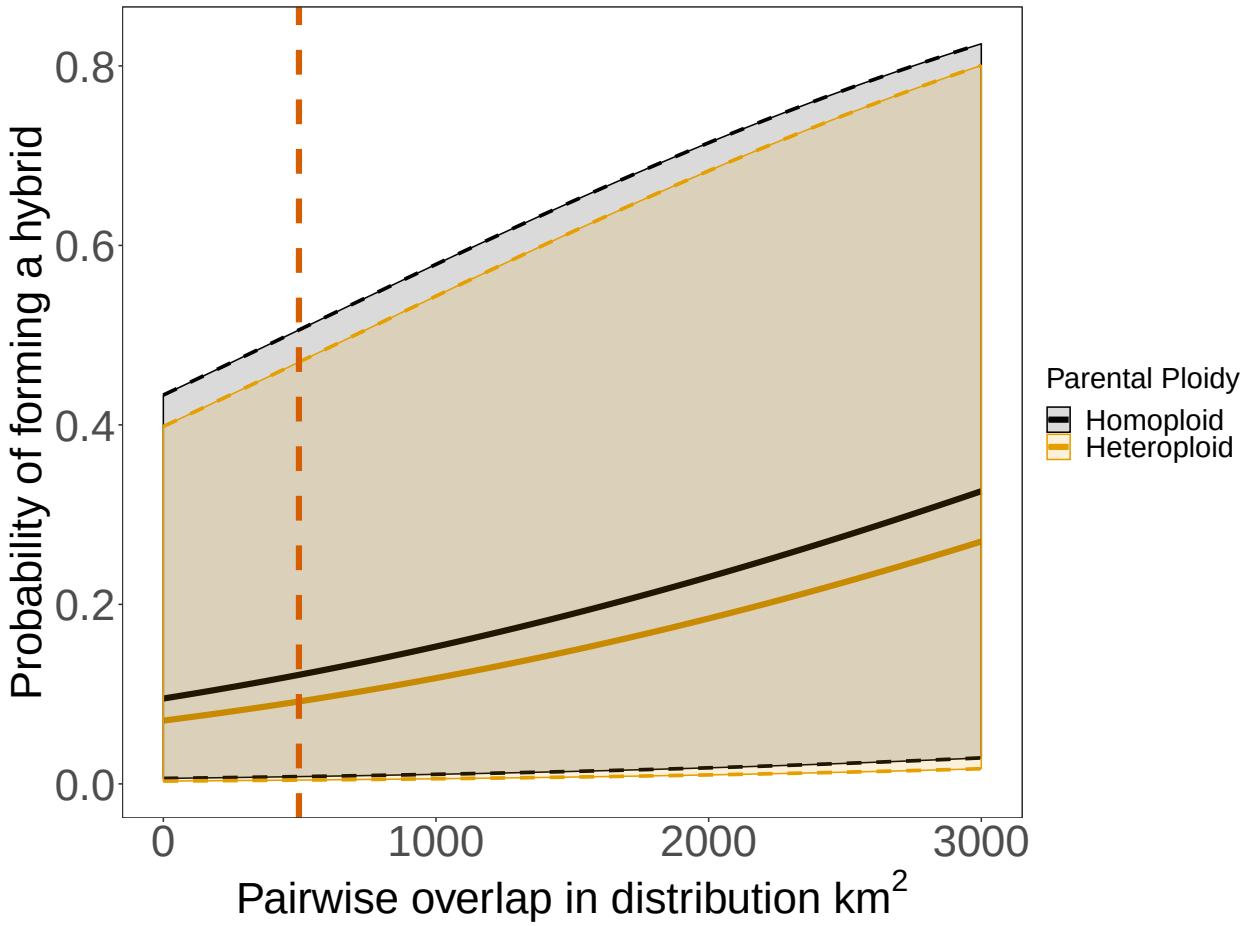


Figure 5: Predicted fit of probability of hybridisation given branch length between parental species and ploidy difference of parental species from Model 2. Homoploid indicates parental species of the same ploidy level, and heteroploidy indicates parental species of different ploidy levels. Dashed lines indicate the 95% Credible Intervals, and the bold lines represent the posterior mode of the coefficients of congeneric pairs of species hybridising as a function of pairwise branch length, conditional on parental ploidy status. The effect is visualised at mean hectad sharing for annual-perennial parent combinations and accounting for phylogenetic effects. The red dashed line indicates mean pairwise branch length between all pairs of species.

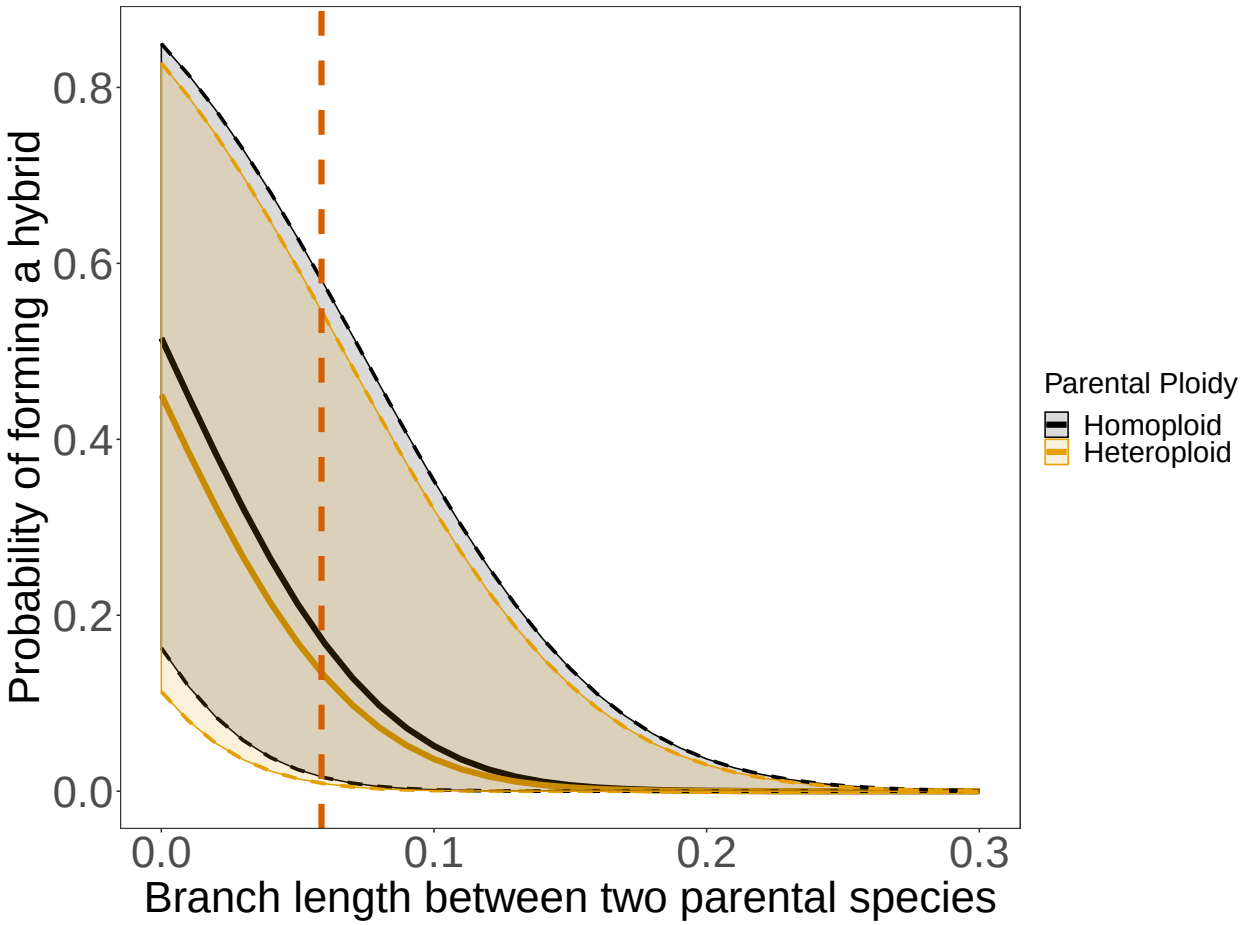


Figure 6: Predicted fit of probability of hybridisation given branch length between parental species from Model 1. Black dashed lines are the 95% Credible Intervals, bold line is the posterior mean of the coefficient for the probability of congeneric pairs of species hybridising as a function of branch length. This effect is visualised at mean hectad sharing, for annual-perennial parent combinations and accounting for phylogenetic effects. The bold red dashed line indicates mean genus level genetic distance between pairs of species.

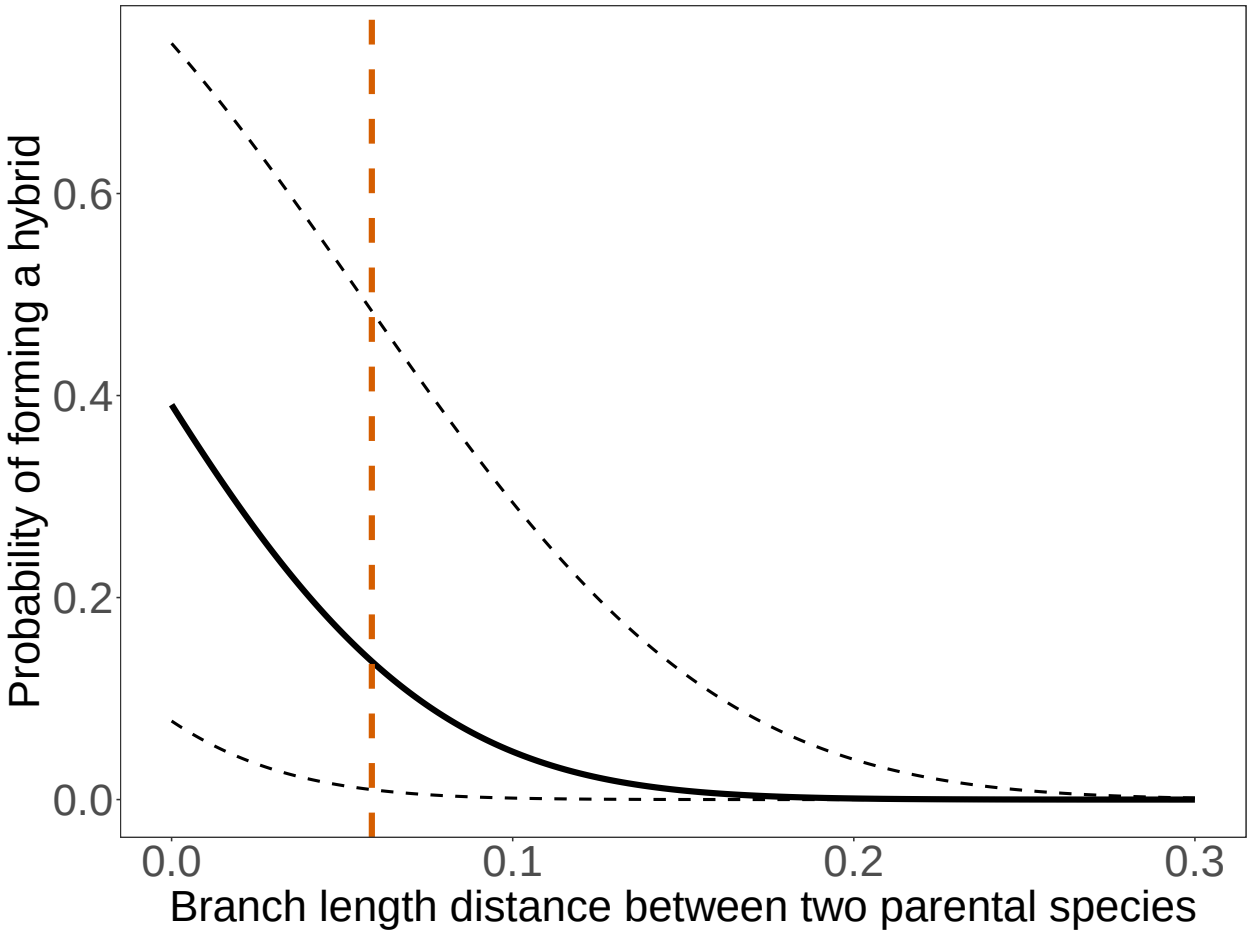


Table 2: Model 1: Probability of hybridisation on the probit scale with genetic distance, hectads shared and life history of parental species as fixed covariates. The posterior mean of the distribution of each coefficient is given, along with lower and upper 95% Credible Intervals. The p-value (pMCMC) is also reported and given in bold where significant. Annual-perennial and perennial-perennial levels are jointly tested using a Wald test in the main text.

Covariate	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	-1.31	-3.76	0.60	1000	0.22
Branch length between species pairs	-59.75	-66.69	-51.98	185.41	0.0010
Hectads shared between species pairs	0.001	0.0007	0.0012	1000	0.0010
Annual-perennial parent pair	-0.12	-0.97	0.66	1000	0.76
Perennial-perennial parent pair	0.64	-0.25	1.58	1000	0.16
Genus size	-0.0014	-0.041	0.031	1107	0.92

Table 3: Phylogenetic signal of probability of hybridisation and the species variance independent of phylogenetic effects on the probit scale. 95% Credible Intervals of the variances are also presented. See Methods in Chapter 3 for calculation.

Variance Component	Posterior Mode	Lower Credible Interval	Upper Credible Interval
Model 1 Phylogenetic Variance	0.62	0.32	0.77
Model 1 Species Variance	0.33	0.18	0.58
Model 2 Phylogenetic Variance	0.61	0.30	0.82
Model 2 Species Variance	0.34	0.084	0.44

Table 4: Model 2: Probability of hybridisation on the probit scale with ploidy, genetic distance, hectads shared and life history of parental species as covariates. The posterior mean of the distribution of each coefficient is given, along with lower and upper 95% Credible Intervals. The p-value (pMCMC) is also reported and given in bold where significant.

Covariate	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	-0.11	-2.41	2.12	813	0.93
Branch length between species pairs	-74.93	-88.38	-63.46	319	0.0010
Crosss ploidy effect	-0.73	-1.02	-0.40	1000	0.0010
Hectads shared between species pairs	0.0013	0.0009	0.0016	883	0.0010
Annual-perennial parent pair	0.093	-1.15	1.23	836	0.89
Perennial-perennial parent pair	0.82	-0.40	1.96	836	0.16
Genus size	-0.029	-0.084	0.031	621	0.32

Appendix 3: Chapter 4

Figure 7: Distributions of parameters fitted to the models with constant gene flow (blue), secondary contact (green), and without gene flow (grey) in the demographic simulation software, $\delta a \delta I$. The model with historic gene flow (orange) had poor AIC values, and so is omitted here.

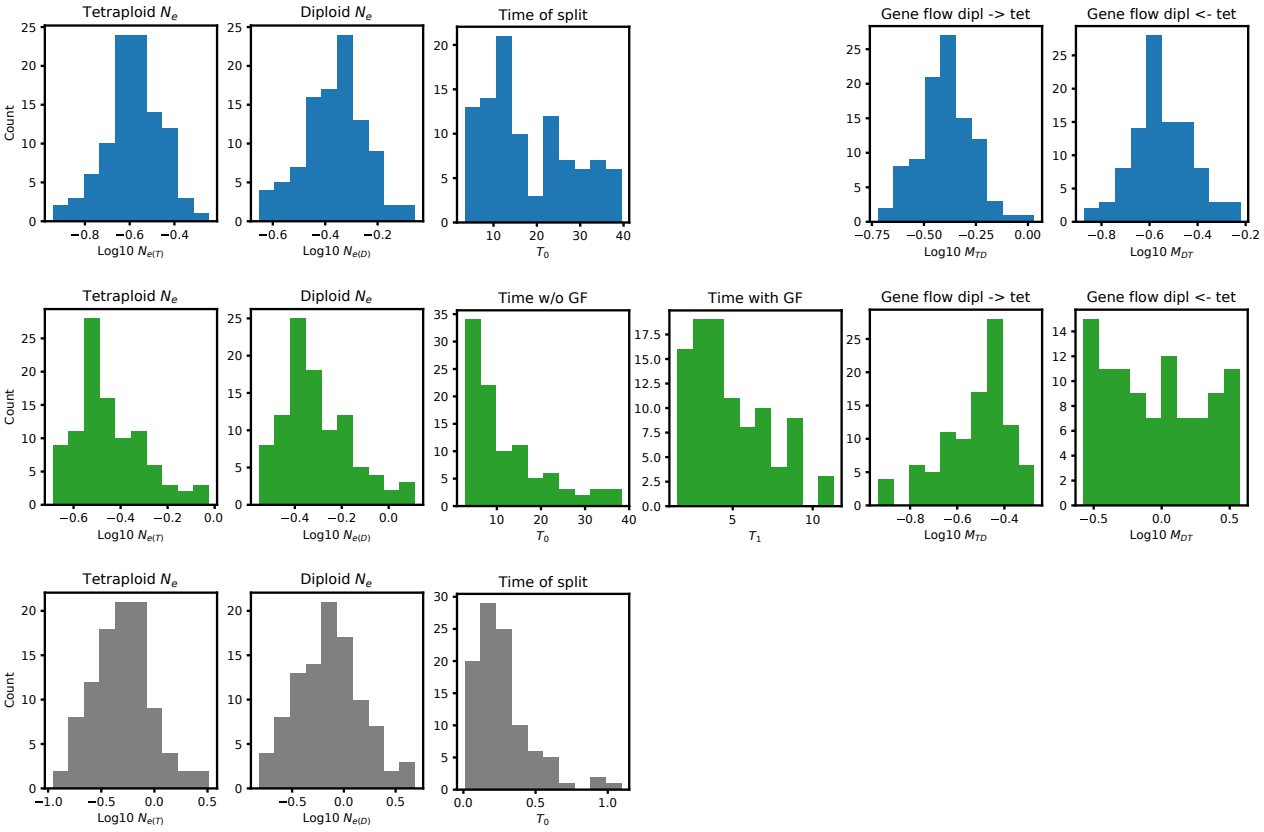


Table 5: Primers and PCR conditions used to amplify the *rpL32*-*trnL*$_{\mathrm{UAG}}$ plastid marker in *Euphrasia* species.

Primer	Orientation	Sequence (5'-3')	Reagents (1 reaction)	PCR conditions	References
rpL32-F	Forward	CAGTTCCAAAAAAACGTACTTC	12.5μM Taq 2X Master Mix, 0.5μL Bovine Serum Albumen, 0.5μL forward and reverse primers at 10μM, 10.5μL water, 1μL sample DNA	5 min at 94°C, 35× (30 s at 94°C, 45 s at 50°C, 40 s at 72°C), 5 min at 72°C	(Wang et al., 2018)
trnL$_{\mathrm{UAG}}$	Reverse	CTGCTTCCTAAGAGCAGCGT			

Table 6: Primers and PCR conditions used to amplify the ITS1 nuclear marker in *Euphrasia* species.

Primer	Orientation	Sequence (5'-3')	Reagents (1 reaction)	PCR conditions	References
ITS4	Forward	TCCTCCGCTTATTGATATGC	12.5μM Taq 2X Master Mix, 0.5μL Bovine Serum Albumen, 0.5μL forward and reverse primers at 10μM, 10.5μL water, 1μL sample DNA	5min at 94°C, 30 x (30s at 94°C, 30s at 54°C, 2min at 72°C), 10 min at 72°C.	(Wang et al., 2018)
ITS5	Reverse	GGAAGTAAAAGTCGTAACAAGG			

Appendix 4: Chapter 6

Table 7: Host species used in the common garden experiment in Chapter 6. The species along with the taxonomic family they belong to, their ecological functional group and the source of the seeds are also given. Commercial seed stocks list the original collection where known.

Common name	Species name	Family	Functional group (informal)	Seed source
Thale cress	*Arabidopsis thaliana*	Brassicaceae	Herb	Laboratory stock
Field horsetail	*Equisetum arvense*	Equisetaceae	Fern	Wild collected in Edinburgh (GPS coordinates: 55.9679, -3.2129)
Red fescue	*Festuca rubra*	Poaceae	Grass	Commerical: Emorsgate seeds (Yorkshire + Dorset)
Yorkshire fog	*Holcus lanatus*	Poaceae	Grass	Commerical: Emorsgate seeds
Common liverwort	*Marchantia polymorpha*	Marchantiaceae	Bryophyte	Wild collected in Edinburgh (GPS coordinates: 55.9679, -3.2129)
Ribwort plantain	*Plantago lanceolata*	Plantaginaceae	Herb	Commerical: Emorsgate seeds (Somerset + Wiltshire)
Scots pine	*Pinus sylvestris*	Pinaceae	Tree	Commerical: Scotia Seeds
White clover	*Trifolium repens*	Fabaceae	Herb	Commerical: Emorsgate seeds (Yorkshire + Wiltshire)

Table 8: Collection details for *Euphrasia* species used in the common garden experiment. *Population also used in the multiple host phenotypic plasticity experiment.

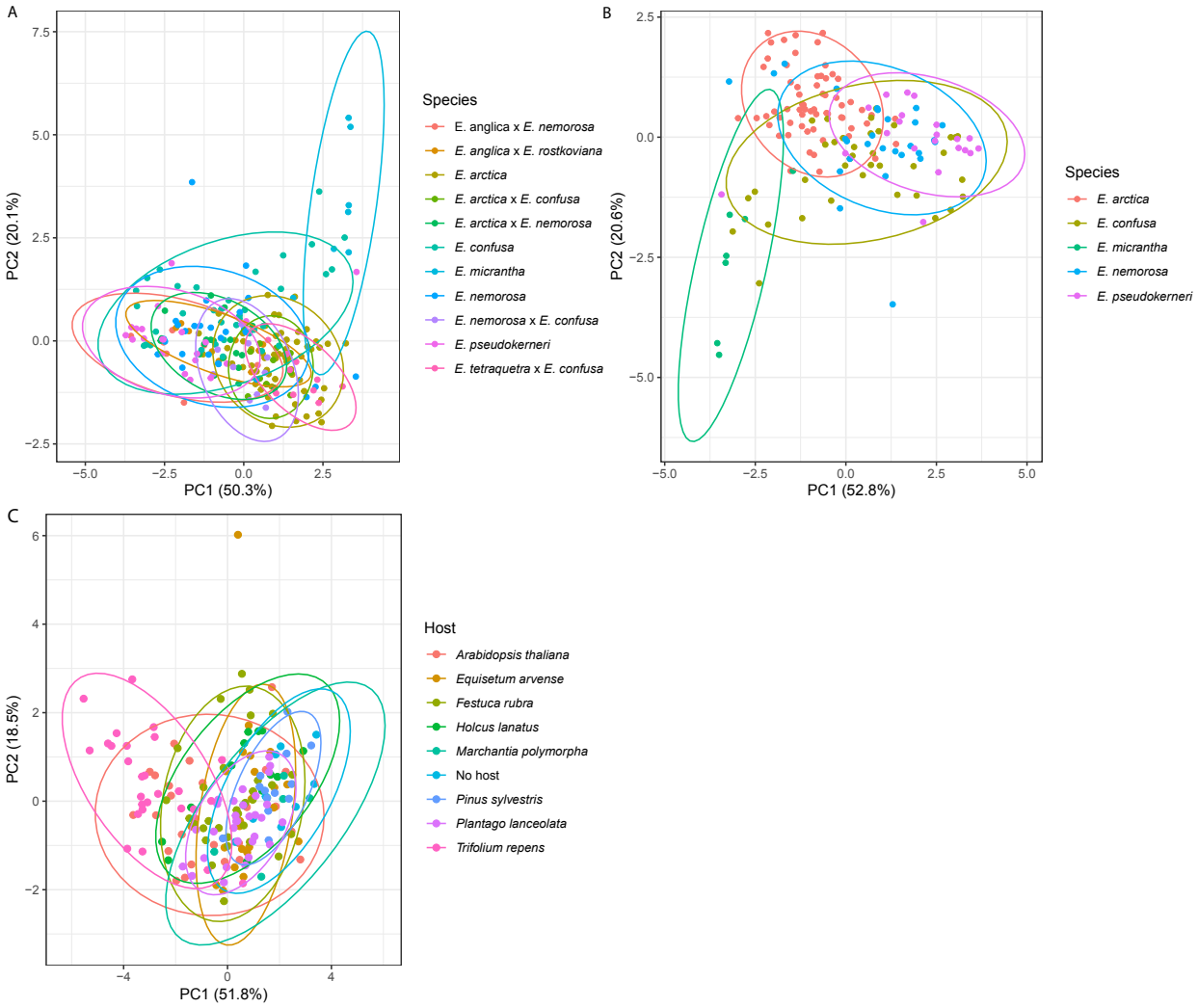
Collection number	Taxon	Locality	Latitude	Longitude	Collector
E4E0138	*E. arctica*	Fintallick, Glen Ledock, Comrie, Perthshire	56.41318	–4.03085	Dot Hall
E4E0144	*E. arctica*	Balachuirn, Isle of Raasay	57.38996	–6.06877	S.J. Bungard
E4E0032	*E. arctica*	South Links, Burray, Orkney	58.85275	–2.88701	John Crossley
E4E0139	*E. arctica*	Dalreoch Farm, Enochdhu	56.74199	–3.53350	Martin Robinson
E4E0049	*E. arctica*	Ouaisne, Jersey	49.17707	–2.18293	Anne Haden
E4E0247	*E. arctica*	Elsdon. Newcastle upon Tyne	55.22770	–2.10234	Stephanie Miles
NBer001*	*E. arctica*	North Berwick Glenn, East Lothian	56.05696	–2.70456	Alex Twyford
E4E0038	*E. confusa*	Oldbury, near Hartshill, Warwickshire	52.55285	–1.53980	John and Monika Walton
E4E0114	*E. confusa*	Trethew Mill, Bodmin, Cornwall 50.39585	–4.709558	Rosemary Parslow	
E4E0095	*E. confusa*	North Anston Grassland, South Yorkshire	53.34738	–1.20803	Graeme Coles
E4E0009	*E. confusa*	Devil's Hole Blowout, Ravenmeols Local Nature Reserve, Merseyside	53.54062	–3.09041	Philip H. Smith
E4E0188	*E. micrantha*	Meall a Bathaich, Glen Garry, East Perthshire	56.82082	–4.182812	Alistair Godfrey
E4E0064	*E. nemorosa*	Castle Hill Local Nature Reserve, East Sussex	50.7842	0.052719	David Harris

Collection number	Taxon	Locality	Latitude	Longitude	Collector
E4E0069	*E. nemorosa*	Meridian Business Park, Leicester	52.60857	–1.19809	Geoffrey Hall
E4E0123	*E. nemorosa*	Bloody Oaks Triangle, Tickercote, Rutland	52.68950	–0.56263	Geoffrey Hall
E4E0029	*E. pseudokerneri*	Levin Down, Sussex	50.91346	–0.74150	Elizabeth Sturt
E4E0112	*E. pseudokerneri*	Beeston Common, Norfolk	52.93442	1.220071	Francis Farrow
E4E0027	*E. anglica* x *E. nemorosa*	West Dean Woods, Sussex	50.93212	–0.79735	Elizabeth Sturt
E4E0016	*E. anglica* x *E. rostkoviana*	Straduff Rathcabbin, Co. Tipperary	53.11902	–8.02454	David Nash
E4E0033	*E. arctica* x *E. confusa*	Nr Quoyorally, South Ronaldsay, Orkney	58.75897	–2.93473	John Crossley
E4E0145	*E. arctica* x *E. nemorosa*	Kylfakin, Wof, Skye	57.26685	–5.76042	S.J. Bungard
E4E0021	*E. arctica* x *E. nemorosa*	Dunamase, Co. Laois	53.03153	–7.21015	David Nash
E4E0031	*E. nemorosa* x *E. confusa*	Dolebury Fort, Somerset	51.32605	–2.79432	C.W. Hurfurt
E4E0143	*E. tetraquetra* x *E. confusa*	Ballyteige Burrow, Co Wexford, Ireland	52.20268	–6.64325	Jim Hurley

Table 9: Summary of trait values for many *Euphrasia* species and hybrids grown on a clover host (i.e. the species differences experiment). Values are means ±1 SE. Length measurements are in millimeters. Note: Date of first flower not recorded.

Taxon	Corolla length	Height	Internode ratio	Julian days to flower	Lower floral leaf teeth	Nodes to flower	Number of branches
E. arctica	8.0 ± 0.2	82.9 ± 4.4	1.1 ± 0.1	195.2 ± 1.5	4.4 ± 0.1	8.6 ± 0.2	*4.56 ± 0.2
E. confusa	6.9 ± 0.2	134.4 ± 7.2	1.6 ± 0.1	200.2 ± 2.4	5.3 ± 0.2	11.1 ± 0.4	7.26 ± 0.5
E. micrantha	5.6 ± 0.2	70.6 ± 8.1	3.0 ± 0.4	—	2.4 ± 0.3	8.3 ± 0.2	0.57 ± 0.4
E. nemorosa	7.7 ± 0.1	127.4 ± 8.1	1.4 ± 0.1	206.6 ± 1.7	5.1 ± 0.2	11.9 ± 0.5	7.67 ± 0.5
E. pseudokerneri	8.8 ± 0.4	176.4 ± 15.6	1.4 ± 0.1	205.1 ± 2.0	5.5 ± 0.2	13.2 ± 0.4	8.67 ± 0.6
E. anglica x *E. nemorosa*	9.1 ± 0.5	148.1 ± 11.8	1.4 ± 0.1	195.7 ± 1.9	6.0 ± 0.3	12.0 ± 0.6	10.00 ± 1.0
E. anglica x *E. rostkoviana*	7.9 ± 0.2	122.6 ± 8.3	1.3 ± 0.1	192.3 ± 12.3	5.9 ± 0.3	10.6 ± 0.5	7.44 ± 0.7
E. arctica x *E. confusa*	9.5 ± 0.2	100.3 ± 4.3	1.4 ± 0.1	193.4 ± 3.2	3.8 ± 0.1	7.8 ± 0.3	5.70 ± 0.4
E. arctica x *E. nemorosa*	8.0 ± 0.2	132.2 ± 14.5	1.3 ± 0.1	205.3 ± 2.4	6.0 ± 0.3	11.3 ± 0.4	6.50 ± 0.4
E. arctica x *E. nemorosa*	7.9 ± 0.2	92.5 ± 5.9	1.0 ± 0.1	199.3 ± 2.8	5.1 ± 0.2	9.8 ± 0.3	7.00 ± 0.5
E. confusa x *E. tetraquetra*	7.2 ± 0.2	57.4 ± 5.8	0.7 ± 0.1	194.1 ± 2.7	4.2 ± 0.2	7.6 ± 0.4	4.00 ± 0.3

Figure 8: Principal component analysis of morphological variation of *Euphrasia* in a common garden. Panels show (A) five species and six hybrids grown with a single clover host, (B) five species grown with a clover host omitting hybrids, and (C) *E. arctica* with nine host treatments. Points represent individuals, and ellipses represent the standard error of the (weighted) average of scores.



Table(s) 10: The first five principal components extracted from the principal component analysis, with the contribution of variance of each trait to each principal component. The last two rows of each table show the standard deviation and the proportion of variance explained by the principal component.

Species differences (including hybrids)	PC1	PC2	PC3	PC4	PC5
Branches	0.229	0.053	0.071	0.252	0.094
Corolla length	0.089	0.262	0.369	0.032	0.136
Height	0.211	0.115	0.149	0.047	0.379
Internode ratio	0.005	0.441	0.186	0.030	0.190
Leaf teeth	0.213	0.056	0.097	0.428	0.128
Nodes to flower	0.224	0.093	0.126	0.181	0.081
Standard deviation	1.738	1.099	0.964	0.616	0.533
Proportion of variance	0.503	0.201	0.155	0.063	0.047

Species differences (excluding hybrids)	PC1	PC2	PC3	PC4	PC5
Branches	0.226	0.024	0.096	0.233	0.017
Corolla length	0.100	0.269	0.361	0.082	0.141
Height	0.214	0.128	0.151	0.063	0.367
Internode ratio	0.029	0.434	0.202	0.000	0.171
Leaf teeth	0.214	0.032	0.064	0.424	0.159
Nodes to flower	0.217	0.113	0.125	0.198	0.145
Standard deviation	1.780	1.111	0.932	0.612	0.433
Proportion of variance	0.528	0.206	0.145	0.062	0.031

Phenotypic plasticity	PC1	PC2	PC3	PC4	PC5
Branches	0.183	0.065	0.032	0.098	0.220
Corolla length	0.139	0.001	0.252	0.340	0.030
Height	0.179	0.150	0.016	0.065	0.128
Internode ratio	0.070	0.301	0.274	0.119	0.146
Julian days to flower	0.158	0.198	0.056	0.077	0.191
Leaf teeth	0.178	0.024	0.090	0.153	0.166
Nodes to flower	0.093	0.262	0.280	0.147	0.119
Standard deviation	1.904	1.137	0.924	0.725	0.586
Proportion of variance	0.518	0.185	0.122	0.075	0.049

Figure 9: Pairwise differences in trait value of *Euphrasia* species grown with clover in a common garden experiment. Tukey comparisons are presented between each pair of species, with significant comparisons shown in bold. Point estimates are the mean difference of the comparison, and error bars are +/- one standard error, calculated from the species differences model using the emmeans R package. *** p < 0.001, ** p < 0.01, * p < 0.05.

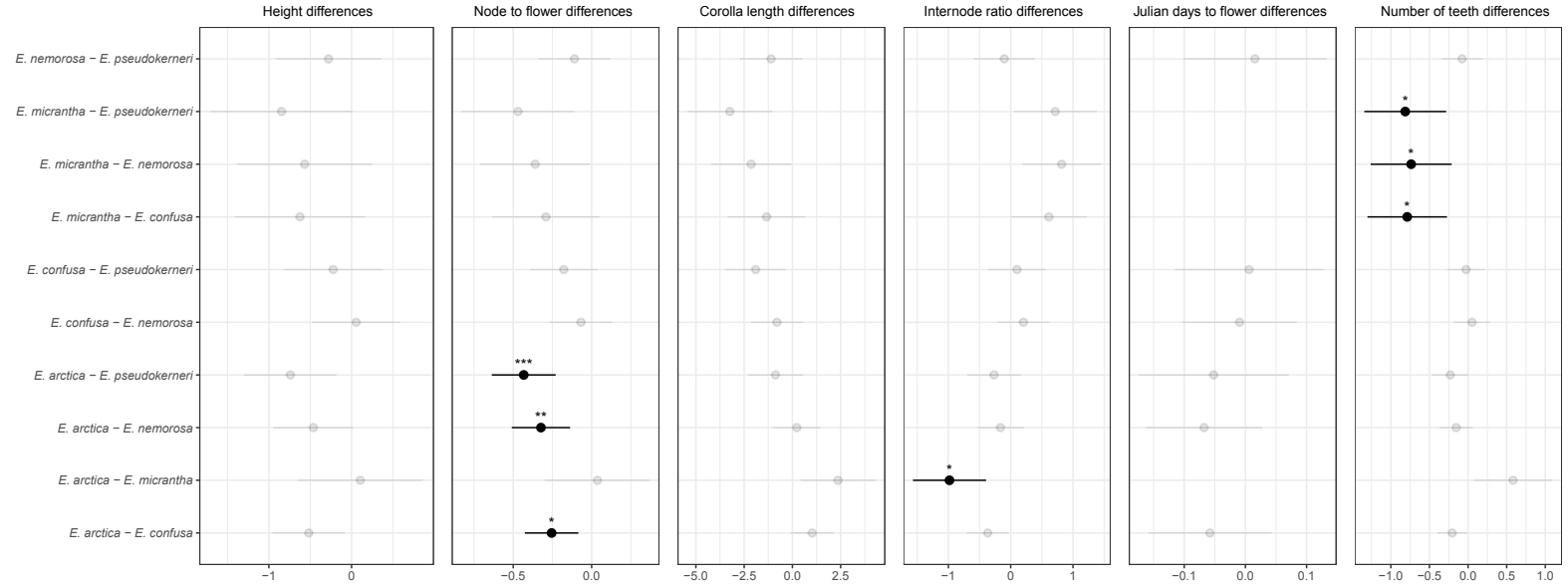


Table 11: Summary of trait values for *Euphrasia arctica* grown on many different hosts. Values are mean +/- one standard error. Length and height measurements are in millimeters.

Early season	At first flowering							End of season	
Host	Height	Corolla length	Height	Internode ratio	Julian days to flower	Lower floral leaf teeth	Nodes to flower	Number of branches	Height
A. thaliana	12.8 ± 1.1	6.1 ± 0.3	19.2 ± 1.6	2.4 ± 0.1	201.6 ± 4.3	3.2 ± 0.1	8.8 ± 0.3	2.1 ± 0.4	30.0 ± 3.2
E. arvense	6.1 ± 0.4	5.9 ± 0.3	15.1 ± 1.1	2.6 ± 0.2	215.3 ± 4.6	2.4 ± 0.1	9.3 ± 0.3	0.4 ± 0.1	35.6 ± 4.8
F. rubra	6.7 ± 0.4	6.3 ± 0.1	19.5 ± 1.4	2.6 ± 0.2	216.5 ± 4.4	2.8 ± 0.2	9.6 ± 0.3	0.8 ± 0.3	39.6 ± 4.1
H. lanatus	7.1 ± 1.3	6.3 ± 0.1	16.0 ± 1.6	2.4 ± 0.2	224.5 ± 7.0	2.5 ± 0.2	9.8 ± 0.4	0.8 ± 0.4	33.8 ± 6.8
M. polymorpha	6.3 ± 0.7	5.5 ± 0.4	9.6 ± 1.3	2.9 ± 0.4	222.6 ± 17.0	1.7 ± 0.3	9.7 ± 0.5	0	11.3 ± 2.5
No host	5.9 ± 0.3	5.3 ± 0.2	11.2 ± 1.1	2.8 ± 0.2	241.3 ± 7.9	1.9 ± 0.3	9.9 ± 0.5	0	9.7 ± 2.0
P. lanceolata	7.5 ± 0.5	6.1 ± 0.1	14.1 ± 0.8	2.8 ± 0.1	211.2 ± 3.7	2.9 ± 0.1	10.4 ± 0.3	0.4 ± 0.1	28.3 ± 3.4
P. sylvestris	6.2 ± 0.6	5.7 ± 0.3	12.2 ± 1.3	2.9 ± 0.2	233.8 ± 6.1	1.9 ± 0.2	9.2 ± 0.3	0	17.2 ± 2.6
T. repens	12.9 ± 1.4	7.4 ± 0.2	39.4 ± 2.6	2.1 ± 0.2	189.8 ± 2.0	3.9 ± 0.1	8.7 ± 0.3	4.7 ± 0.4	143.2 ± 8.6

Table 12: Comparison of *E. arctica* traits in the phenotypic plasticity common garden experiment. Tukey comparisons are presented between *E. arctica* traits with two different host treatments. Point estimates are the mean difference of the comparison, calculated from the phenotypic plasticity model using the emmeans R package. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Host 1	Host 2	Corolla length	Height	Internode ratio	Julian days to flower	Nodes to flower	Number of leaf teeth
Arabidopsis thaliana	No host	1.065*	0.49*	0.102	-0.178***	-0.116	0.508
Equisetum arvense	No host	0.946*	0.304	0.041	-0.112***	-0.066	0.212
Festuca rubra	No host	1.04*	0.529**	0.073	-0.112***	-0.034	0.397
Holcus lanatus	No host	1.05*	0.332	0.077	-0.063	-0.009	0.258
Marchantia polymorpha	No host	0.25	-0.181	0.07	-0.031	-0.025	-0.136
Pinus sylvestris	No host	0.481	0.067	0.015	-0.03	0.051	0.01
Plantago lanceolata	No host	0.879	0.246	0.016	-0.137***	-0.071	0.419
Trifolium repens	No host	2.102***	1.241***	0.180*	-0.244***	-0.134	0.711
Equisetum arvense	*Arabidopsis thaliana*	-0.119	-0.186	-0.061	0.066*	0.05	-0.296
Festuca rubra	*Arabidopsis thaliana*	-0.024	0.039	-0.029	0.065**	0.082	-0.111
Holcus lanatus	*Arabidopsis thaliana*	-0.015	-0.158	-0.025	0.114***	0.107	-0.25
Marchantia polymorpha	*Arabidopsis thaliana*	-0.815	-0.671***	-0.032	0.147***	0.091	-0.644
Pinus sylvestris	*Arabidopsis thaliana*	-0.584	-0.423*	-0.087	0.148***	0.167	-0.498
Plantago lanceolata	*Arabidopsis thaliana*	-0.186	-0.244	-0.086	0.041	0.044	-0.089
Trifolium repens	*Arabidopsis thaliana*	1.037***	0.751***	0.077	-0.066	-0.018	0.204
Festuca rubra	*Equisetum arvense*	0.095	0.225	0.031	0	0.032	0.185
Holcus lanatus	*Equisetum arvense*	0.104	0.028	0.035	0.049	0.057	0.046
Marchantia polymorpha	*Equisetum arvense*	-0.696	-0.486*	0.029	0.081	0.041	-0.348
Pinus sylvestris	*Equisetum arvense*	-0.465	-0.237	-0.025	0.082**	0.117	-0.202
Plantago lanceolata	*Equisetum arvense*	-0.067	-0.059	-0.024	-0.025	-0.006	0.207
Trifolium repens	*Equisetum arvense*	1.156***	0.937***	0.138*	-0.132***	-0.068	0.499*
Holcus lanatus	*Festuca rubra*	0.01	-0.197	0.003	0.049	0.025	-0.139
Marchantia polymorpha	*Festuca rubra*	-0.79	-0.71***	-0.002	0.081	0.009	-0.533
Pinus sylvestris	*Festuca rubra*	-0.56	-0.462**	-0.057	0.083**	0.085	-0.387
Plantago lanceolata	*Festuca rubra*	-0.161	-0.283	-0.056	-0.025	-0.038	0.022
Trifolium repens	*Festuca rubra*	1.062***	0.712***	0.106	-0.132***	-0.1	0.315
Marchantia polymorpha	*Holcus lanatus*	-0.8	-0.513*	-0.006	0.033	-0.016	-0.394

Host 1	Host 2	Corolla length	Height	Internode ratio	Julian days to flower	Nodes to flower	Number of leaf teeth
Pinus sylvestris	*Holcus lanatus*	-0.569	-0.265	-0.061	0.034	0.06	-0.248
Plantago lanceolata	*Holcus lanatus*	-0.171	-0.086	-0.06	-0.074**	-0.063	0.161
Trifolium repens	*Holcus lanatus*	1.052**	0.909***	0.102	-0.18***	-0.125	0.454
Pinus sylvestris	*Marchantia polymorpha*	0.231	0.248	-0.055	0.001	0.076	0.146
Plantago lanceolata	*Marchantia polymorpha*	0.629	0.427	-0.054	-0.106***	-0.047	0.555
Trifolium repens	*Marchantia polymorpha*	1.852***	1.423***	0.109	-0.213***	-0.109	0.847*
Plantago lanceolata	*Pinus sylvestris*	0.398	0.178	0.001	-0.107***	-0.123	0.409
Trifolium repens	*Pinus sylvestris*	1.621***	1.174***	0.164*	-0.214***	-0.185	0.701*
Trifolium repens	*Plantago lanceolata*	1.223***	0.996***	0.163*	-0.107***	-0.063	0.292

Table 13: Analysis of deviance for each trait in the phenotypic plasticity experiment with *E. arctica* grown with many different hosts, assuming a Poisson distribution. For each model, we report the change in degrees of freedom (df), deviance, residual degrees of freedom, residual deviance, and p-value generated from the χ^2 distribution. Factor host, where the model includes all host species, is compared to the intercept model where no hosts are fitted.

Trait	Factor	df	Deviance	Resid. df	Resid. Dev	Pr($> \chi^2$)
Julian days to flower	Host	8	192.390	184	419.1153	2.56E-37
	(Intercept)		192	611.5053		
Nodes to flower	Host	8	5.020	185	38.47252	0.755416
	(Intercept)		193	43.49272		
Number of leaf teeth	Host	8	26.793	185	41.37748	0.000767
	(Intercept)		193	68.17096		

Table 14: ANOVAs for traits measured in the phenotypic plasticity experiment with *E. arctica* grown with many different hosts, assuming Gaussian distributed residuals. For each model, we report the degrees of freedom (df), sums of squares (SS), mean squares (MS), F-statistic, and p-value.

Trait	Factor	df	SS	MS	F	p
Corolla length	Host	8	49.469	6.184	9.854565	3.00E-11
	Residuals	173	108.555	0.6275		
Height	Host	8	27.021	3.378	23.139	2.52E-24
	Residuals	185	27.009	0.146		
Internode ratio	Host	8	0.562	0.070	3.362213	0.001275
	Residuals	184	3.845	0.0209		

Table 15: Summary of generalised linear models for the phenotypic plasticity experiment with *Euphrasia arctica* grown on many hosts in a common garden. All models compare *E. arctica* grown with a particular host to the intercept of no host. Generalised linear models assuming Poisson residuals with log link function were used in Julian days to flower, nodes to flower and number of leaf teeth, while all others assumed Gaussian residuals. The model coefficient is reported with standard error in brackets. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Term	Corolla length	Height (log)	Internode ratio	Julian days to flower	Nodes to flower	Number of leaf teeth
(Intercept)	5.250 (0.250)***	2.363 (0.115)***	0.353 (0.043)***	5.489 (0.02)***	2.293 (0.095)***	0.646 (0.218)**
Arabidopsis thaliana	1.064 (0.293)***	0.489 (0.135)***	0.102 (0.051)*	-0.177 (0.024)***	-0.115 (0.114)	0.507 (0.241)*
Equisetum arvense	0.945 (0.300)**	0.304 (0.138)*	0.041 (0.052)	-0.111 (0.024)***	-0.065 (0.116)	0.212 (0.254)
Festuca rubra	1.040 (0.288)***	0.529 (0.134)***	0.073 (0.050)	-0.112 (0.023)***	-0.033 (0.111)	0.396 (0.242)
Holcus lanatus	1.050 (0.323)**	0.331 (0.147)*	0.077 (0.055)	-0.063 (0.025)*	-0.008 (0.123)	0.257 (0.267)
Marchantia polymorpha	0.250 (0.433)	-0.181 (0.171)	0.070 (0.064)	-0.03 (0.029)	-0.024 (0.143)	-0.135 (0.338)
Pinus sylvestris	0.480 (0.333)	0.067 (0.153)	0.015 (0.058)	-0.029 (0.026)	0.051 (0.126)	0.010 (0.290)
Plantago lanceolata	0.879 (0.288)**	0.245 (0.134)	0.016 (0.05)	-0.136 (0.023)***	-0.071 (0.112)	0.419 (0.242)
Trifolium repens	2.101 (0.293)***	1.241 (0.136)***	0.180 (0.051)***	-0.243 (0.024)***	-0.133 (0.115)	0.711 (0.239)**

Figure 10: Relationship between growth-related traits and end of season height for *E. arctica* grown with eight hosts and no host. (A) height at first flowering, (B) height 6-weeks after germination, (C) Julian days to flower, (D) number of branches. Length measurements are reported in mm.

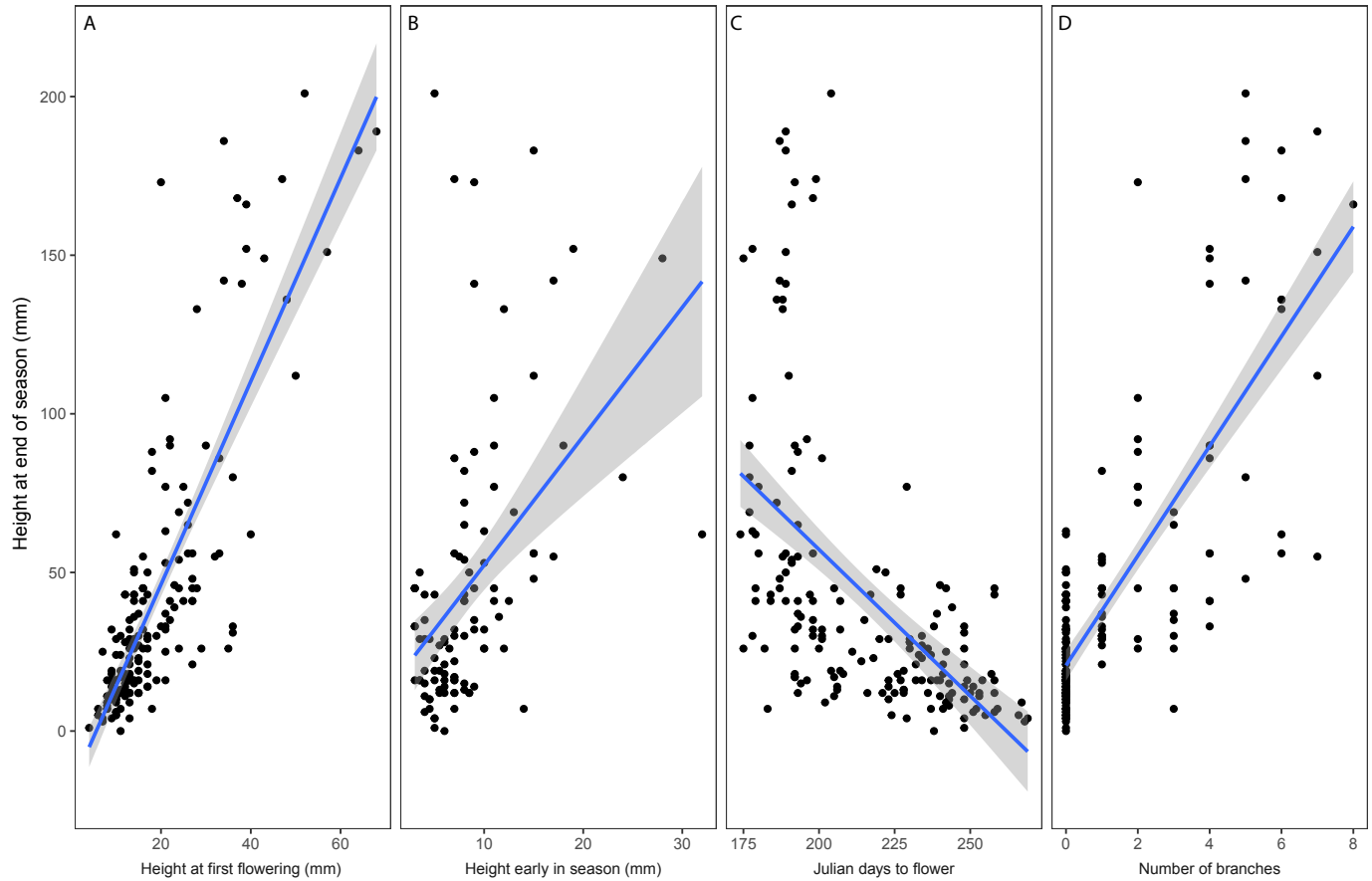


Figure 11: Comparison between trait values for wild-collected herbarium specimens and common garden plants of diverse *Euphrasia* species for (A) nodes to flower, (B) corolla length (mm), (C) number of leaf teeth, (D) internode ratio. Points are for *Euphrasia* population means, with bars representing the standard error of measurements.

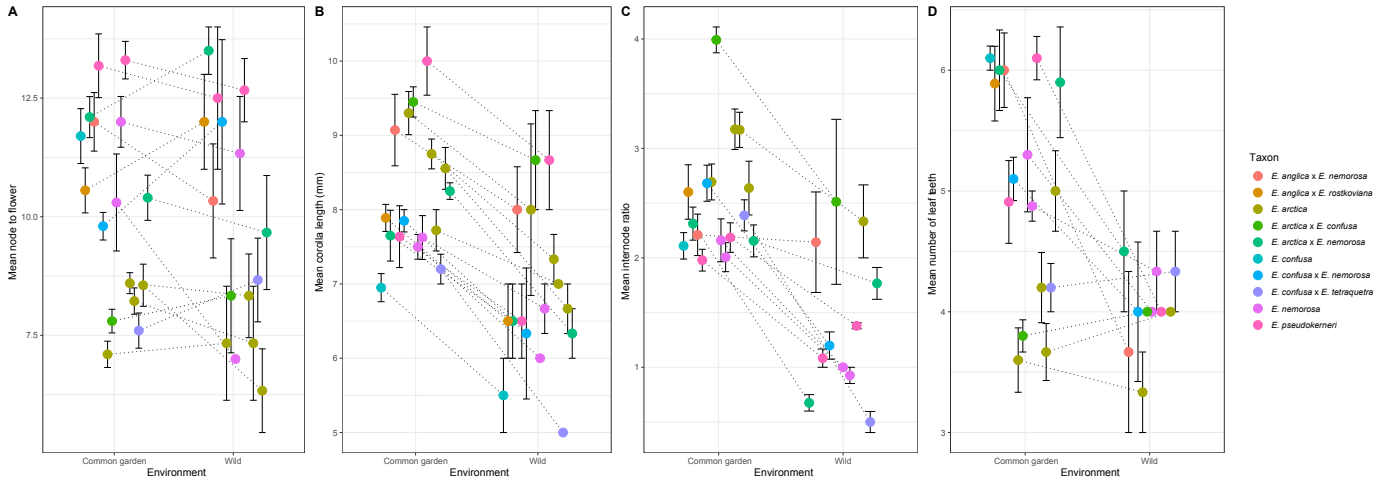


Table 16: Model output from MCMCglmm comparing traits for the wild collected *Euphrasia* specimens to the baseline of the common garden data (Intercept). The posterior means are reported along with the lower and upper 95% credible intervals, as well as the p-value (pMCMC) for the effect.

Trait	Factor	Posterior mean	Lower credible interval	Upper credible interval	pMCMC
Branches	(Intercept)	1.863	1.682	2.086	0.001
	Wild collected	-0.457	-0.619	-0.290	0.001
Internode ratio	(Intercept)	2.533	2.118	2.920	0.001
	Wild collected	-1.008	-1.206	-0.823	0.001
Corolla	(Intercept)	8.182	7.477	8.756	0.001
	Wild collected	-1.363	-1.650	-1.032	0.001
Nodes	(Intercept)	2.322	2.189	2.465	0.001
	Wild collected	-0.016	-0.135	0.086	0.800
Teeth	(Intercept)	1.616	1.485	1.722	0.001
	Wild collected	-0.187	-0.369	-0.004	0.050

Appendix 5: Chapter 7

Figure 12: *Euphrasia* reproductive output over time showing differences in reproductive trajectories, data from Experiment 1. Values represent mean reproductive nodes at a particular time point ± one standard error. Eleven species of host are shown, along with the average host where points are the mean of all hosts in the experiment.

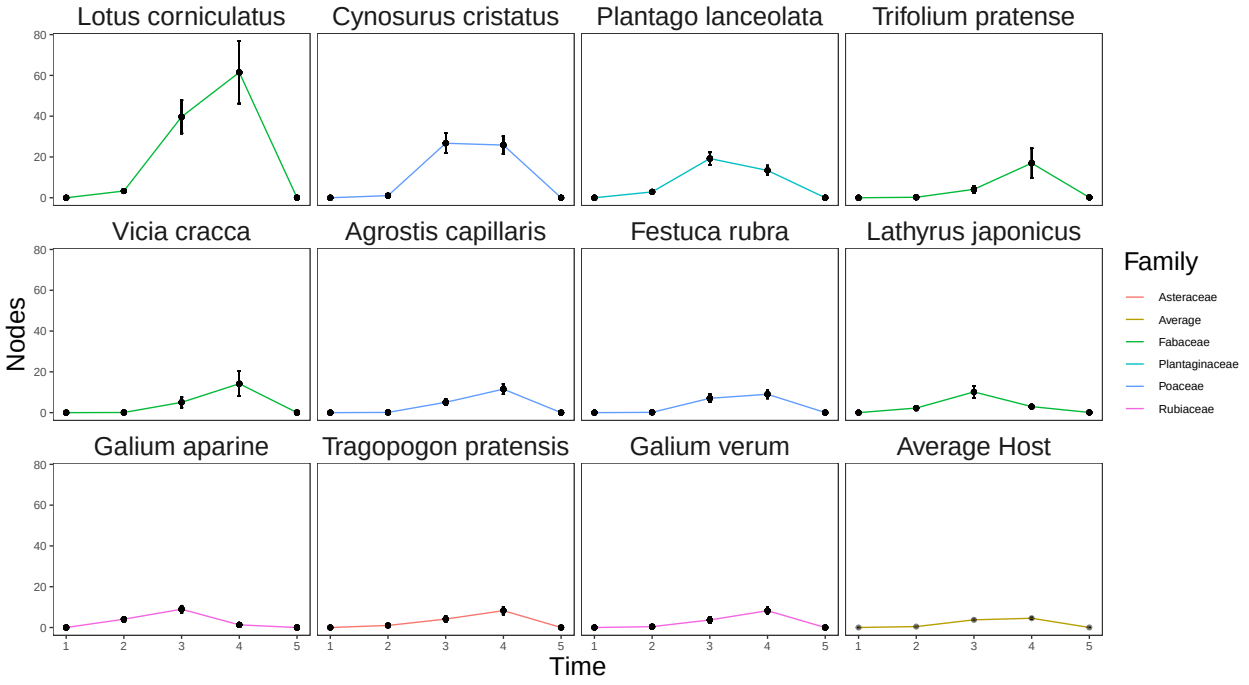


Figure 13: Posterior distributions of the phylogenetic signal for the models from Experiment 1, where 45 different host species were grown with *Euphrasia arctica*. The distributions of phylogenetic signal are shown for three *Euphrasia* traits: survival, total reproductive output at the end of the season, and days to flower. Total reproductive output shows both the highest and least variable estimate of phylogenetic signal, however all are significant as the distributions are not overlapping zero.

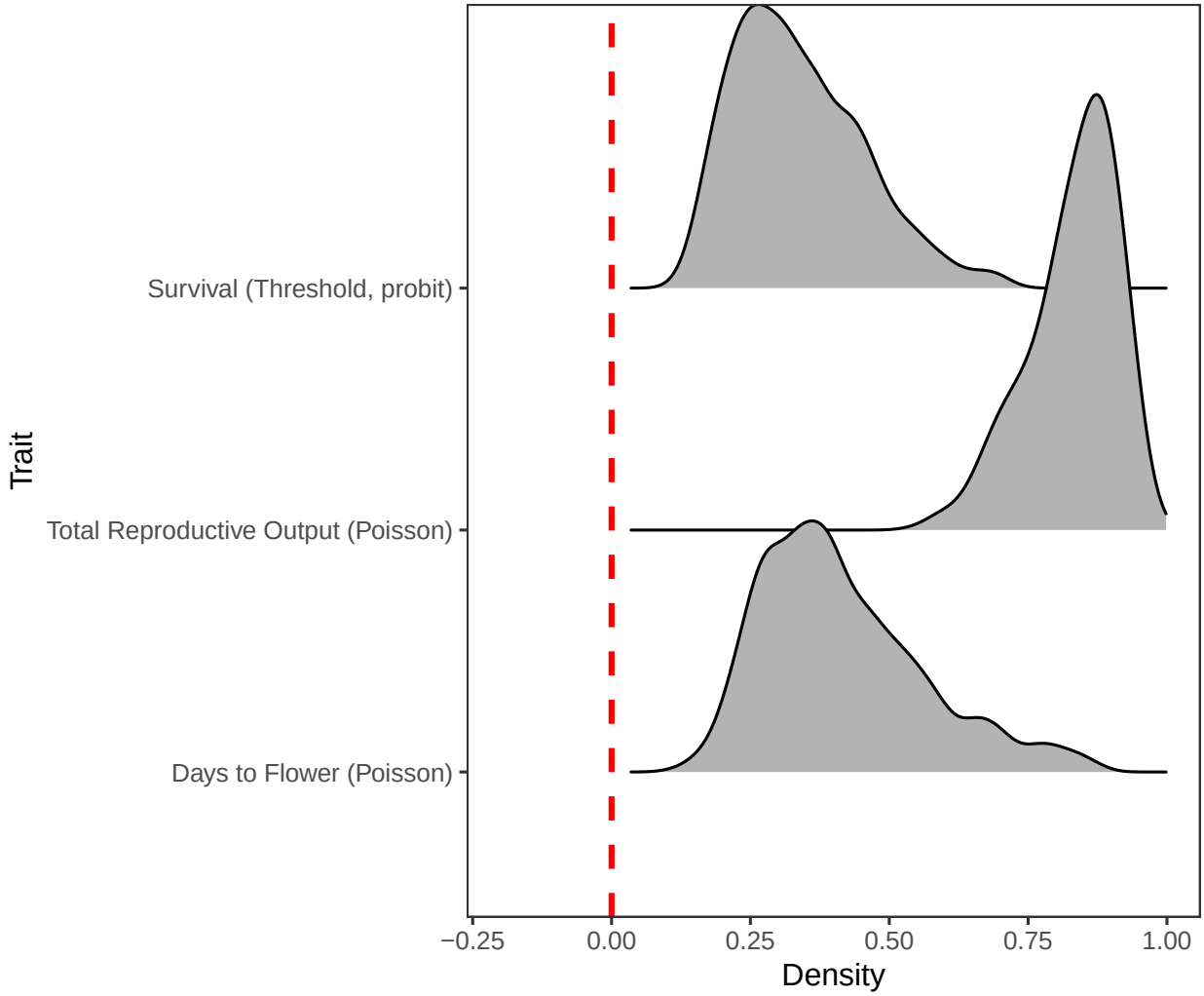


Figure 14. Posterior distribution of the variance for random effects in the model fitted for Experiment 2, where four species of *Euphrasia* were grown on thirteen different species of host. The random effects are the *Euphrasia*-host interaction, the sole effect of host species, and the residual variance. Although the residual variance is the explaining most variation, both the host-parasite interaction and hosts themselves are estimated to be significantly way from zero.

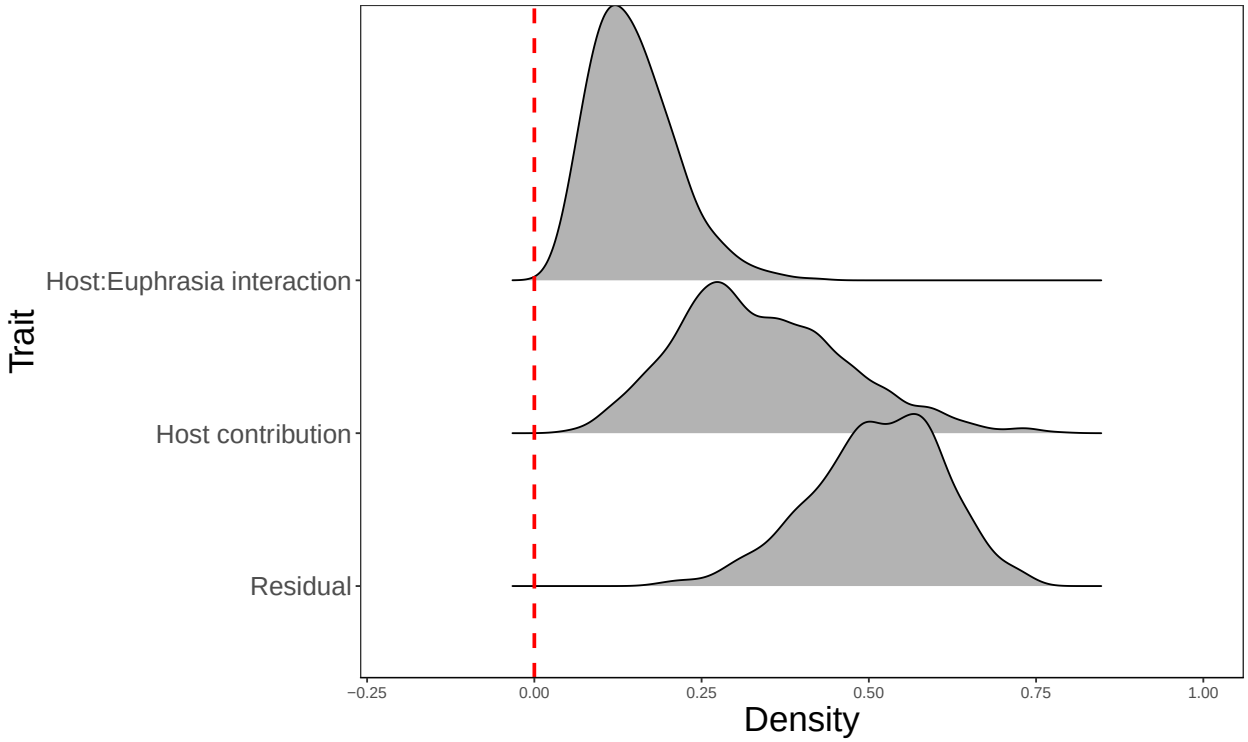


Figure 15. Performance of four species of *Euphrasia* on thirteen different species of host plants measured as cumulative reproductive nodes. Each panel represents a unique *Euphrasia* population (a = A1766, b = T1761, c = V1761, d = M1767, e = M1768, f = M1769), coloured by species. Two populations, (e) and (f) co-occur. Host species are ranked by average performance conferred to a *Euphrasia* species, where HPU = *Hypericum pulchrum*, CVU = *Calluna vulgaris*, HLA = *Holcus lanatus*, OVU = *Origanum vulgare*, UGA = *Ulex gallii*, PMA = *Plantago maritima*, PLA = *Plantago lanceolata*, VCH = *Veronica chamaedrys*, FOV = *Festuca ovina*, DFL = *Deschampsia flexuosa*, ACU = *Agrostis curtisii*, LPE = *Lolium perenne* and LCO = *Lotus corniculatus*. Y-axis values are the log of the mean cumulative reproductive nodes ± one standard error.

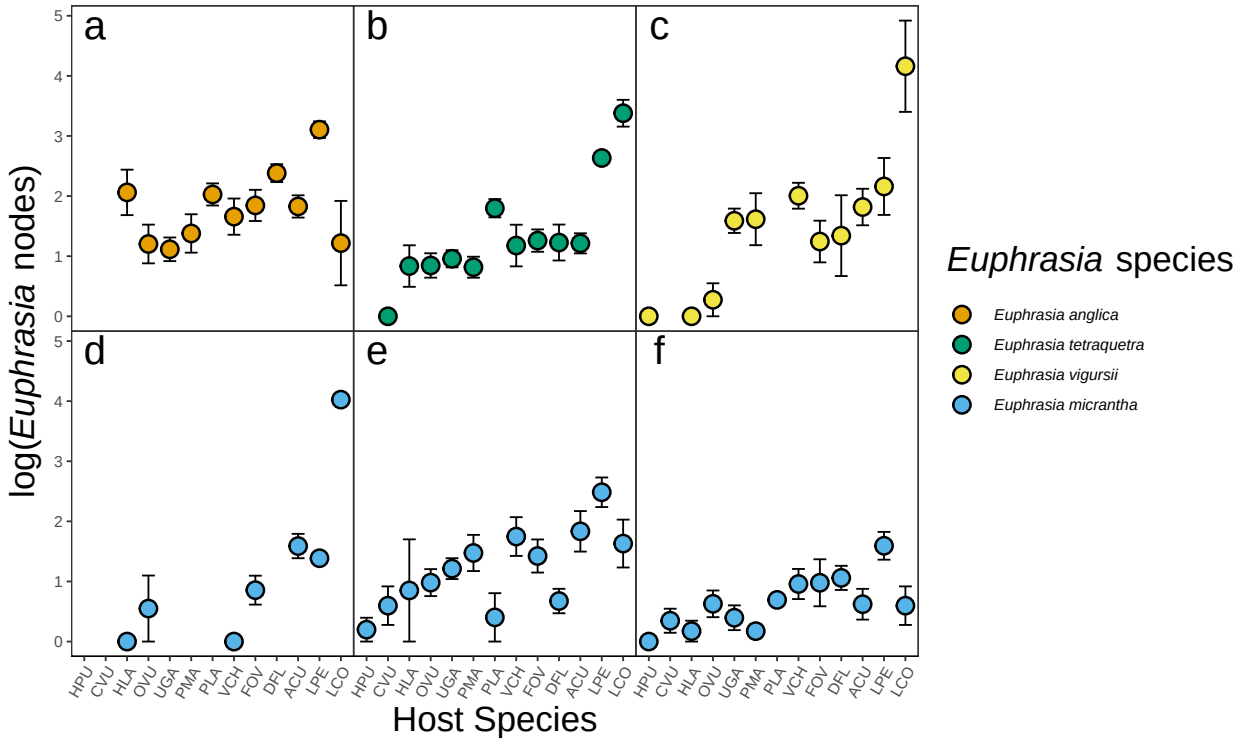


Table 17: Plant names, attributes and collection sources for host species used in Experiment 1.

Host species	Authority	Functional group	Life History	Seed source
No host	-	-	-	-
Agrostis capillaris	L.	Grass	Perennial	Emorsgate
Allium ursinum	L.	Forb	Perennial	RBGE
Anthriscus sylvestris	(L.) Hoffm.	Forb	Perennial	Emorsgate
Arabidopsis thaliana	(L.) Heynh.	Forb	Annual	Inbred lines University of Edinburgh
Centaurea nigra	L.	Forb	Perennial	Emorsgate
Centranthus ruber	(L.) DC.	Forb	Perennial	Chiltern Seeds
Chenopodium album	L.	Forb	Annual	Author collections
Chenopodium bonus-henricus	L.	Forb	Perennial	Surplus seed RBGE
Cynosurus cristatus	L.	Grass	Perennial	Emorsgate
Cystopteris dickeniana	R. Sim	Fern	Perennial	RBGE
Dactylorhiza purpurella	(T.Stephenson & T.A.Stephenson) Soó	Forb	Perennial	RBGE
Equisetum arvense	L.	Fern	Perennial	RBGE
Erica tetralix	L.	Woody	Perennial	RBGE
Festuca rubra	L.	Grass	Perennial	Emorsgate
Fragaria vesca	L.	Forb	Perennial	Scotia seeds
Galanthus nivalis	L.	Forb	Perennial	RBGE
Galium aparine	L.	Forb	Annual	Author collection, Upper Halliford, Surrey, Engalnd, 11/16
Galium verum	L.	Forb	Perennial	Emorsgate
Helianthemum nummularium	(L.) Mill.	Forb	Perennial	Scotia seeds
Holcus lanatus	L.	Grass	Perennial	Emorsgate
Hordeum vulgare	L.	Grass	Annual	Wiggly Wigglers
Hyacinthoides non-scripta	(L.) Chouard ex Rothm.	Forb	Perennial	RBGE
Lagurus ovatus	L.	Grass	Annual	www.wildflowershop.co.uk
Lathyrus japonicus	Willd.	Legume	Perennial	RBGE
Leucanthemum vulgare	(Vaill.) Lam.	Forb	Perennial	Emorsgate
Lotus corniculatus	L.	Legume	Perennial	Emorsgate
Meum athamanticum	Jacq.	Forb		RBGE
Mimulus guttatus	DC.	Forb	Perennial	Author collections

Host species	Authority	Functional group	Life History	Seed source
Ononis spinosa	L.	Legume	Perennial	Emorsgate & Wild Flower Shop
Papaver rhoeas	L.	Forb	Annual	Emorsgate
Phleum pratense	L.	Grass	Perennial	Wild Flower Shop
Pinus sylvestris	L.	Woody	Perennial	Scotia seeds
Plantago lanceolata	L.	Forb	Perennial	Emorsgate
Pteridium aquilinum	L. (Kuhn)	Fern	Perennial	British Pteridological Society spore exchange
Rumex acetosella	L.	Forb	Perennial	Scotia seeds
Senecio vulgaris	L.	Forb	Annual	RBGE
Silene dioica	(L.) Clairv.	Forb	Perennial	D. Charlseworth, Univ. Edinburgh
Silene latifolia	Poir.	Forb	Perennial	D. Charlseworth, Univ. Edinburgh
Thymus polytrichus	A.Kern. ex Borbás	Woody	Perennial	Emorsgate
Sorbus aucuparia	L.	Woody	Perennial	RBGE
Tragopogon pratensis	L.	Forb	Perennial	Scotia seeds
Trifolium pratense	L.	Legume	Perennial	Chiltern Seeds & Wild Flower Shop
Ulex europaeus	L.	Legume/Woody	Perennial	Tree Seed Online Ltd
Vicia cracca	L.	Legume	Perennial	Emorsgate
Zea mays	L.	Grass	Annual	Chiltern Seeds

Table 18: Plant names, attributes and collection sources for host species used in Experiment 2.

Host species	Authority	Source/Location	Plant status
Agrostis curtisii	Kerguélen	Millenium Seed Bank, Kew Gardens	Seed
Calluna vulgaris	(L.) Hull	RBGE	Seed, but small plants from cuttings
Deschampsia (*Avenella*) *flexuosa*	(L.) Trin.	Chiltern Seeds	Seed
Festuca ovina	L.	Emorsgate	Seed
Holcus lanatus	L.	Emorsgate	Seed
Hypericum pulchrum	L.	Scotia Seeds	Seed
Lotus corniculatus	L.	Emorsgate	Seed
Lolium perenne	L.	Emorsgate	Seed
Origanum vulgare	L.	Emorsgate	Seed
Plantago lanceolata	L.	Emorsgate	Seed
Plantago maritima	L.	Scotia Seeds	Seed
Ulex gallii	Planch.	Millenium Seed Bank, Kew Gardens	Seed
Veronica chamaedrys	L.	Scotia Seeds	Seed

Table 19: Euphrasia species collections across both experiments in Chapter 7.

Experiment	Euphrasia species	Location	Grid Reference
1	*E. arctica*	Inverkeithing, Scotland	NT 1389 82312
2	*E. anglica*	(A1766)	Cheddar, Somerset
2	*E. vigursii*	(V1761)	St Agnes Head, Cornwall
2	*E. tetraquetra*	(T1761)	St Agnes Head, Cornwall
2	*E. micrantha*	(M1767)	Borrowdale, Cumbria
2	*E. micrantha*	(M1768)	Alness, Scotland
2	*E. micrantha*	(M1769)	Orkney, Scotland

Table 20: Model output from MCMCglmm for the event history analysis (survival) model in Experiment 1. The intercept represents the latent probit estimate of mean *Euphrasia* survival on a perennial grass transplanted at the earliest date, measured at the first time point. The posterior means are reported along with the lower and upper 95% credible intervals as well as the effective sample size and p-value for the effect (pMCMC).

Covariates	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	3.0348	1.8630	4.1519	1000	<0.001
Time	-1.0533	-1.1164	-0.9912	1000	<0.001
AnnPerAnn	0.1390	-0.2489	0.6076	1000	0.5300
Normalised transplant date	-0.0164	-0.0213	-0.0117	1000	<0.001
Functional group fern	-0.2583	-1.5117	1.0171	1000	0.6520
Functional group forb	-0.3076	-0.9687	0.3844	1000	0.3700
Functional group legume	-0.0828	-1.0457	0.7646	1000	0.8500
Functional group woody	-0.6675	-1.4986	0.1819	1000	0.0980

Table 21: Model output from MCMCglmm for the days to flower model in Experiment 1. The intercept represents the log of the mean days to flower since germination of *Euphrasia* on a perennial grass transplanted at the earliest date. The posterior means are reported along with the lower and upper 95% credible intervals as well as the effective sample size and p-value for the effect (pMCMC).

Covariates	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	4.6197	4.1765	5.0536	1000	<0.001
AnnPerAnn	-0.1380	-0.2703	0.0043	1188	0.0560
Functional group fern	-0.1127	-0.5410	0.3556	1000	0.6000
Functional group forb	-0.0879	-0.3087	0.1793	1106	0.3780
Functional group legume	-0.0650	-0.3307	0.3032	860.9	0.6160
Functional group woody	0.0991	-0.2964	0.4466	1000	0.5520
Normalised transplant date	0.0034	0.0008	0.0060	1000	0.0160

Table 22: Model output from MCMCglmm for the number of reproductive nodes over time model in Experiment 1. The intercept represents log of the mean number of reproductive nodes of *Euphrasia* on a perennial grass transplanted at the earliest date, measured at the first time point. The posterior means are reported along with the lower and upper 95% credible intervals as well as the effective sample size and p-value for the effect (pMCMC).

Covariates	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	-4.1298	-17.0773	5.4805	550	0.3420
Time3	2.3713	1.5862	3.2031	773.2	<0.001
Time4	3.0630	2.1378	3.9166	1000	<0.001
AnnPerAnn	0.7872	-1.2385	2.8500	1000	0.4460
Functional group fern	-4.3612	-16.8977	6.6709	789.8	0.3960
Functional group forb	-2.3178	-9.4309	3.7584	793.8	0.4420
Functional group legume	-2.3657	-10.7235	5.1473	756.9	0.5760
Functional group woody	-7.6673	-15.5032	-1.0839	549.4	0.0180
Normalised transplant date	-0.0760	-0.0919	-0.0625	1000	<0.001
Time3:AnnPerAnn	-0.9448	-2.0965	0.1002	1000	0.0920
Time4:AnnPerAnn	-2.3383	-3.6057	-0.8897	1000	0.0040

Table 23: Model output from MCMCglmm for the cumulative reproductive nodes at the end of the season model in Experiment 1. The intercept represents the log of the mean cumulative reproductive nodes at the end of the season of *Euphrasia* on a perennial grass transplanted at the earliest date. The posterior means are reported along with the lower and upper 95% credible intervals as well as the effective sample size and p-value for the effect (pMCMC).

Covariates	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	-0.4637	-9.8823	9.4058	1093	0.9240
AnnPerAnn	-0.3610	-2.9028	2.1730	886.5	0.7720
Functional group fern	-3.6600	-15.1134	6.8501	1000	0.4660
Functional group forb	-2.9965	-8.8016	2.1653	1097	0.2340
Functional group legume	-2.0488	-9.1675	4.6899	1000	0.5500
Functional group woody	-7.5786	-14.1020	-1.0165	633.3	0.0100
Normalised transplant date	-0.0762	-0.0945	-0.0570	1000	<0.001

Table 24: Model output from MCMCglmm for the number of cumulative reproductive nodes of *Euphrasia* individuals at the end of the season from Experiment 2. The intercept represents log of the mean cumulative number of reproductive nodes of *Euphrasia anglica*, population A1766, on a host that was transplanted at the earliest date. The posterior means are reported along with the lower and upper 95% credible intervals as well as the effective sample size and p-value for the effect (pMCMC).

Covariates	Posterior mean	l-95% CI	u-95% CI	Effective sample size	pMCMC
(Intercept)	1.7842	1.2210	2.2714	787.7	0.0010
Euphrasia micrantha	-1.2795	-1.7479	-0.8284	1000	0.0010
Euphrasia tetraquetra	-0.3702	-0.8160	-0.0076	873.2	0.0620
Euphrasia vigursii	-0.2457	-0.7758	0.2138	1000	0.3340
Population: M1767	0.3269	-0.2098	0.9299	846.7	0.2760
Population: M1768	0.7931	0.4788	1.0699	1000	0.0010
Normalised transplant date	0.0059	-0.0084	0.0237	1208	0.4820

Appendix 6: Horticultural protocols for experimental studies of eyebrights (*Euphrasia*, Orobanchaceae)

Introduction

Parasitic plants are a diverse group of 4,500 species that are defined by possessing a parasitic feeding organ called a haustorium that can attach and steal nutrients from a host plant (Nickrent and Musselman 2017). Some of the most familiar parasitic plants include the crop pest witchweed (*Striga*, Orobanchaceae), mistletoes such as *Viscum album* (Santalaceae), the common grassland wildflower yellow rattle (*Rhinanthus*, Orobanchaceae), and the species possessing the largest flower in nature, *Rafflesia arnoldii* (Rafflesiaceae; Twyford 2018). The diversity of parasitic plants, with parasitism described from 12 plant families (Westwood et al. 2010), is matched by the diversity of growing conditions necessary to succeed in cultivating these plants. Even related parasitic plant species can be found in contrasting conditions and it is important that these are matched in cultivation (e.g. the Orobanchaceae; Joel, Gressel, and Musselman 2013). There are also a number of specific horticultural issues associated with growing parasitic plants that must be overcome (e.g. germination stimulants; Yoneyama et al. 2010, and host specificity; Wicaksono et al. 2016), though a wide variety of parasitic plants have been and continue to be cultivated (Pignone and Hammer 2016). Parasitic plants are a particular challenge for cultivation as they require conditions suitable for the parasite, the host, and their interaction. Many parasites require host-specific cues in order to germinate, and in many cases the parasite must be added at an appropriate time for the parasite to establish (e.g. Yoder 1999).

In this article, we describe our experience of optimising horticultural protocols for eyebrights (*Euphrasia*, Orobanchaceae). The genus *Euphrasia* contains approximately 263 species (Daniel Nickrent, pers. comms.) distributed throughout temperate areas of the northern and southern hemisphere, and in montane regions of tropical South East Asia (Gussarova et al. 2008). It includes both perennial and annual species (e.g. Yeo 1973). *Euphrasia* are generalist hemiparasites, meaning they are photosynthetically competent and can grow without a host, but perform much better when grown with one of many potential hosts (grasses, forbs, legumes; Yeo 1964). We are currently developing British *Euphrasia* as a study system for understanding plant parasitism, and for investigating evolutionary questions related to hybridisation, polyploidy, and self-fertilisation. There are 21 British native species and these show rich variation in habitat, associated species, ploidy (there are diploids and tetraploids; Yeo 1956) and mating system (there are selfing and outcrossing species; French et al. 2005; Metherell and Rumsey 2018). They are known to hybridise extensively in the field and produce a diverse array of local hybrids as well as stable hybrid species (Stace and Crawley 2015; Metherell and Rumsey 2018). Here, we focus on protocols for experimental studies under laboratory conditions, in pot trials, and under field conditions.

General considerations for cultivating *Euphrasia*

Most experimental work on plants focuses on species that are simple to grow, are small in size at maturity, and rapidly complete their life cycle, such as the thale-cress *Arabidopsis thaliana*, which is a widely used model system (Weinkoop, Baginsky, and Weckwerth 2010). While British native *Euphrasia* are also small annuals, their cultivation is made difficult due to specific germination requirements and the need for a host. Firstly, *Euphrasia* species require a period of cold before they are able to germinate (Liebst and Schneller 2008). In cultivation, seeds therefore need to be stratified. In our experimental work we have achieved this in two ways: leaving seeds outside over winter, and forcing germination through placing seeds in the fridge. Other than requiring stratification, *Euphrasia* species require no special methods for germination, with wild collected seeds planted without removing abortive seeds usually giving 40-50% germination success (Chapters 6 and 7). The probability of germination depends on the condition of the seed. In a seed quality experiment in February 2019, we sowed 20 seeds of *E. arctica* from each of three categories (shrivelled, intermediate, and

plump, see Figure 1) onto moist soil in seed trays that were kept in cold frames. The seeds had been collected in September of the preceding year, kept dry, and stored at 4°C overwinter. Germination differed between categories. By mid-June none of the shrivelled seeds had germinated, but six intermediate ones, and ten plump ones. It is likely that the shrivelled seeds have been aborted by the parental plant, either because of developmental problems, genetic abnormalities, or limited resources (Stephenson 1981).

Figure 1: Three categories of seed quality used to assess the germination probability: shrivelled (left), intermediate (centre), and plump (right).

Additionally, *Euphrasia* species require a host for acceptable growth and this requirement must be met within a few days of germination to ensure growth is not hindered (Yeo 1961). In pots, a host can be transplanted carefully with minimal mortality, otherwise *Euphrasia* can be sown with host seed at the same time. Sowing *Euphrasia* into pre-existing vegetation is possible, however *Euphrasia* is a poor competitor and the vegetation must be sufficiently low or sparse for successful establishment. *Euphrasia* seeds can be stored by routine drying (e.g. at room temperature for one week) and then refrigerating or freezing, with frozen seeds surviving for at least three years (Chapman, Miles, and Trivedi 2019).

Laboratory conditions

For detailed studies of plant development, it is necessary to grow plants under controlled laboratory conditions. *Euphrasia* seeds will readily germinate on moist filter paper under sterile conditions. Ethanol should be used to sterilise the plates and seeds, then the filter paper placed inside with the seeds on top. The lid should then be placed on top and the plate sealed to avoid contamination. Seeds should be maintained in a fridge at ~4°C until germination (no supplemental light required). We have tested two seed collections this way, an unidentified diploid species sourced from Wales by commercial supplier Emorsgate, and tetraploid *E. arctica* wild-collected from Inverkeithing in Scotland. Only the second genotype germinated, after six weeks. This method is suitable for growing young seedlings, such as those required for cytology, but further refinement is necessary to make this suitable for growing plants to a larger growth stage. For more detailed developmental studies and for genetic manipulation (such as virus-induced gene silencing) plants can be grown on sterilised plates. Seeds will germinate after ~10 days at 4°C on 1/4 Hoagland media, a widely used hydroponic nutrient solution used in other parasitic plant research (Delavault et al. 1998).

Pot trials

Growing *Euphrasia* in pots has the benefit of plants being in a soil-like substrate where they can form more natural host interactions than in the laboratory. Such conditions are preferred when studying differences between species, because less natural conditions used in the lab may trigger growth that is not normally observed. When different species are grown under common conditions, phenotypic differences between individuals and between species have to be due to genetic differences (e.g. Riihimäki and Savolainen 2004). Reduced differentiation under common conditions relative to the wild, however, shows that the phenotypic differences observed in nature were mainly due to environmental differences. This is the basis for common garden experiments, which have been extensively used in ecology, evolution and genetics to understand 'nature vs nurture'.

We have performed four experimental common garden studies with *Euphrasia* plants grown in pots. Our initial experiment took place in a glasshouse at the nursery at RBGE in 2016, and aimed to study the different growth patterns of five different *Euphrasia* species (and multiple hybrids), and individuals of *E. arctica* grown on eight different hosts (and without a host; see Chapter 6). We found that host species impacted on the phenotype of *E. arctica* for some traits (e.g. height) but not for others (e.g. nodes to flower) and that certain *Euphrasia* species overlapped in many traits (e.g. *E. arctica*/*confusa*/*nemorosa*), while others were relatively distinct (e.g. *E. micrantha*). While successful, the most notable challenge was the relatively high mortality of individuals, and issues with high temperatures under glass on hot summer days which caused flower buds to abort. In the second and third experiments (see Chapter 7), which took place over two years (2017/18), used a new, better-ventilated and climatically controlled glasshouse for the *Euphrasia* post germination. In the first year, we measured the same species, *E. arctica*, but this time on 45 different hosts, and found that survival and fitness varies greatly between *Euphrasia* on different host species. In the second year, we measured fitness across four different *Euphrasia* species on thirteen different hosts, which revealed host-parasite interactions

in specific *Euphrasia*-host combinations. The fourth experiment (Becher et al. 2020) looked to investigate different morphologies of three tetraploid *Euphrasia* species collected from Fair Isle (*E. arctica*, *E. foulaensis*, and *E. micrantha*; Becher et al. 2020).

The Fair Isle experiment used seeds from two populations of each of the three *Euphrasia* species, grown with twelve host species, including wild-collected heather (*Calluna vulgaris*) and juniper (*Juniperus communis*) from Fair Isle. This experiment was performed in an outside seed frame (seen in Figure 2a), which is a well-ventilated structure that protects from damage by animals or wind. While there are benefits to growing plants outside (such as less-vigorous growth more similar to that seen in the wild), these plants grew relatively poorly, due to the partial shade of the side of the seed frames. We also found the soil mix, RBGE 1, has a tendency to become waterlogged, which creates problems after heavy or extended wet periods, but can also dry out rapidly in hot weather. This was less of a problem in the glasshouse where watering can be more easily controlled.

Each of these four experiments followed a similar protocol. All experiments aimed for at least 30 pots of each population-host combination and allowed for approximately 50% germination success. A single seed is planted in the centre of the pot, with this placement helping to identify it from any contaminant weed seeds. Planting pots of 9x9x9.5 cm are filled with a loose bark-based substrate (RGBE1). After sowing plants are lightly top dressed with sieved soil, and the pots moved to a seed frame where they remained until germination (Figure 2a). Host seeds are sown into trays with RGBE1 in February and introduced after the *Euphrasia* germinates.

Proper introduction of a host plant is critical in the establishment of a connection between *Euphrasia* and the host. We developed a protocol where the *Euphrasia* did not have to be transplanted, as early trials indicated this resulted in high mortality. Instead, we transplant young host plants ($<$ 2 weeks post germination) into a pot containing a *Euphrasia* individual in the centre (Figure 2b). The transplanted individual is placed equidistant between the *Euphrasia* individual and one of the four corners of the pot. In common garden trials with many plants, this allows us to keep a consistent distance between the *Euphrasia* and the host plant so there is no effect of distance to host. Placing a host too close to the *Euphrasia* could lead to either very early attachment (beneficial) or high levels of competition (detrimental), and vice versa.

In April, each germinant pot received one host plant, the survival of which was generally high. Plants are then moved to their final growing conditions (the glasshouse, or outside; Figure 2c). During the course of the summer, host plants had to be trimmed to avoid shading of neighbouring pots. Pots were randomised monthly to minimse block effects (e.g. plants on one bench growing better than others). Every day, plants were checked to measure traits on the day of first flowering, which represented a standardised time point to measure traits between individuals for use in statistical analyses.

Watering is necessary to prevent pots drying out. We have watered when required rather than as a matter of routine. We have found watering by hand to be more reliable for experimental work than automated irrigation, which can be patchy and may lead to uneven growth. *Euphrasia* favours drier over damper growing conditions, although this sometimes resulted in sub-optimal conditions for the hosts.

Supplementary feeding has proven necessary for vigorous *Euphrasia* growth in small pots. Our feeding regime begins in May before the transplantation of hosts had been completed. When *Euphrasia* flower, feeding is increased from fortnightly to weekly. Liquid feed was diluted at 1.5% by a Dosatron when watering with a fine rose. While the vigour of some of the hosts visibly improved after feeding, there seemed to be less of a direct correlation between feeding and increased *Euphrasia* vigour.

In addition to the previously mentioned trimming of the hosts, the pots were regularly weeded. Special attention must be paid at the start of the process, before *Euphrasia* seeds have apparently germinated, so that a *Euphrasia* individual does not begin to parasitise a weed. Moss and liverworts also need to be periodically removed when they threaten to smother the *Euphrasia*. Great care has to be taken to not disturb the roots of either the *Euphrasia* or host when weeding, therefore all weeding is best done with a pair of tweezers.

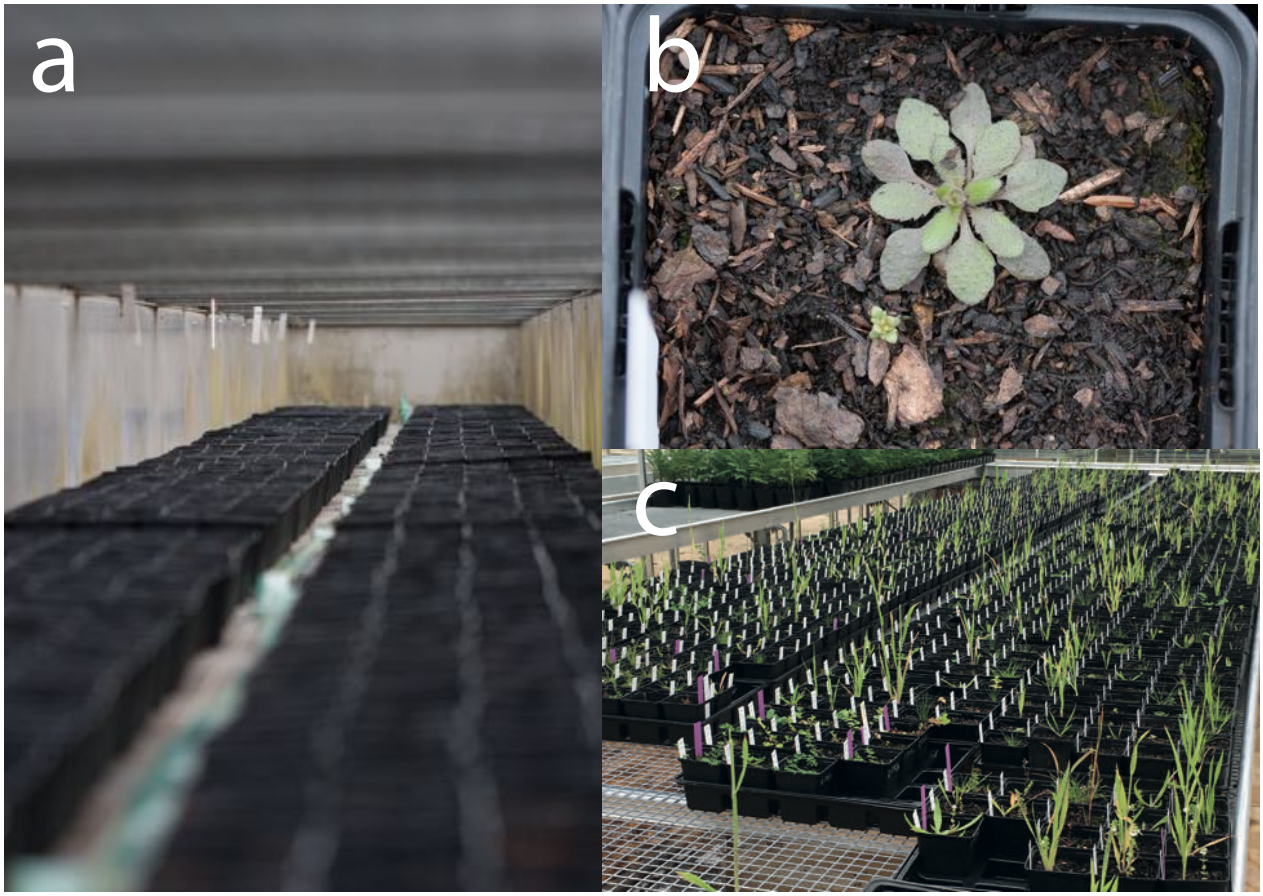


Figure 2: Pot trials in *Euphrasia* plants. a – thousands of *Euphrasia* seeds placed in pots, which are located in an outside cold frame throughout the winter (2016) to stimulate germination the following spring (2017). b – close-up of a pot with a *Euphrasia* individual (bottom left) parasitising an *Arabidopsis thaliana* individual (2017). c – a full scale growth experiment in progress with hundreds of mature *Euphrasia* individuals parasitising a multitude of host plants (2018).

There are a few species of pest which attack *Euphrasia* in cultivation. The most serious pests are aphids (Aphididae), which attack the upper stem and leaves. In heavy infestations, leaves can fall off the plant, and in some cases aphid damage can be fatal. The effect of aphids can be alleviated by spraying a soapy solution on the plants to suffocate the aphids, or if *Euphrasia* individuals are kept in glasshouses, to keep air movement and ventilation. Another common pest is a species of rust (*Coleosporium*; likely alternate host of pine; Ellis and Ellis 1985), which although an alarming orange colour, has an unknown effect on *Euphrasia*. Lastly, some caterpillar species in the Lepidopteran genus *Perizoma* attack *Euphrasia* by spinning and eating the leaves (Fitter and Peat 1994).

As *Euphrasia* are annual plants, all individuals die at the end of the season, which may last until late September. After germination, if a *Euphrasia* individual is unable to find a host then it either remains small in stature for months before dying, or dies quickly - usually within two weeks. Host species can influence survival of *Euphrasia* dramatically. Fast growing hosts which *Euphrasia* cannot attach to compete with the *Euphrasia* seedling for light, and increase the probability of *Euphrasia* mortality. In general, leguminous or grass host species confer higher probabilities of survival for *Euphrasia* than forbs or woody plants.

Field trials

Yellow rattle (*Rhinanthus minor*) has widely been exploited in meadows for its properties of parasitising and reducing the vigour of surrounding plants, thus reducing the need for mowing and for maintenance (Westbury and Dunnett 2007; Ameloot, Verheyen, and Hermy 2005). *Euphrasia*, as a related hemiparasite, could be used for similar purposes, with the wide-range of habitats of different *Euphrasia* species making it potentially useful in habitats where *Rhinanthus* does not survive. To produce seeds for large-scale planting requires *Euphrasia* to be cultivated on a field scale. In a collaboration with Scotia Seeds (http://www.scotiaseeds.co.uk/) we set up some field plots with a view to understand how feasible it would be to cultivate *Euphrasia* at scale. In late October 2018, a 200m^2 plot was cleared to allow the sowing of host and *Euphrasia* species, with 24 2m^2 plots used for planting. Two different populations of *Euphrasia* arctica were trialled, one from North Berwick collected in 2016 and one from Inverkeithing collected in 2017. Sowing densities for *Euphrasia* were at 500 seeds per square metre (0.625g) and host densities were around 2g per metre square. We used four different host treatments in combination with *Euphrasia*: *Lotus corniculatus*, *Cynosurus cristatus*, *Plantago lanceolata* and Mavisbank Meadow Mix (see details of mix on website: (https://www.scotiaseeds.co.uk/shop/mavisbank-mix/). In early September 2019 the mature plants were hand collected from each plot. Ten random individuals from each plot were sampled in order to determine vigour of the *Euphrasia* plants in each plot.

Preliminary results indicate that cultivation of *Euphrasia* arctica on a field scale yields vigorous plants in excess of 20cm in height (Figure 3). From experimental data from pot trials, which used the same species of *Euphrasia*, plants around 20cm tall produced ~150 reproductive nodes and therefore around 1800 seeds (extrapolated from models in Chapter 7, and assuming six seeds per capsule). For harvesting efficiency it is recommended that either *L. corniculatus* or *P. lanceolata* is used as grass species cause *Euphrasia* to form flexuous stem bases, which are difficult to harvest. *Euphrasia* arctica therefore could be managed on a field scale and give suitable yields for Scotia Seeds.

Reciprocal transplant experiments

While the protocols outlined above emulate aspects of natural conditions, planting *Euphrasia* in the wild is the 'gold standard' for experimental studies as it is the only way to include all biotic and abiotic stressors found in nature. Reciprocal transplant experiments involve translocating individuals between natural environments, and can be used to distinguish between genetic and environmental effects. If after the transplant, each transplanted sample shows the same traits as at their 'home' site, it can be assumed that the trait differences

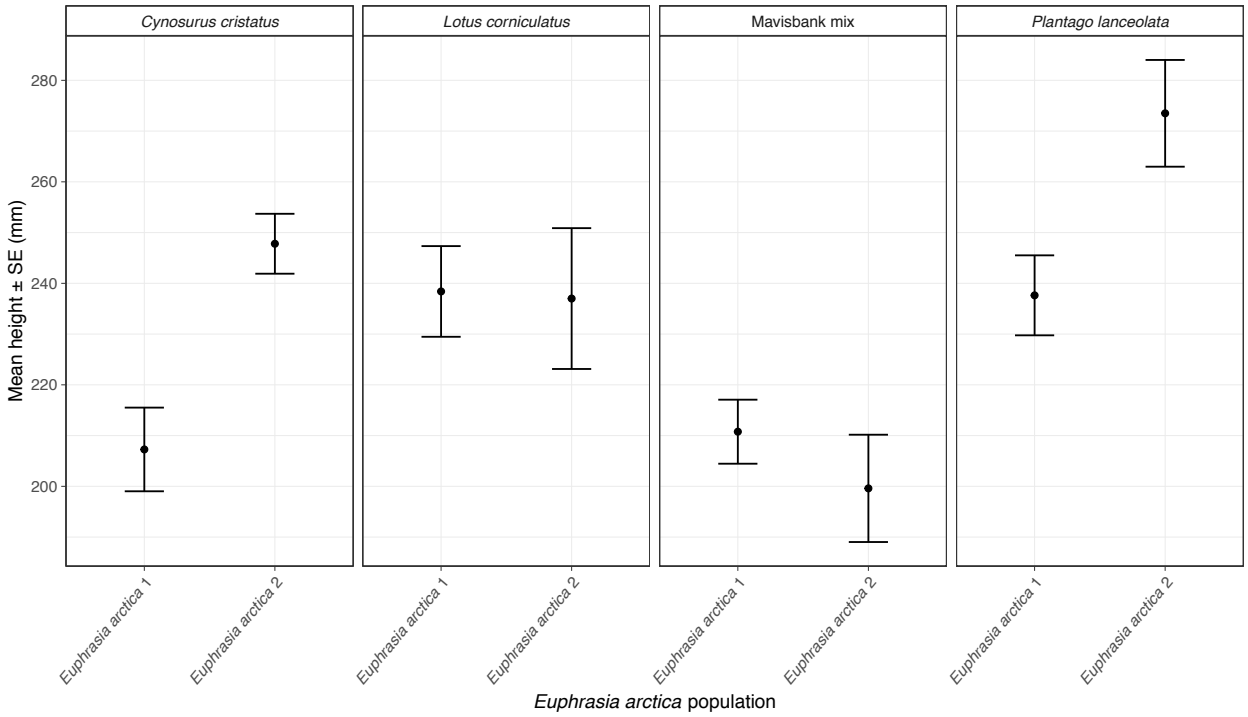


Figure 3: Mean heights and standard errors of both *Euphrasia arctica* populations from plots with each dominant host. Measurements were pooled from three replicate plots for *E. arctica* genotype 1 and one plot for *E. arctica* genotype 2.

have a genetic base. If on the contrary, the growing site determines some trait of interest, then the plants react plastically to their environment, and their genes do not determine the trait in question. It may well be that the result of a reciprocal transplant experiment is intermediate – many traits are affected by both genetic and environmental factors. Reciprocal transplants are particularly useful for investigating local adaptation, such as whether plants produce more seeds in their home site.

Conducting a transplant with hemiparasitic *Euphrasia* bears unique challenges. It is well-established now that the performance of *Euphrasia* plants depends critically on whether there are host plants available, which species they are, and on the timing of haustorium attachment. This means that, unlike in other large transplant experiments, clearing the local vegetation is not an option. We have therefore tested two approaches to transplants in *Euphrasia*.

To test the suitability of different substrates and planting containers, a preliminary field trail was carried out near Inverkeithing, Scotland, in 2018. We buried in the ground compostable plant pots (filled with local soil) and inflated "Jiffy" peat pellets (Figure 4). In each, we placed one *Euphrasia* seed. On our next visit, approximately four months later, we found that the compostable pots had persisted virtually unchanged and the outer mesh of the "Jiffy" pellets had not decomposed. Some pellets had lost their mesh, which we found in the nearby vegetation. While the surrounding vegetation was in good condition, our planting containers appeared dry and we recorded hardly any germination. 2018 was a very dry year, which may have affected the decomposition process, but the materials tested did not seem appropriate to be used in the wild in larger experiments.

Figure 4: A small *Euphrasia* plant germinated inside a "Jiffy" pellet. After four months in the ground, the pellet's outer mesh remained virtually unchanged. Photo: Hannes Becher.

On our second attempt, this time in field sites on Fair Isle, we decided to make planting holes and to fill them with sterile substrate. We used commercial compost (John Innes). Small planting holes were filled with the sterile compost in order to exclude local plants. These holes are small enough for surrounding plants to root through them in a short time, but they reduced the possibility of a local *Euphrasia* seeding directly

onto the planting spot. Planting was carried out in mid-September. Sites were chosen in three habitat types: heathland, coastal turf, and grassland. Woody vegetation, chiefly *Calluna vulgaris* and *Empetrum nigrum*, was cut back to allow access to the ground. Planting holes were dug with a bulb planter with the planting holes arranged in grids for ease of recoverability. Each hole was filled with compost to soil level. A single *Euphrasia* seed was then placed in the centre of each circular compost patch and a light top dressing of composts was applied. Each planting hole was individually labelled with a pencil-marked plastic tag. The label positions were recorded so that finding one label allowed to locate all other planting holes in a grid. Each label was sunk into the ground completely. While in many respects this type of experiment under wild conditions provides the most natural settings, it comes with extensive challenges. The ease of digging into substrate depended on the location; heather bog was especially challenging to produce neat holes to sow *Euphrasia* into. Sowing is also very difficult in windy conditions, even with the help of tweezers. Finding the holes months later required good mapped locations of each grid, as some were remarkably well hidden. Some birds also removed the labels and sheep were liable to trample on the holes made, both of which resulted in missing data. One of the biggest challenges was the low percent germination, which although observed in common garden experiments, is even lower in the wild. Despite these challenges, it is still possible to carry out these kinds of experiments in natural settings.

Conclusions and future directions

The genus *Euphrasia* represents an excellent study system to investigate a wide variety of topics, from the evolution of parasitism, to the role of polyploidy, hybridisation, and mating system in taxonomic complexity. *Euphrasia* can easily be brought into cultivation in petri dishes, and in pots, both in the glasshouse and outside. In addition, *Euphrasia* can be grown on many different species of host plant, making it an ideal system to investigate host effects on *Euphrasia* and vice versa. Field trials have been met with success on a commercial scale, however reciprocal transplant experiments in the wild are difficult and require many thousands of replicates for statistically robust inference.

There are many possibilities for future research building on these protocols. The laboratory protocols for fine scale developmental, genetic and host-parasite interface work should be developed to understand the nature of haustoria formation in *Euphrasia* on different host plants (as has been done in *Rhinanthus* Rümer et al. 2007). Further, transcriptome sequencing of haustoria may reveal the genes underlying parasitism in *Euphrasia* (Yang et al. 2015). Investigation of below ground host-*Euphrasia* interactions in pots will open up an area of research into the number of haustorial connections made to host plants, and may reveal host preferences. Further large scale reciprocal transplant experiments should be made to understand host preferences and the extent of local adaptation in the genus.

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