

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.

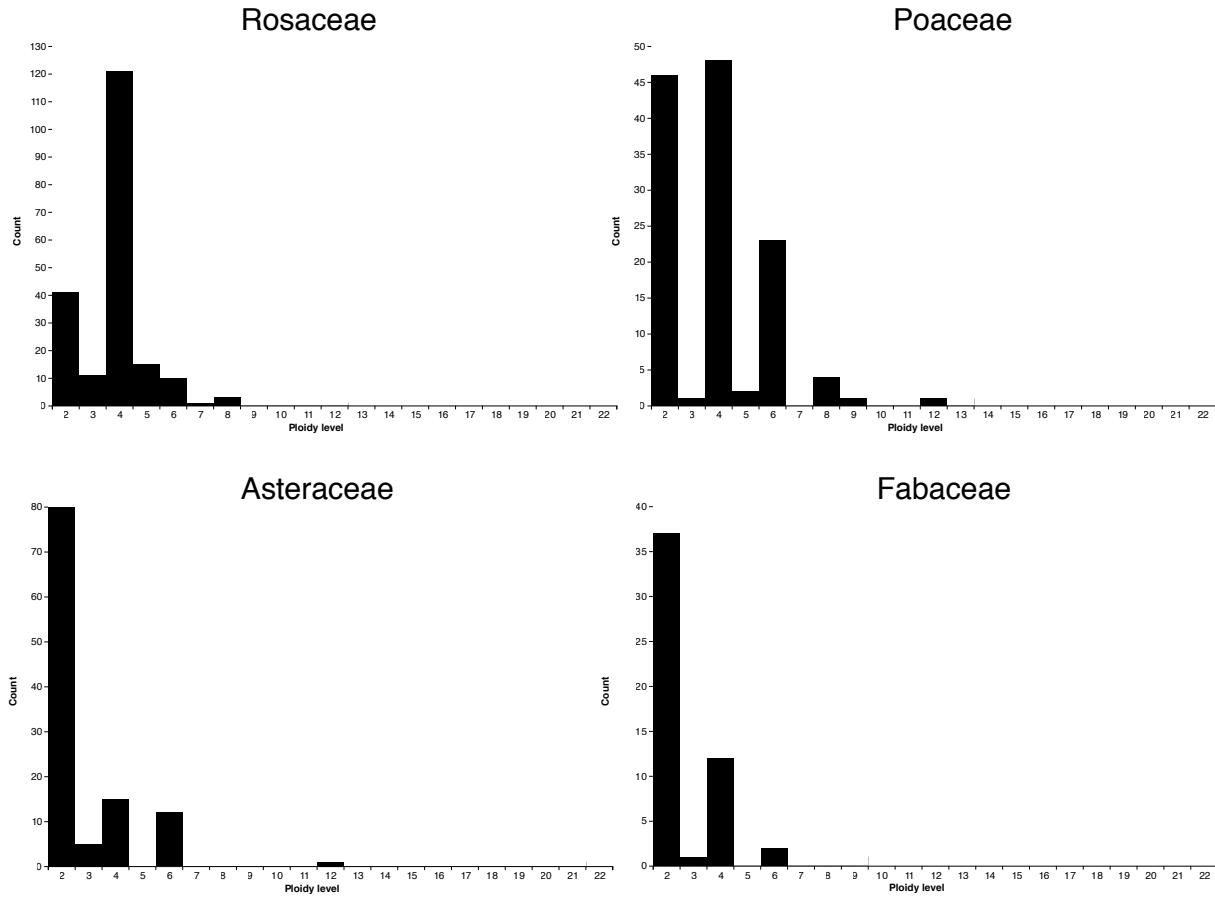


Table 1: Search strings for Google Scholar searches used to generate the list of examples of cross-ploidy hybrids in Chapter 2. Note that other examples were added if they were deemed to be important and/or well known.

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

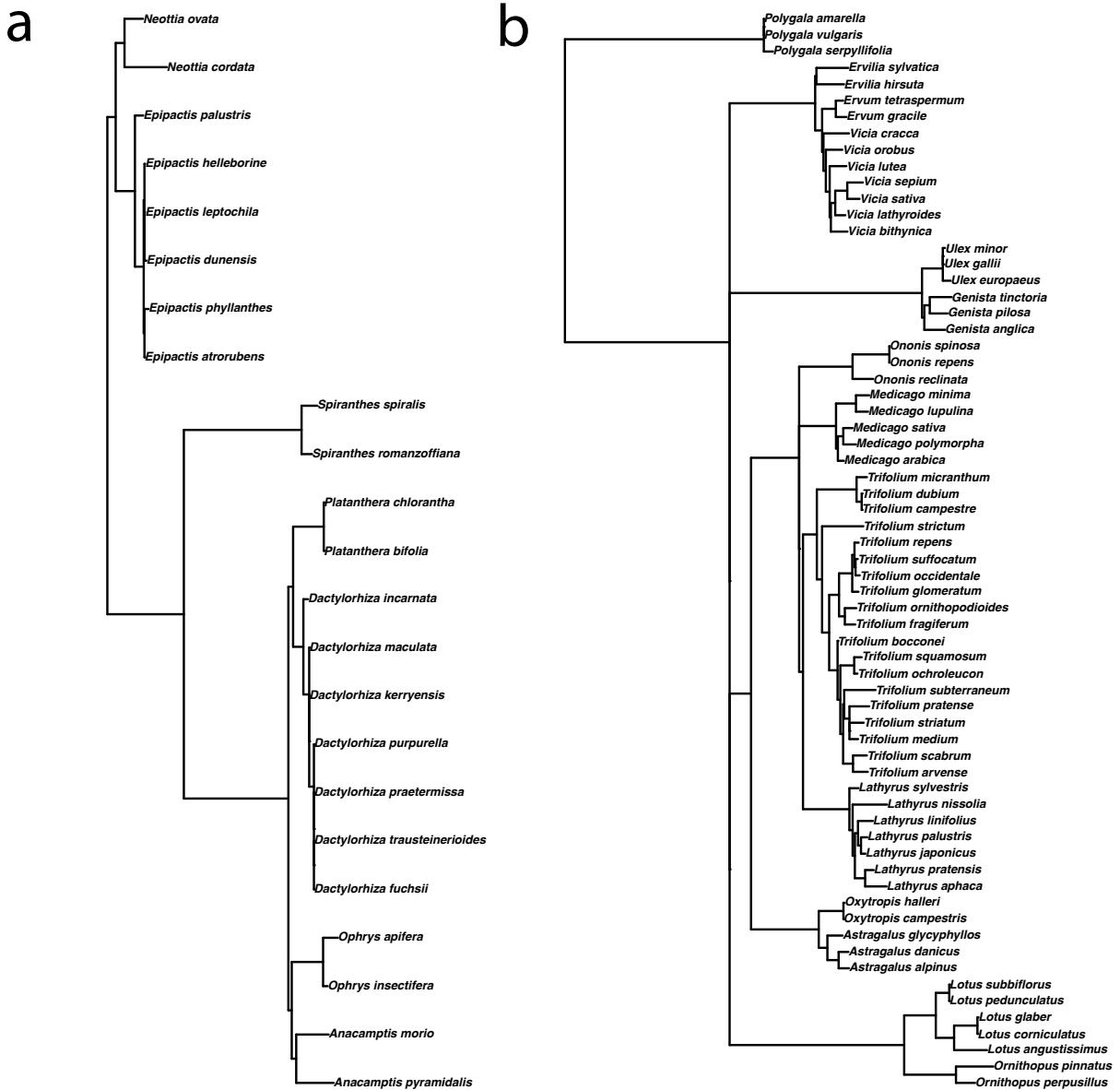


Figure 3: The joint probability of hybridisation between two parental species give both branch length between species (tree based genetic distance) and geographical overlap between parental species (measured as overlap in occupancy of 10x10km grid squares in the UK). The degree of shading in the scale 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.

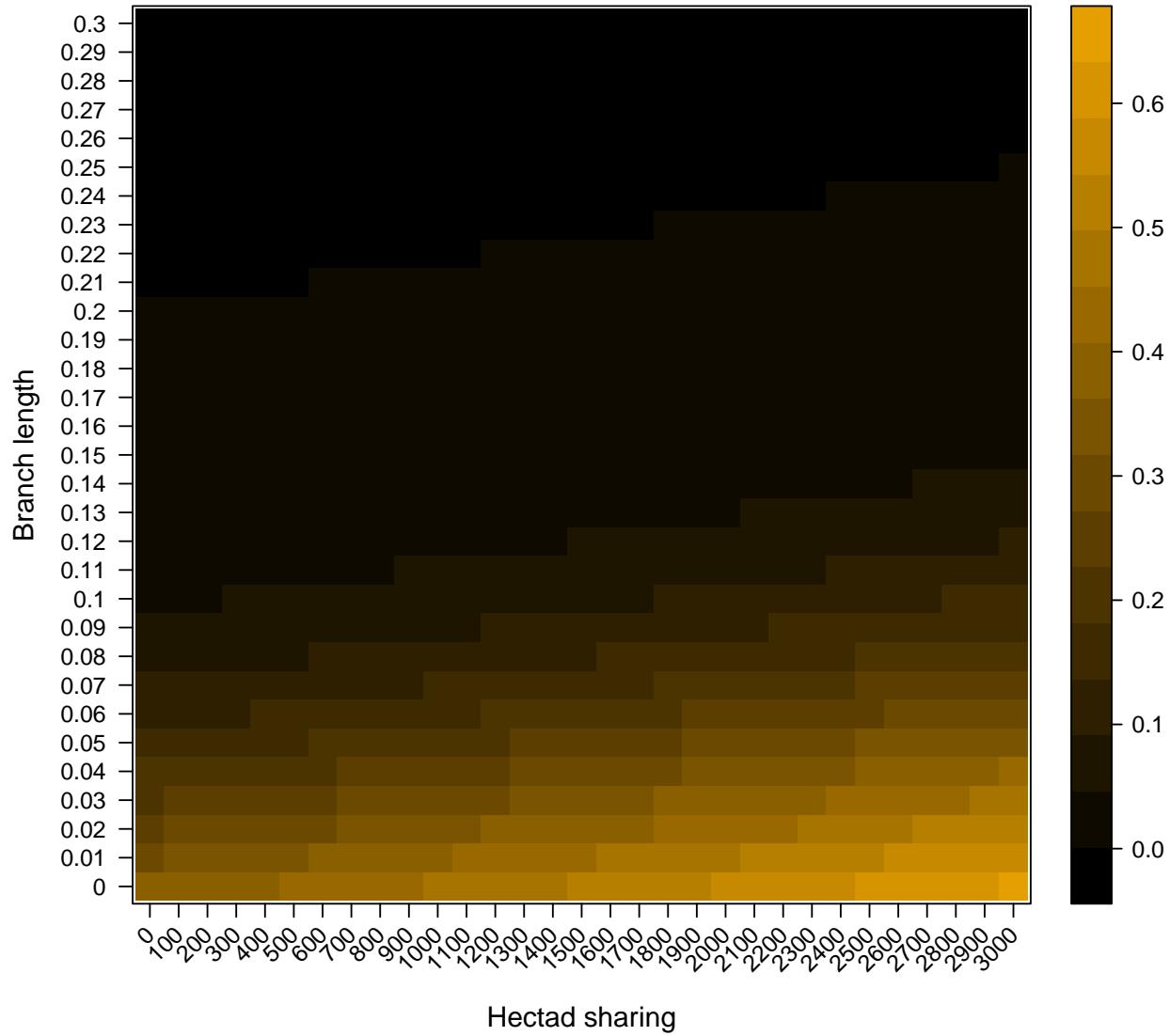


Figure 4: Predicted fit of probability of hybridisation given hectad sharing and ploidy difference of parental species from Model 2. Dashed lines indicate the 95% Credible Intervals, and the bold lines represent the posterior mode of the coefficients of congeneric pairs of species hybridising as a function 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 ($10 \times 10 \text{ km}^2$).

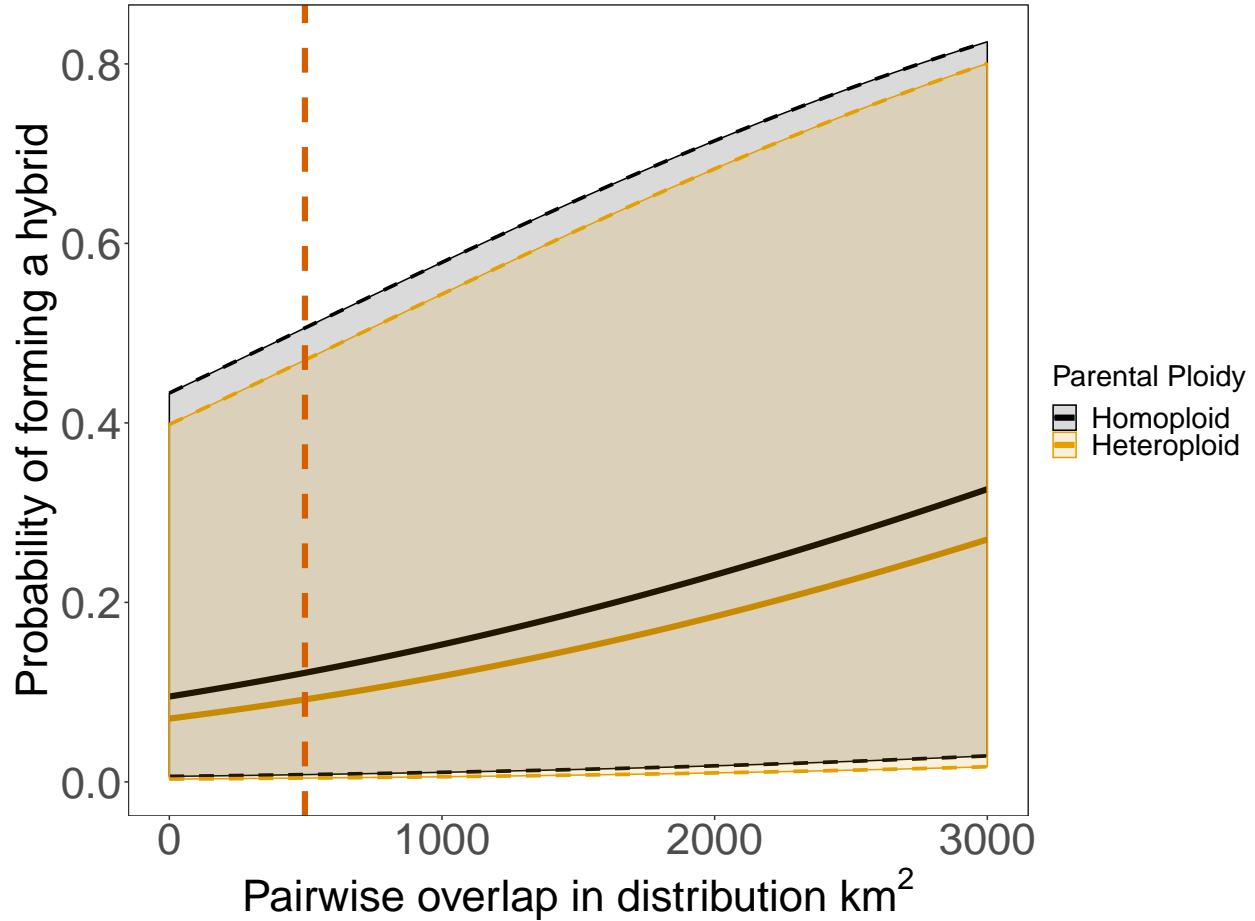


Figure 5: Predicted fit of probability of hybridisation given branch length between parental species and ploidy difference of parental species from Model 2. Homoploid indicates parental species of the same ploidy level, and heteroploid indicates parental species of different ploidy levels. Dashed lines 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.

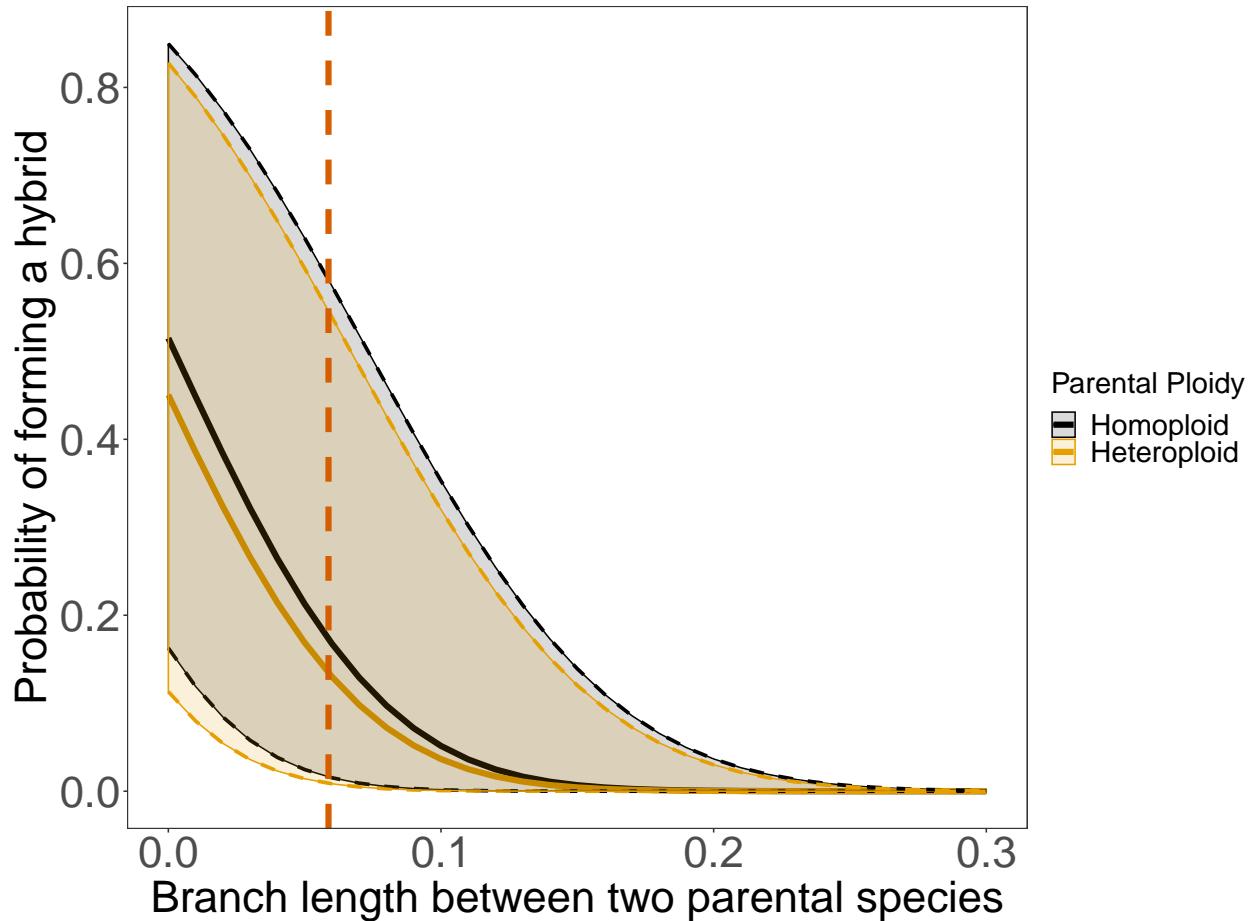


Figure 6: Predicted fit of probability of hybridisation given branch length between parental species from Model 1. Black dashed lines are the 95% Credible Intervals, bold line is the posterior mean of the coefficient for the probability of congeneric pairs of species hybridising as a function of branch 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.

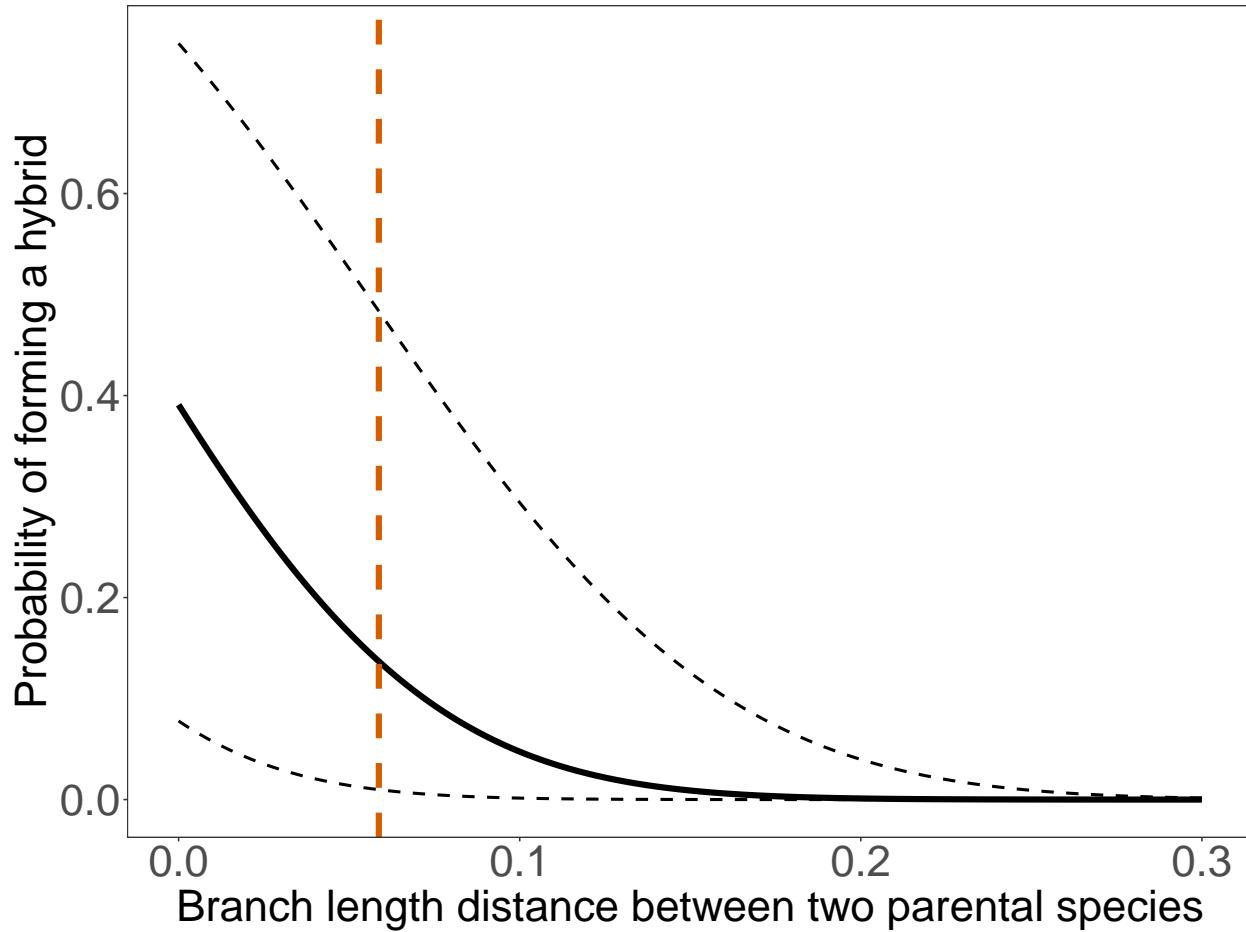


Table 2: Model 1: Probability of hybridisation on the probit scale with genetic distance, hectads shared and life history of parental species as fixed covariates. The posterior mean of the distribution of each coefficient is given, along with lower and upper 95% Credible Intervals. The p-value (pMCMC) 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, $\delta\alpha\delta I$. The model with historic gene flow (orange) had poor AIC values, and so is omitted here.

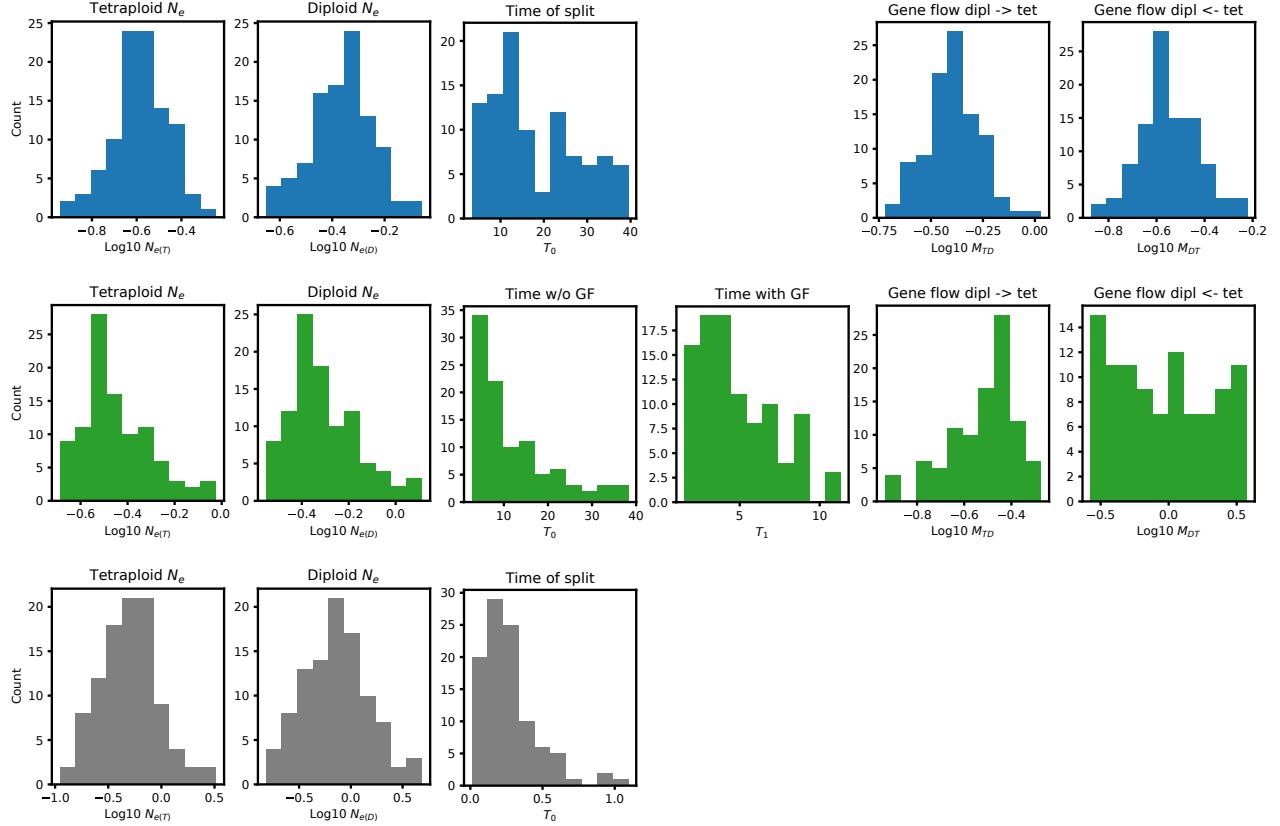


Table 5: Primers and PCR conditions used to amplify the *rpL32-trnLUAG* plastid marker in *Euphrasia* species.

Primer	Sequence Orientation (5' to 3')	Reagents (1 reaction)	PCR conditions	References
<i>rpL32</i> -Forward	CAGTTCCAAAAT ^A T ^G TC ^T CA ^C T ^X	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)
<i>trnLUAG</i> -Reverse	CTGCTTCCTAAGAGCAGCGT			

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

Sequence Primer (5' to 3') Orientation	Reagents (1 reaction)	PCR conditions	References
ITS4Forward TCCTCCGCTTATTCATATGC ITS4Reverse GGAAGTAAAGTCGTAACAAGG	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)

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	<i>Arabidopsis thaliana</i>	Brassicaceae	Herb	Laboratory stock
Field horsetail	<i>Equisetum arvense</i>	Equisetaceae	Fern	Wild collected in Edinburgh (GPS coordinates: 55.9679, -3.2129)
Red fescue	<i>Festuca rubra</i>	Poaceae	Grass	Commerical: Emorsgate seeds (Yorkshire + Dorset)
Yorkshire fog	<i>Holcus lanatus</i>	Poaceae	Grass	Commerical: Emorsgate seeds
Common liverwort	<i>Marchantia polymorpha</i>	Marchantiaceae	Bryophyte	Wild collected in Edinburgh (GPS coordinates: 55.9679, -3.2129)
Ribwort plantain	<i>Plantago lanceolata</i>	Plantaginaceae	Herb	Commerical: Emorsgate seeds (Somerset + Wiltshire)
Scots pine	<i>Pinus sylvestris</i>	Pinaceae	Tree	Commerical: Scotia Seeds
White clover	<i>Trifolium repens</i>	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.

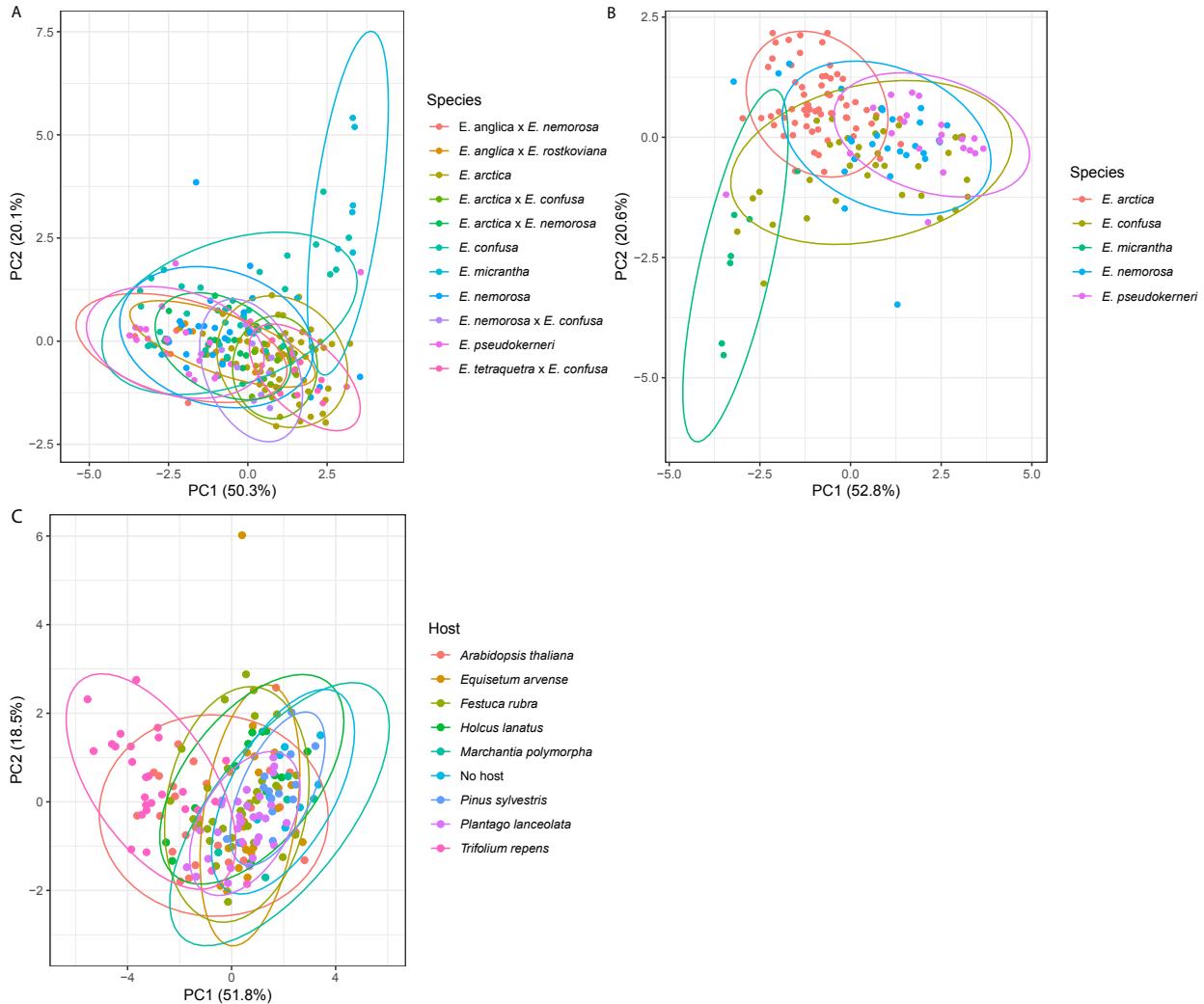
Collection number	Taxon	Locality	Latitude	Longitude	Collector
E4E0138	<i>E. arctica</i>	Fintallick, Glen Ledock, Comrie, Perthshire	56.41318	-4.03085	Dot Hall
E4E0144	<i>E. arctica</i>	Balachuirn, Isle of Raasay	57.38996	-6.06877	S.J. Bungard
E4E0032	<i>E. arctica</i>	South Links, Burray, Orkney	58.85275	-2.88701	John Crossley
E4E0139	<i>E. arctica</i>	Dalreoch Farm, Enochdhu	56.74199	-3.53350	Martin Robinson
E4E0049	<i>E. arctica</i>	Ouaisne, Jersey	49.17707	-2.18293	Anne Haden
E4E0247	<i>E. arctica</i>	Elsdon. Newcastle upon Tyne	55.22770	-2.10234	Stephanie Miles
NBer001*	<i>E. arctica</i>	North Berwick Glenn, East Lothian	56.05696	-2.70456	Alex Twyford
E4E0038	<i>E. confusa</i>	Oldbury, near Hartshill, Warwickshire	52.55285	-1.53980	John and Monika Walton
E4E0114	<i>E. confusa</i>	Trethew Mill, Bodmin, Cornwall	50.39585	–	Rosemary Parslow
E4E0095	<i>E. confusa</i>	North Anston Grassland, South Yorkshire	53.34738	-1.20803	Graeme Coles
E4E0009	<i>E. confusa</i>	Devil's Hole Blowout, Ravenmeols Local Nature Reserve, Merseyside	53.54062	-3.09041	Philip H. Smith
E4E0188	<i>E. micrantha</i>	Meall a Bathaich, Glen Garry, East Perthshire	56.82082	-4.182812	Alistair Godfrey
E4E0064	<i>E. nemorosa</i>	Castle Hill Local Nature Reserve, East Sussex	50.7842	0.052719	David Harris
E4E0069	<i>E. nemorosa</i>	Meridian Business Park, Leicester	52.60857	-1.19809	Geoffrey Hall
E4E0123	<i>E. nemorosa</i>	Bloody Oaks Triangle, Tickercote, Rutland	52.68950	-0.56263	Geoffrey Hall
E4E0029	<i>E. pseudokerneri</i>	Levin Down, Sussex	50.91346	-0.74150	Elizabeth Sturt
E4E0112	<i>E. pseudokerneri</i>	Beeston Common, Norfolk	52.93442	1.220071	Francis Farrow
E4E0027	<i>E. anglica</i> x <i>E. nemorosa</i>	West Dean Woods, Sussex	50.93212	-0.79735	Elizabeth Sturt
E4E0016	<i>E. anglica</i> x <i>E. rostkoviana</i>	Straduff Rathcabbin, Co. Tipperary	53.11902	-8.02454	David Nash
E4E0033	<i>E. arctica</i> x <i>E. confusa</i>	Nr Quoyorally, South Ronaldsay, Orkney	58.75897	-2.93473	John Crossley
E4E0145	<i>E. arctica</i> x <i>E. nemorosa</i>	Kylfakin, Wof, Skye	57.26685	-5.76042	S.J. Bungard

Collection number	Taxon	Locality	Latitude	Longitude	Collector
E4E0021	<i>E. arctica</i> x <i>E. nemorosa</i>	Dunamase, Co. Laois	53.03153	-7.21015	David Nash
E4E0031	<i>E. nemorosa</i> x <i>E. confusa</i>	Dolebury Fort, Somerset	51.32605	-2.79432	C.W. Hurlfurt
E4E0143	<i>E. tetraquetra</i> x <i>E. confusa</i>	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
<i>E. arctica</i>	8.0 \pm 0.2	82.9 \pm 4.4	1.1 \pm 0.1	195.2 \pm 1.5	4.4 \pm 0.1	8.6 \pm 0.2	*4.56 \pm 0.2
<i>E. confusa</i>	6.9 \pm 0.2	134.4 \pm 7.2	1.6 \pm 0.1	200.2 \pm 2.4	5.3 \pm 0.2	11.1 \pm 0.4	7.26 \pm 0.5
<i>E. micrantha</i>	5.6 \pm 0.2	70.6 \pm 8.1	3.0 \pm 0.4	—	2.4 \pm 0.3	8.3 \pm 0.2	0.57 \pm 0.4
<i>E. nemorosa</i>	7.7 \pm 0.1	127.4 \pm 8.1	1.4 \pm 0.1	206.6 \pm 1.7	5.1 \pm 0.2	11.9 \pm 0.5	7.67 \pm 0.5
<i>E. pseudokernerri</i>	8.8 \pm 0.4	176.4 \pm 15.6	1.4 \pm 0.1	205.1 \pm 2.0	5.5 \pm 0.2	13.2 \pm 0.4	8.67 \pm 0.6
<i>E. anglica</i> x <i>E. nemorosa</i>	9.1 \pm 0.5	148.1 \pm 11.8	1.4 \pm 0.1	195.7 \pm 1.9	6.0 \pm 0.3	12.0 \pm 0.6	10.00 \pm 1.0
<i>E. anglica</i> x <i>E. rostkoviana</i>	7.9 \pm 0.2	122.6 \pm 8.3	1.3 \pm 0.1	192.3 \pm 12.3	5.9 \pm 0.3	10.6 \pm 0.5	7.44 \pm 0.7
<i>E. arctica</i> x <i>E. confusa</i>	9.5 \pm 0.2	100.3 \pm 4.3	1.4 \pm 0.1	193.4 \pm 3.2	3.8 \pm 0.1	7.8 \pm 0.3	5.70 \pm 0.4
<i>E. arctica</i> x <i>E. nemorosa</i>	8.0 \pm 0.2	132.2 \pm 14.5	1.3 \pm 0.1	205.3 \pm 2.4	6.0 \pm 0.3	11.3 \pm 0.4	6.50 \pm 0.4
<i>E. arctica</i> x <i>E. nemorosa</i>	7.9 \pm 0.2	92.5 \pm 5.9	1.0 \pm 0.1	199.3 \pm 2.8	5.1 \pm 0.2	9.8 \pm 0.3	7.00 \pm 0.5
<i>E. confusa</i> x <i>E. tetraquetra</i>	7.2 \pm 0.2	57.4 \pm 5.8	0.7 \pm 0.1	194.1 \pm 2.7	4.2 \pm 0.2	7.6 \pm 0.4	4.00 \pm 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.

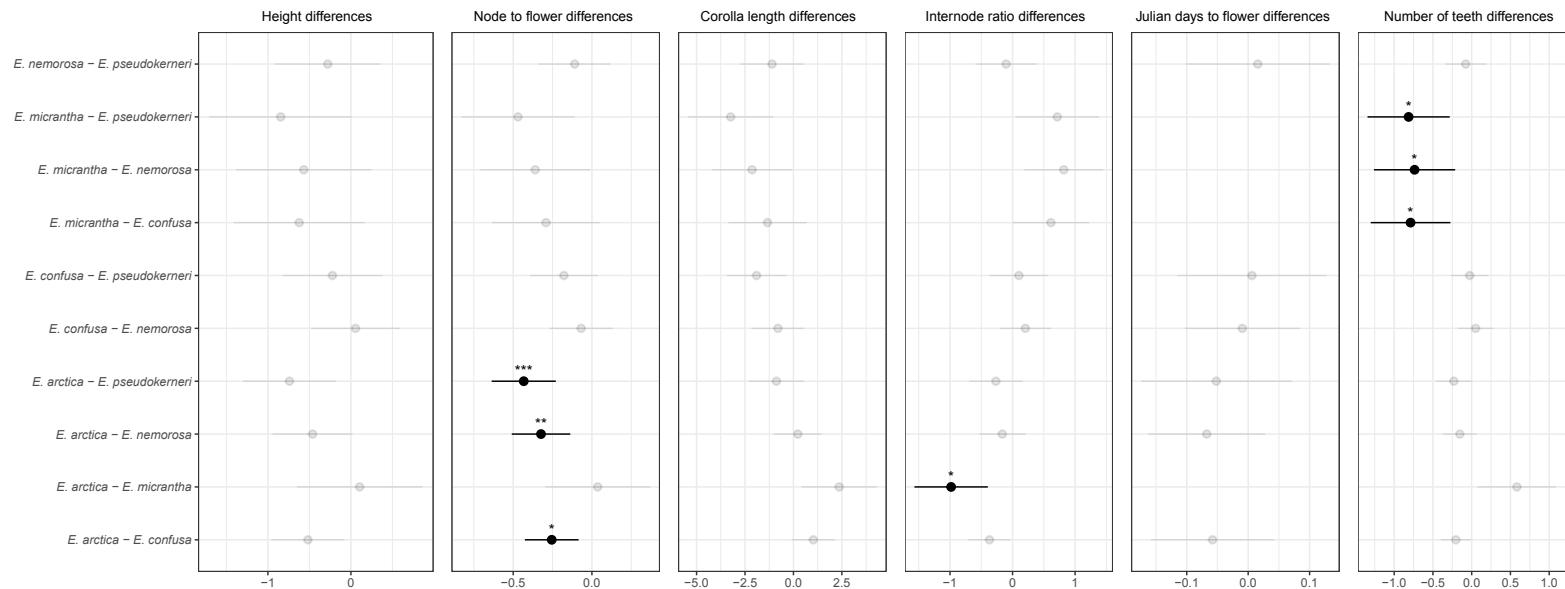


Table 11: Summary of trait values for *Euphrasia arctica* grown on many different hosts. Values are mean +/- one standard error. Length and height measurements are in millimeters.

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
<i>A. thaliana</i>	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
<i>E. arvense</i>	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
<i>F. rubra</i>	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
<i>H. lanatus</i>	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
<i>M. polymorpha</i>	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
<i>P. lanceolata</i>	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
<i>P. sylvestris</i>	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
<i>T. repens</i>	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
<i>Arabidopsis thaliana</i>	No host	1.065*	0.49*	0.102	-0.178***	-0.116	0.508
<i>Equisetum arvense</i>	No host	0.946*	0.304	0.041	-0.112***	-0.066	0.212
<i>Festuca rubra</i>	No host	1.04*	0.529**	0.073	-0.112***	-0.034	0.397
<i>Holcus lanatus</i>	No host	1.05*	0.332	0.077	-0.063	-0.009	0.258
<i>Marchantia polymorpha</i>	No host	0.25	-0.181	0.07	-0.031	-0.025	-0.136
<i>Pinus sylvestris</i>	No host	0.481	0.067	0.015	-0.03	0.051	0.01
<i>Plantago lanceolata</i>	No host	0.879	0.246	0.016	-0.137***	-0.071	0.419
<i>Trifolium repens</i>	No host	2.102***	1.241***	0.180*	-0.244***	-0.134	0.711
<i>Equisetum arvense</i>	<i>Arabidopsis thaliana</i>	-0.119	-0.186	-0.061	0.066*	0.05	-0.296
<i>Festuca rubra</i>	<i>Arabidopsis thaliana</i>	-0.024	0.039	-0.029	0.065**	0.082	-0.111
<i>Holcus lanatus</i>	<i>Arabidopsis thaliana</i>	-0.015	-0.158	-0.025	0.114***	0.107	-0.25
<i>Marchantia polymorpha</i>	<i>Arabidopsis thaliana</i>	-0.815	-0.671***	-0.032	0.147***	0.091	-0.644
<i>Pinus sylvestris</i>	<i>Arabidopsis thaliana</i>	-0.584	-0.423*	-0.087	0.148***	0.167	-0.498
<i>Plantago lanceolata</i>	<i>Arabidopsis thaliana</i>	-0.186	-0.244	-0.086	0.041	0.044	-0.089
<i>Trifolium repens</i>	<i>Arabidopsis thaliana</i>	1.037***	0.751***	0.077	-0.066	-0.018	0.204
<i>Festuca rubra</i>	<i>Equisetum arvense</i>	0.095	0.225	0.031	0	0.032	0.185
<i>Holcus lanatus</i>	<i>Equisetum arvense</i>	0.104	0.028	0.035	0.049	0.057	0.046
<i>Marchantia polymorpha</i>	<i>Equisetum arvense</i>	-0.696	-0.486*	0.029	0.081	0.041	-0.348

Host 1	Host 2	Corolla length	Height	Internode ratio	Julian days to flower	Nodes to flower	Number of leaf teeth
<i>Pinus sylvestris</i>	<i>Equisetum arvense</i>	-0.465	-0.237	-0.025	0.082**	0.117	-0.202
<i>Plantago lanceolata</i>	<i>Equisetum arvense</i>	-0.067	-0.059	-0.024	-0.025	-0.006	0.207
<i>Trifolium repens</i>	<i>Equisetum arvense</i>	1.156***	0.937***	0.138*	-0.132***	-0.068	0.499*
<i>Holcus lanatus</i>	<i>Festuca rubra</i>	0.01	-0.197	0.003	0.049	0.025	-0.139
<i>Marchantia polymorpha</i>	<i>Festuca rubra</i>	-0.79	-0.71***	-0.002	0.081	0.009	-0.533
<i>Pinus sylvestris</i>	<i>Festuca rubra</i>	-0.56	-0.462**	-0.057	0.083**	0.085	-0.387
<i>Plantago lanceolata</i>	<i>Festuca rubra</i>	-0.161	-0.283	-0.056	-0.025	-0.038	0.022
<i>Trifolium repens</i>	<i>Festuca rubra</i>	1.062***	0.712***	0.106	-0.132***	-0.1	0.315
<i>Marchantia polymorpha</i>	<i>Holcus lanatus</i>	-0.8	-0.513*	-0.006	0.033	-0.016	-0.394
<i>Pinus sylvestris</i>	<i>Holcus lanatus</i>	-0.569	-0.265	-0.061	0.034	0.06	-0.248
<i>Plantago lanceolata</i>	<i>Holcus lanatus</i>	-0.171	-0.086	-0.06	-0.074**	-0.063	0.161
<i>Trifolium repens</i>	<i>Holcus lanatus</i>	1.052**	0.909***	0.102	-0.18***	-0.125	0.454
<i>Pinus sylvestris</i>	<i>Marchantia polymorpha</i>	0.231	0.248	-0.055	0.001	0.076	0.146
<i>Plantago lanceolata</i>	<i>Marchantia polymorpha</i>	0.629	0.427	-0.054	-0.106***	-0.047	0.555
<i>Trifolium repens</i>	<i>Marchantia polymorpha</i>	1.852***	1.423***	0.109	-0.213***	-0.109	0.847*
<i>Plantago lanceolata</i>	<i>Pinus sylvestris</i>	0.398	0.178	0.001	-0.107***	-0.123	0.409
<i>Trifolium repens</i>	<i>Pinus sylvestris</i>	1.621***	1.174***	0.164*	-0.214***	-0.185	0.701*
<i>Trifolium repens</i>	<i>Plantago lanceolata</i>	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	$\text{Pr}(> \chi^2)$
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)**
<i>Arabidopsis thaliana</i>	1.064 (0.293)***	0.489 (0.135)***	0.102 (0.051)*	-0.177 (0.024)***	-0.115 (0.114)	0.507 (0.241)*
<i>Equisetum arvense</i>	0.945 (0.300)**	0.304 (0.138)*	0.041 (0.052)	-0.111 (0.024)***	-0.065 (0.116)	0.212 (0.254)
<i>Festuca rubra</i>	1.040 (0.288)***	0.529 (0.134)***	0.073 (0.050)	-0.112 (0.023)***	-0.033 (0.111)	0.396 (0.242)
<i>Holcus lanatus</i>	1.050 (0.323)**	0.331 (0.147)*	0.077 (0.055)	-0.063 (0.025)*	-0.008 (0.123)	0.257 (0.267)
<i>Marchantia polymorpha</i>	0.250 (0.433)	-0.181 (0.171)	0.070 (0.064)	-0.03 (0.029)	-0.024 (0.143)	-0.135 (0.338)
<i>Pinus sylvestris</i>	0.480 (0.333)	0.067 (0.153)	0.015 (0.058)	-0.029 (0.026)	0.051 (0.126)	0.010 (0.290)
<i>Plantago lanceolata</i>	0.879 (0.288)**	0.245 (0.134)	0.016 (0.05)	-0.136 (0.023)***	-0.071 (0.112)	0.419 (0.242)
<i>Trifolium repens</i>	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.

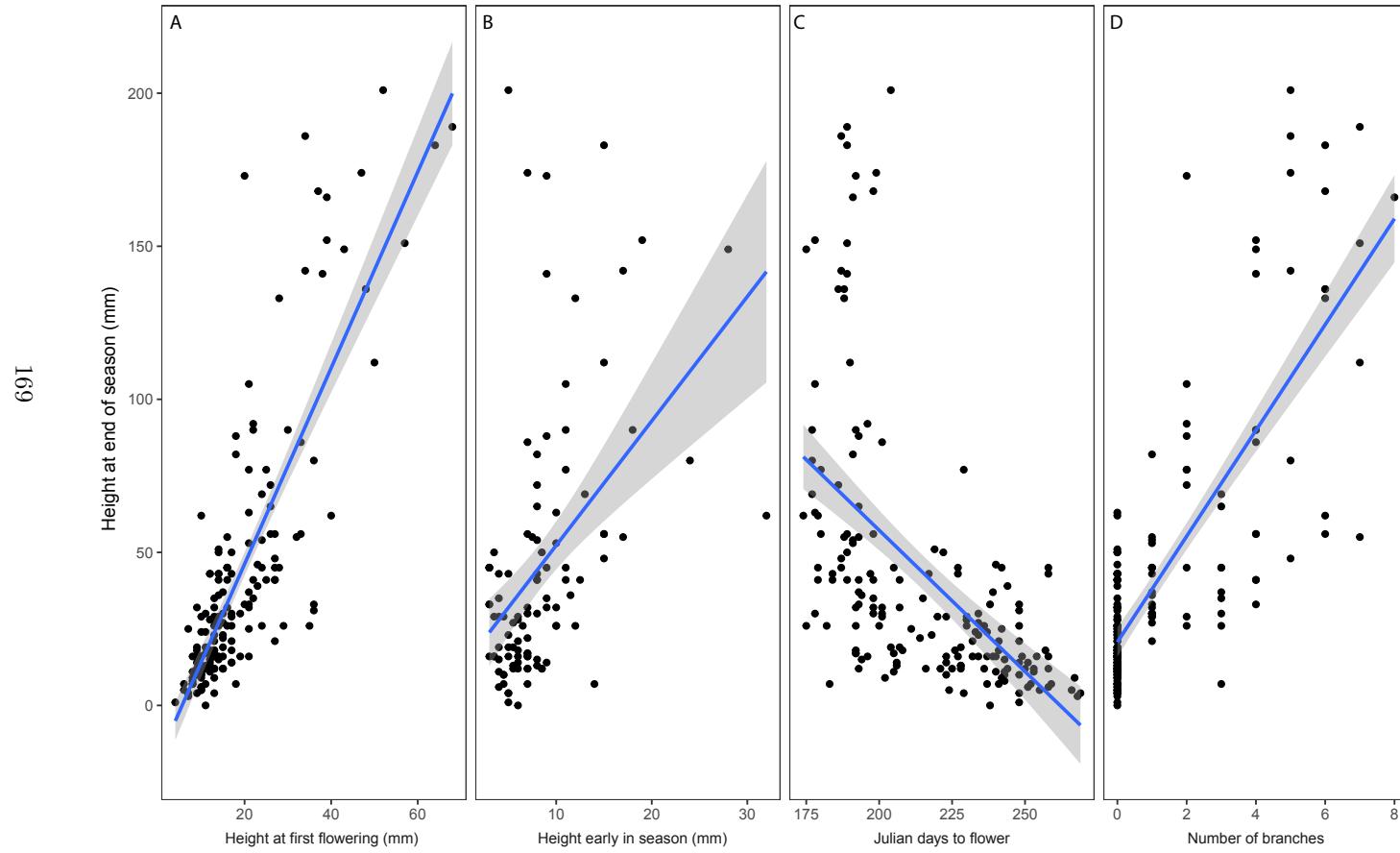


Figure 11: Comparison between trait values for wild-collected herbarium specimens and common garden plants of diverse *Euphrasia* species for (A) nodes to flower, (B) corolla length (mm), (C) number of leaf teeth, (D) internode ratio. Points are for *Euphrasia* population means, with bars representing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

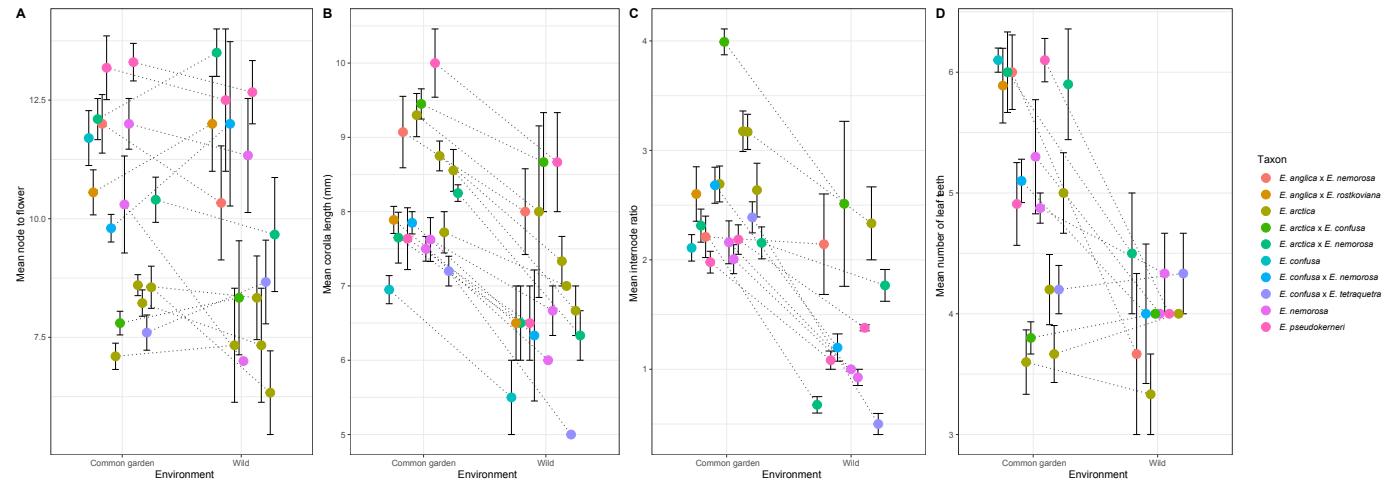


Table 16: Model output from MCMCglmm comparing traits for the wild collected *Euphrasia* specimens to the baseline of the common garden data (Intercept). The posterior means are reported along with the lower and upper 95% credible intervals, as well as the p-value (pMCMC) for the effect.

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

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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 \pm 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.

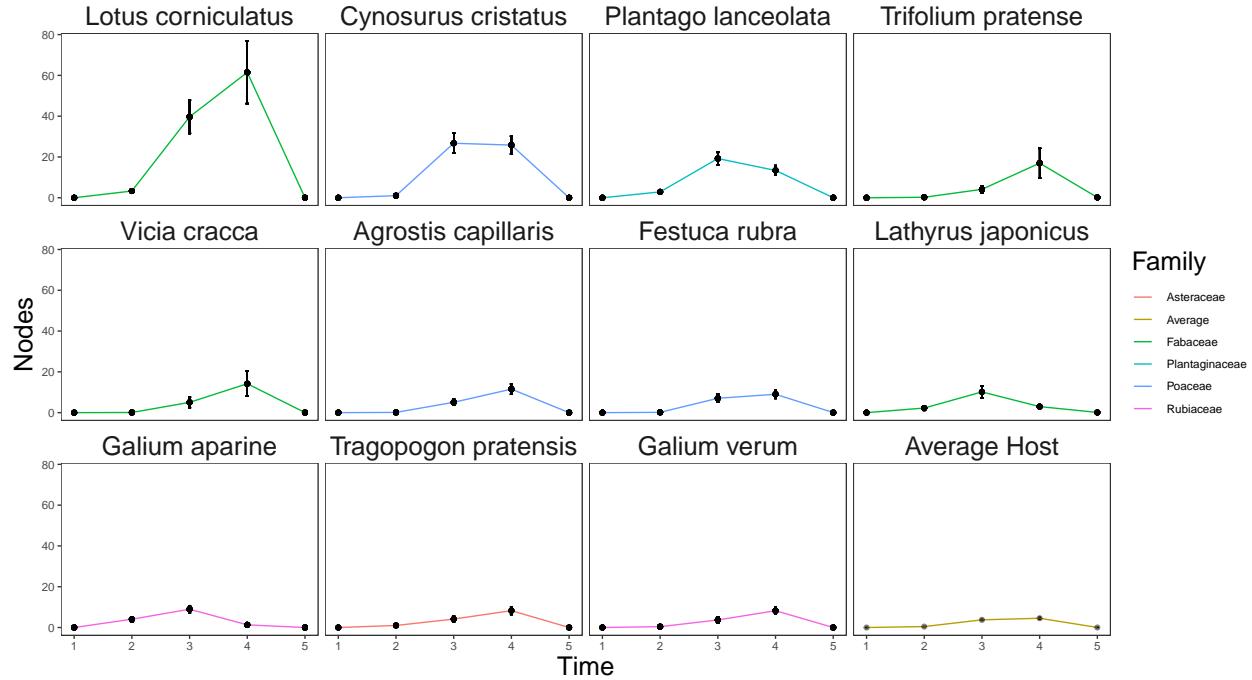


Figure 13: Posterior distributions of the phylogenetic signal for the models from Experiment 1, where 45 different host species were grown with *Euphrasia arctica*. The distributions of phylogenetic signal are shown for three *Euphrasia* traits: survival, total reproductive output at the end of the season, 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.

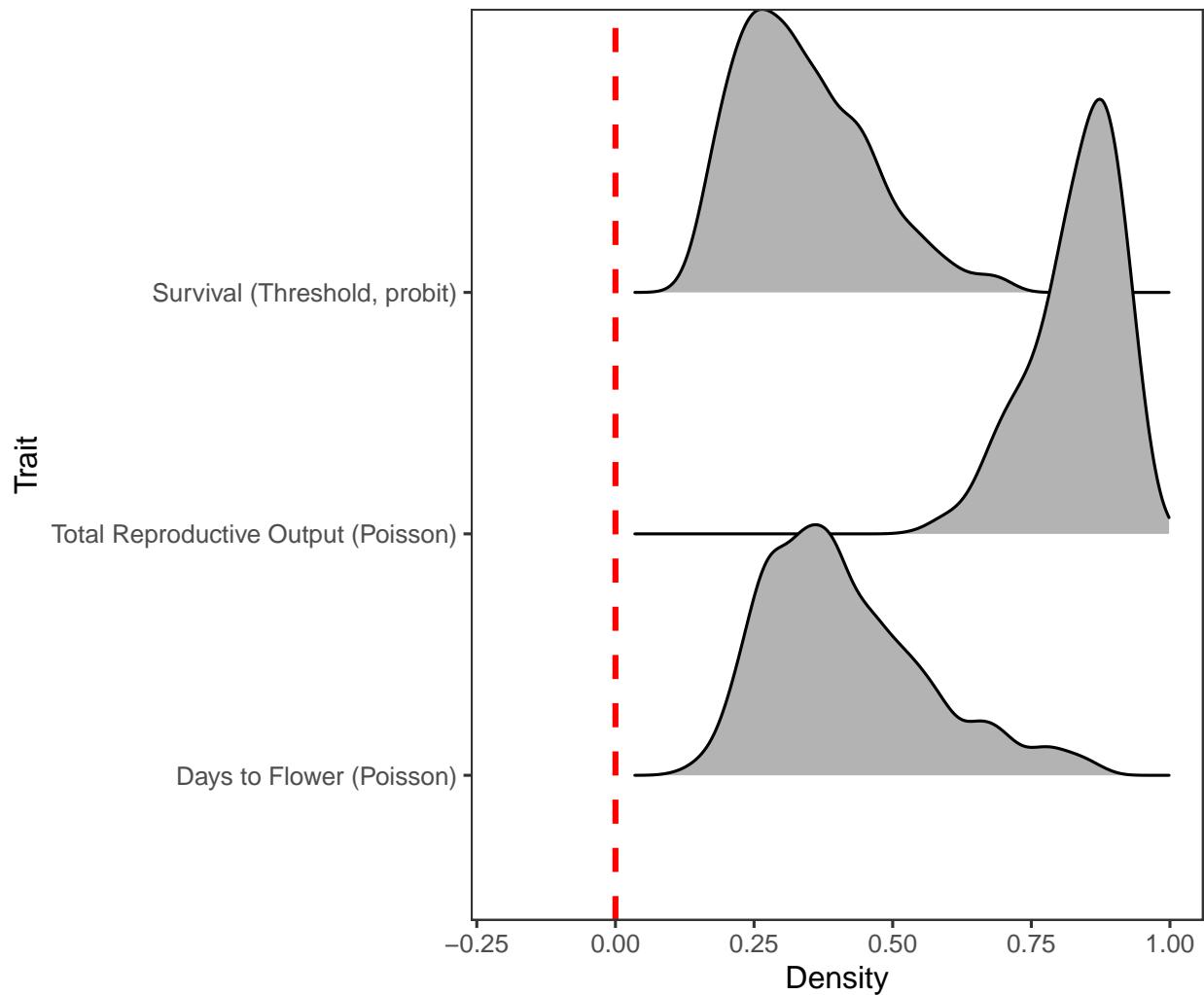


Figure 14. Posterior distribution of the variance for random effects in the model fitted for Experiment 2, where four species of *Euphrasia* were grown on thirteen different species of host. The random effects are the *Euphrasia*-host interaction, the sole effect of host species, and the residual variance. 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.

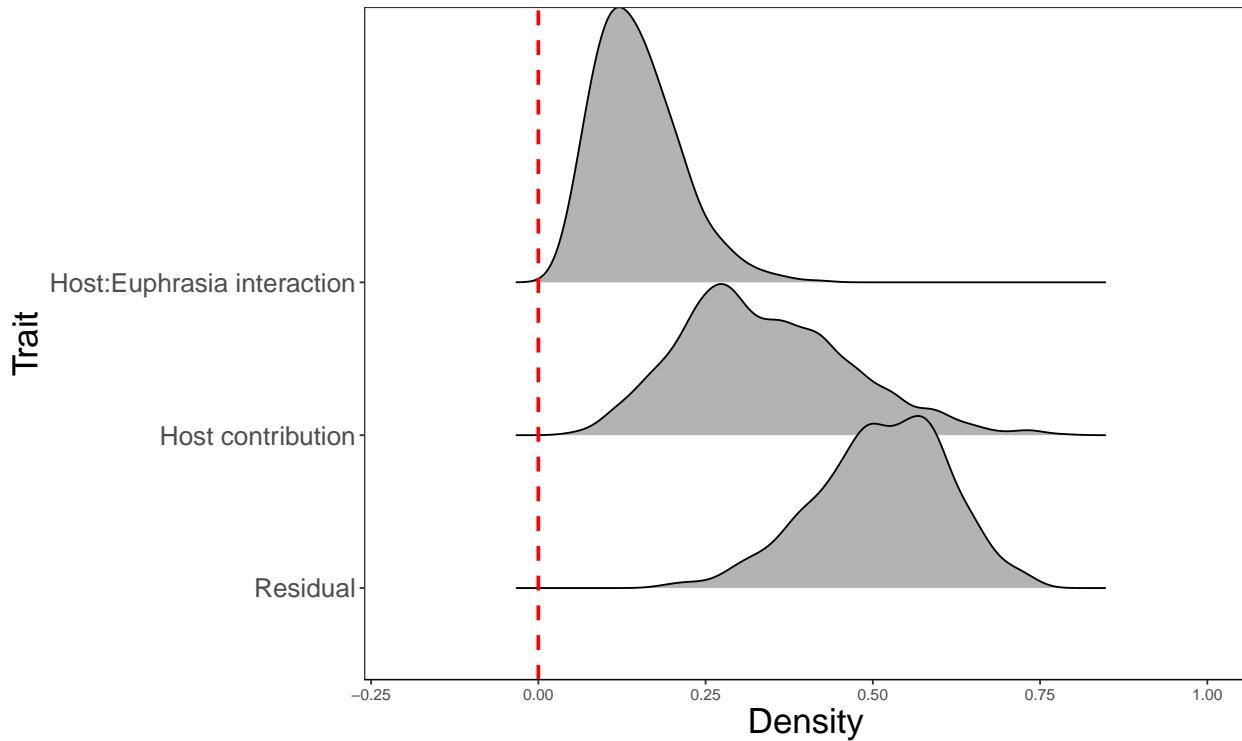


Figure 15. Performance of four species of *Euphrasia* on thirteen different species of host plants measured as cumulative reproductive nodes. Each panel represents a unique *Euphrasia* population (a = A1766, b = T1761, c = V1761, d = M1767, e = M1768, f = M1769), coloured by species. Two populations, (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 \pm one standard error.

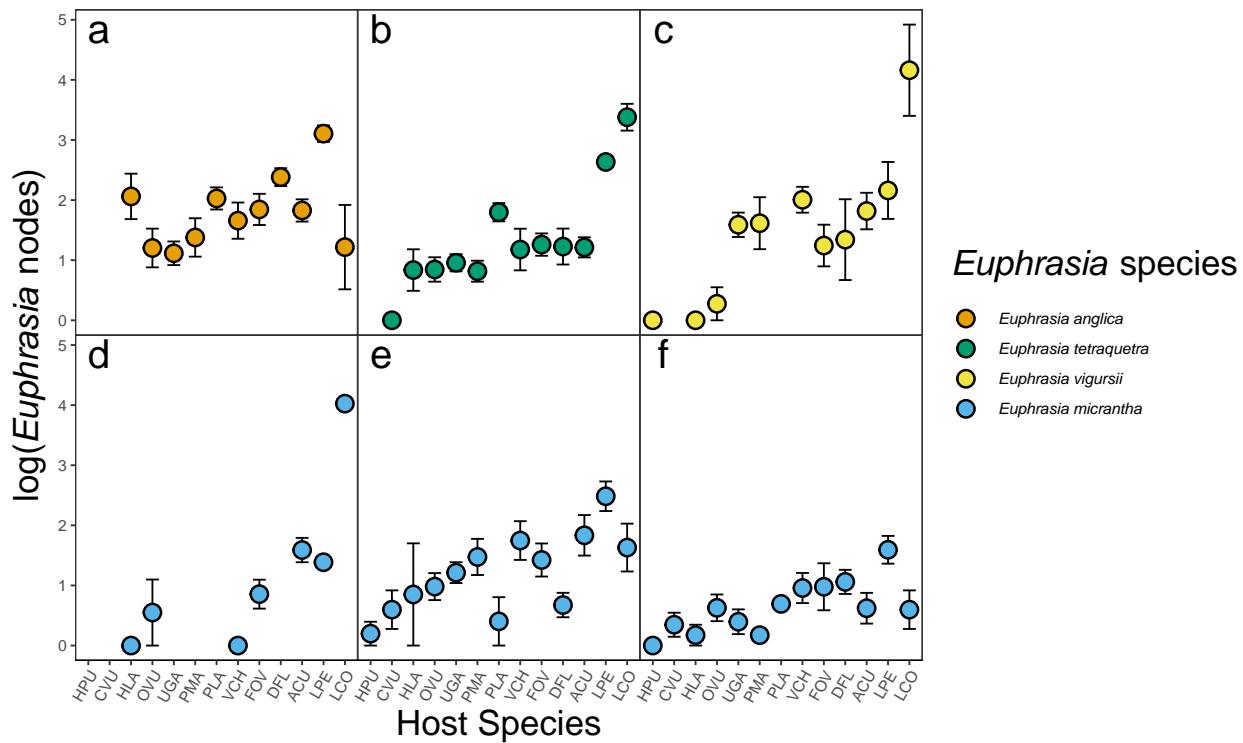


Table 17: Plant names, attributes and collection sources for host species used in Experiment 1.

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

Host species	Authority	Functional group	Life history		Seed source
<i>Mimulus guttatus</i>	DC.	Forb	Perennial		Author collections
<i>Ononis spinosa</i>	L.	Legume	Perennial		Emorsgate & Wild Flower Shop
<i>Papaver rhoeas</i>	L.	Forb	Annual		Emorsgate
<i>Phleum pratense</i>	L.	Grass	Perennial		Wild Flower Shop
<i>Pinus sylvestris</i>	L.	Woody	Perennial		Scotia seeds
<i>Plantago lanceolata</i>	L.	Forb	Perennial		Emorsgate
<i>Pteridium aquilinum</i>	L. (Kuhn)	Fern	Perennial	British Pteridological Society spore exchange	
<i>Rumex acetosella</i>	L.	Forb	Perennial		Scotia seeds
<i>Senecio vulgaris</i>	L.	Forb	Annual		RBGE
<i>Silene dioica</i>	(L.) Clairv.	Forb	Perennial	D. Charlsworth, Univ. Edinburgh	
<i>Silene latifolia</i>	Poir.	Forb	Perennial	D. Charlsworth, Univ. Edinburgh	
<i>Thymus polytrichus</i>	A.Kern. ex Borbás	Woody	Perennial		Emorsgate
<i>Sorbus aucuparia</i>	L.	Woody	Perennial		RBGE
<i>Tragopogon pratensis</i>	L.	Forb	Perennial		Scotia seeds
<i>Trifolium pratense</i>	L.	Legume	Perennial	Chiltern Seeds & Wild Flower Shop	
<i>Ulex europaeus</i>	L.	Legume/Woody	Biennial	Tree Seed Online Ltd	
<i>Vicia cracca</i>	L.	Legume	Perennial		Emorsgate
<i>Zea mays</i>	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
<i>Agrostis curtisii</i>	Kerguélen	Millenium Seed Bank, Kew Gardens	Seed
<i>Calluna vulgaris</i>	(L.) Hull	RBGE	Seed, but small plants from cuttings
<i>Deschampsia (Avenella) flexuosa</i>	(L.) Trin.	Chiltern Seeds	Seed
<i>Festuca ovina</i>	L.	Emorsgate	Seed
<i>Holcus lanatus</i>	L.	Emorsgate	Seed
<i>Hypericum pulchrum</i>	L.	Scotia Seeds	Seed
<i>Lotus corniculatus</i>	L.	Emorsgate	Seed
<i>Lolium perenne</i>	L.	Emorsgate	Seed
<i>Origanum vulgare</i>	L.	Emorsgate	Seed
<i>Plantago lanceolata</i>	L.	Emorsgate	Seed
<i>Plantago maritima</i>	L.	Scotia Seeds	Seed
<i>Ulex gallii</i>	Planch.	Millenium Seed Bank, Kew Gardens	Seed
<i>Veronica chamaedrys</i>	L.	Scotia Seeds	Seed

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

Experiment	<i>Euphrasia</i> species	Location	Grid Reference
1	<i>E. arctica</i>	Inverkeithing, Scotland	NT 1389 82312
2	<i>E. anglica</i>	(A1766)	Cheddar, Somerset
2	<i>E. vigursii</i>	(V1761)	St Agnes Head, Cornwall
2	<i>E. tetraquetra</i>	(T1761)	St Agnes Head, Cornwall
2	<i>E. micrantha</i>	(M1767)	Borrowdale, Cumbria
2	<i>E. micrantha</i>	(M1768)	Alness, Scotland
2	<i>E. micrantha</i>	(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
<i>Euphrasia micrantha</i>	-1.2795	-1.7479	-0.8284	1000	0.0010
<i>Euphrasia tetraquetra</i>	-0.3702	-0.8160	-0.0076	873.2	0.0620
<i>Euphrasia vigursii</i>	-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)

This article has been accepted at the journal Sibbaldia.

Abstract

Parasitic plants are particularly challenging to cultivate as the growth conditions must be suitable for the parasite, the host, and their interaction. Here, we review our progress growing eyebrights (*Euphrasia*), a group of hemiparasitic plants found in diverse habitats in Britain and Ireland. We consider the protocols required to grow them under a range of conditions, including the growth of un-hosted seedlings in the laboratory, mature plants in pot trials, commercial scale quantities in cultivated fields, and the establishment of plants in the wild. We draw on recent research results from pot experiments, and also present new results from preliminary field trials and reciprocal transplant experiments in nature. We find that the growth conditions for *Euphrasia* must use cold stratification to break seed dormancy, use a suitable host species and manage the host to avoid competition, and mimic their natural environment in terms of free draining soil and unshaded conditions. While *Euphrasia* can be successfully grown in different environments, more reliable protocols are required for establishing mature plants under natural conditions.

Introduction

Parasitic plants are a diverse group of approximately 4,500 species that are characterised 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 mirrored in cultivation (Joel, Gressel, and Musselman 2013). There are also a number of specific horticultural issues associated with growing parasitic plants that must be overcome, and the conditions must be suitable for the parasite, the host, and their interaction. In particular, many parasitic plants (particularly obligate parasites) require host-specific cues in order to germinate, and almost all parasitic plants require a host in order to grow vigorously (Albrecht, Yoder, and Phillips 1999). Despite these issues a wide variety of parasitic plants are cultivated (Pignone and Hammer 2016). Recently, the use of parasitic plants in ecological restoration has increased interest in their cultivation and seed production, making the dissemination of cultivation protocols particularly timely.

The genus *Euphrasia* (Orobanchaceae) 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 (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; Brown et al. 2020). We are currently developing *Euphrasia* as a study system for understanding plant parasitism, and for investigating evolutionary questions related to natural hybridisation, genome evolution, and mating system diversity. There are 20 native British *Euphrasia* species, which show rich variation in habitat preference, associated species, ploidy (there are diploids and tetraploids; Yeo 1956; Wang et al. 2018), and mating system (there are selfing and outcrossing species; French et al. 2005; Metherell and Rumsey 2018).

Species of *Euphrasia* are known to hybridise extensively in the field and produce a diversity of hybrids as well as species of hybrid origin (Stace and Crawley 2015; Metherell and Rumsey 2018).

In this article, we describe our experience optimising horticultural protocols for growing British native eyebrights. This builds on the body of work by Peter Yeo during his time as a taxonomist at Cambridge University Botanic Garden. Yeo published extensively on the taxonomy and evolution of European *Euphrasia* (Yeo 1956, 1961, 1964, 1973). Many of his observations were made on plants he grew in cultivation, either from seeds or from turf containing *Euphrasia* he extracted from the wild. Here, we discuss the range of protocols for experimental growth studies under laboratory conditions, in pot trials, under field conditions, and in the wild. We review our general experience and personal observations made while conducting a suite of experimental studies growing *Euphrasia* with different hosts (Brown et al. 2020; Becher et al. 2020; Brown, Moore, and Twyford 2021), and also present preliminary results from field trials, and from reciprocal transplant experiments in the wild.

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 many model plant species like the thale-cress *Arabidopsis thaliana* (Weinkoop, Baginsky, and Weckwerth 2010). British *Euphrasia* are also small annual plants suitable to experimental manipulation, but require more specialised cultivation. *Euphrasia* seeds have dormancy, and cold treatment is required to induce germination (Yeo 1961 ; Liebst and Schneller 2008). This seasonal cue is likely to be important in natural environments to ensure germination is initiated synchronously, at a suitable time in the spring (Rubin and Friedman 2018). In our experimental work, we have broken seed dormancy in either of two ways: leaving seeds outside over winter, or forcing germination in the fridge. The latter method can be achieved by storing seeds on damp tissue paper on sterile plates. In this case, only a couple of drops of water are needed, otherwise mould growth may grow. With these dormancy constraints, we have only ever grown one generation per year, although a shorter generation time may be possible under controlled conditions (see below).

Seed germination rates in *Euphrasia* are variable and often low (Yeo 1964). We have found the germination success of wild collected seeds to be around 40-50% (Brown et al. 2020), though germination can be as low as 20%. The probability of germination depends on the condition of the seed. For example, in a small-scale test of *Euphrasia arctica* seed germination, we found 10/20 seeds considered ‘plump’ successfully germinated, compared to 6/20 in ‘intermediate’ condition, and none that were considered ‘shriveled’ (Becher, Unpublished Results; Fig. 1). It is likely that the shriveled seeds had been aborted by the parent plant, either because of developmental problems, genetic abnormalities, or limited resources (Stephenson 1981). In general, collections late in the season are likely to have the lowest germination as most viable seeds will have dehisced. Seed cleaning by sieving and winnowing, or for larger quantities using machinery such as a gravity separator, can help to remove shriveled or abnormally small seeds and subsequently improve germination rates. *Euphrasia* seeds should be dried (e.g. at room temperature for one week) prior to storage in the fridge or freezer. Seeds frozen at -4 °C were also found to store well, and survive for at least three years (Chapman, Miles, and Trivedi 2019). In general, germination is synchronous, with the majority of seeds in our outdoor pot experiments germinating in a two-week window in April. However, there are likely to be some differences between species and populations (Fig. 2).

Euphrasia require a host for vigorous growth, and this requirement must be met within a few days of germination to ensure growth is not hindered (Wettstein 1896; Yeo 1961). Germinating *Euphrasia* seeds produce relatively large cotyledons and a robust hypocotyl, while the radicle is relatively small (Simpson 1977). Root expansion occurs quickly, as well as notable root hair formation within a week. *Euphrasia* unable to find a host within the first two weeks often die, while some may remain small in stature above ground but



Figure 1: Three categories of *Euphrasia* seed quality: (a) shrivelled, (b) intermediate, and (c) plump. The seeds are approximately 2 mm in length.

develop an extensive below ground root system questing for a host (Yeo 1961). The choice of host species can have a dramatic influence the survival of *Euphrasia*. For example, some fast growing hosts that *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 survival than forbs or woody plants.

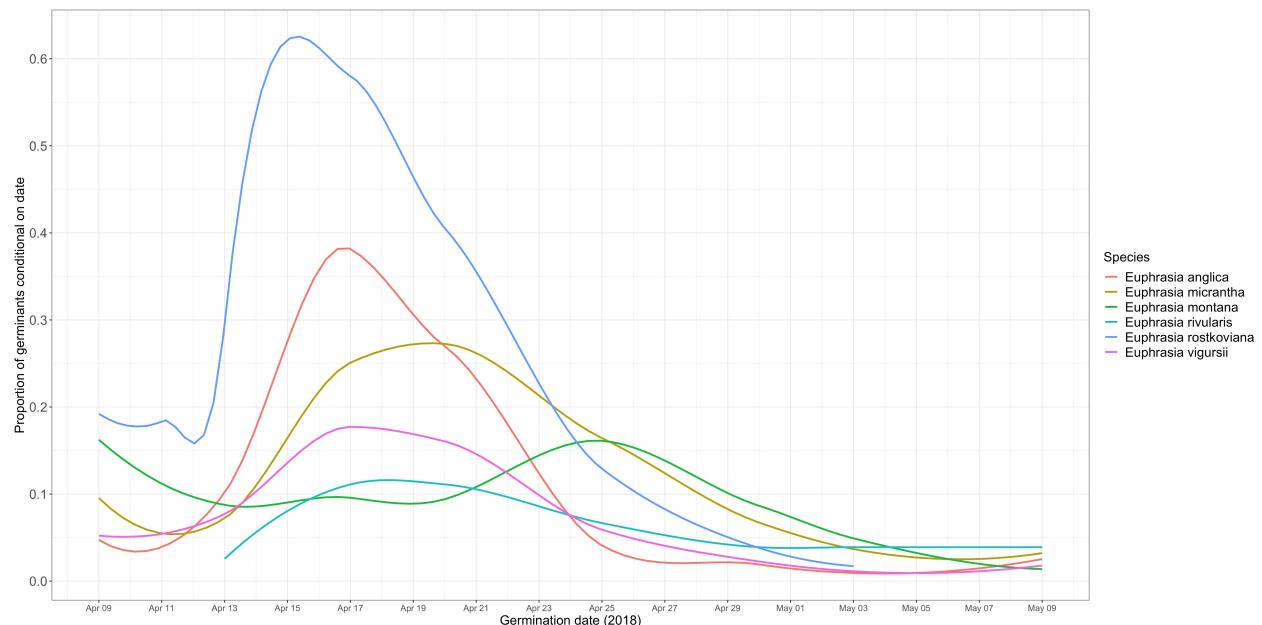


Figure 2: *Euphrasia* seed germination profile at the RBGE. Plot shows germination of six *Euphrasia* species based on monitoring every 2 days in the spring of 2018, for the study of Brown et al. (2021).

In pots, a host can be introduced carefully after *Euphrasia* seeds germinate, with minimal disruption to the *Euphrasia* plant. Otherwise, the host seed can be sown at the same time as the *Euphrasia* seed. However, because *Euphrasia* seed germination is variable and often low (< 50%), this approach wastes many host plants. Moreover, sowing hosts separately in the spring allows germination time to be controlled, whereas planting with *Euphrasia* in the autumn results in asynchronous host germination and growth, adding a confounding variable in controlled experimental studies. More generally, 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 (see Field Trials, below). Subsequently, as British *Euphrasia* are annual plants, all individuals die at the end of the season, which typically lasts until late September.

In cultivation and in the wild, *Euphrasia* may be attacked by a number of pests. The most serious 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 with a soapy solution, or if *Euphrasia* individuals are kept in glasshouses, to keep air movement and ventilation. Another common pest is a species of rust (*Coleosporium* Fig. 3; likely alternate host of *Pinus* species; Ellis and Ellis 1985), which is an alarming orange colour, thought its effect on *Euphrasia* is not known. Lastly, some Lepidopteran caterpillar species in the genus *Perizoma* attack *Euphrasia* by spinning and eating the leaves (Fitter and Peat 1994).

Laboratory conditions

For detailed studies of plant development, it is often necessary to grow plants under controlled laboratory conditions. For such studies, *Euphrasia* seeds can be readily germinated on moist filter paper under sterile conditions, using ethanol to sterilise petri dishes and seeds and sealing the sterile dishes with tape to avoid contamination. Petri dishes should be maintained in a fridge at 4 °C until germination (no supplemental light required). We have grown wild-collected *E. arctica* seeds in this way, with germination observed after a period of six weeks (Brown, Personal Observation). This sterile plate-based method is suitable for obtaining young seedlings, as required for certain applications (such as young root samples for cytogenetic analysis), but further refinement is necessary to make this suitable for growing larger plants. For more detailed developmental studies plants can be grown on an artificial media on sterilised plates. Seeds will germinate after approximately 10 days at 4 °C on 1/4 Hoagland media, a widely used hydroponic nutrient solution used to grow other parasitic plants (Delavault et al. 1998).

Pot trials

Growing *Euphrasia* in pots has the benefit of plants being in a substrate where they can form more natural host interactions than they would on an artificial media in the laboratory. Pot trials are useful for common garden studies, where material of different provenience and/or from different species are grown under common conditions. Any phenotypic differences between individuals, population, and species, which are observed under common conditions can be attributed to heritable (i.e. genetic or some epigenetic) differences (e.g. Riihimäki and Savolainen 2004). Reduced differentiation under common conditions relative to the wild, however, indicates that the phenotypic differences observed in nature were mainly due to environmental differences such as soil, herbivory, or the available host plants. This kind of common garden experiments is used extensively in ecology, evolution, and genetic research to investigate the effects of ‘nature vs nurture’.

We have performed five experimental common garden studies with *Euphrasia* grown in pots at the Royal Botanic Garden Edinburgh (RBGE) nursery (see Table 1). All of our experiments have involved germinating the seeds in pots outside in the bark-based substrate RBGE1, before (in the first four experiments) moving pots with seedlings to a greenhouse environment for the growing season.



Figure 3: *Coleosporium* sp. on *Euphrasia* (Pitlochry, Perthshire, UK, September 2020).

Table 1. Summary of *Euphrasia* common garden experiments conducted at the RBGE nursery.

Aim of experiment	<i>Euphrasia</i> species and hosts	Growth conditions	Reference
1 - Understand morphological differences between diverse <i>Euphrasia</i> species	222 individuals from 11 <i>Euphrasia</i> taxa, grown with a clover host	Pot experiment in a Hartley Botanic Glasshouse, 2016	(Brown et al. 2020)
2 - Study how host species impacts on <i>Euphrasia</i> morphology	194 individuals of <i>E. arctica</i> on 8 different host species, and without a host	Pot experiment in a Hartley Botanic Glasshouse, 2016	(Brown et al. 2020)
3 - Quantify <i>Euphrasia</i> performance and survival with diverse hosts	1379 individuals of <i>E. arctica</i> on 45 different hosts	Pot experiment in a Venlo Glasshouse, 2017	(Brown, Moore, and Twyford 2021)
4 - Investigate <i>Euphrasia</i> -host interactions	1259 individuals from 6 different <i>Euphrasia</i> populations on 13 different hosts	Pot experiment in a Venlo Glasshouse, 2018	(Brown, Moore, and Twyford 2021)
5 - Investigate differences of tetraploid <i>Euphrasia</i> species from an isolated island	2124 individuals from 2 populations from each of 3 <i>Euphrasia</i> species from Fair Isle, Shetland, grown with 12 host species	Pot experiment in an outside frame, 2019	(Becher et al. 2020)

Our initial experiments aimed to study the growth patterns of different *Euphrasia* species, and the impact of different hosts and plants grown without a host (Brown et al. 2020). We found that host species affected some *Euphrasia* traits (e.g. height), but not others (e.g. nodes to flower), and that certain *Euphrasia* species overlapped in many traits (e.g. the related *E. arctica*, *E. confusa* and *E. nemorosa*), while others were relatively distinct (e.g. *E. micrantha* and other *Euphrasia* species tested). In the third and fourth experiments, we measured the same species, *E. arctica*, but this time grown on a wider range of different host species, and found that survival and fitness varied greatly between *Euphrasia* on different hosts. In 2018, we measured for the first time the fitness of different *Euphrasia* species on a range of different hosts, to investigate host-parasite interactions in specific *Euphrasia*-host combinations. With the fifth experiment, we investigated adaptation and morphology in species from different habitats on the isolated island of Fair Isle, Shetland (Becher et al. 2020). Here our aim was to investigate whether the species are morphologically distinct when grown under standardised conditions, using grassland *E. arctica*, coastal *E. foulensis*, and heathland *E. micrantha*. We found that they did retain different morphologies in the common garden, albeit to lesser extent than in their natural environment.

All five experiments aimed for at least 30 pots of each *Euphrasia* population-host combination and allowed for approximately 50% germination success. A single seed was planted in the centre of the pot, with this placement helping to identify it from any contaminant weed seeds. Nine-centimetre planting pots were filled with the potting mix. After sowing, plants were lightly top dressed with sieved soil, and moved to an outside seed frame where they remained until germination (Fig. 4A). Careful introduction of a host plant is critical to establish a connection between *Euphrasia* and the host. Host seeds were sown into trays filled with RGBE1

potting mix in February. In April, we transplanted young host plants (< 2 weeks post-germination) into a pot containing *Euphrasia*. 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 allowed us to keep a consistent distance between the *Euphrasia* and the host plant so there was no effect of distance to host. Placement of a host too close to the *Euphrasia* could lead to either very early attachment (which would be beneficial) or high levels of competition (which would be detrimental). After host introduction pots were moved to their final growing conditions (the glasshouse; Fig. 4B., or outside). During the course of the summer host plants had to be trimmed, to avoid shading the *Euphrasia*, and to avoid spreading species rooting in adjacent pots. This was most important for species with a prostrate or spreading growth habit, such as vigorously growing clover. Pots were randomised monthly to minimise block effects (e.g. plants on one bench growing better than others). We checked daily to see if any *Euphrasia* had newly flowered. All trait measurements were made the day of first flowering as this is a standardised time point allowing comparisons between individuals, while fitness measures were made throughout the season.



Figure 4: Growth frames at the RBGE nursery used for *Euphrasia* germination and the Fair Isle *Euphrasia* experiment of (Becher et al. 2020). Panels (A) and (B) show growth frame with pots in trays of 20. Panels (C) and (D) show fungal growth on the soil surface after waterlogging following the extremely wet spring of 2019.

Host species selection is crucial for vigorous *Euphrasia* growth; without a host *Euphrasia* grow very poorly, remain small, and are unlikely to flower (Brown et al. 2020). We routinely used clover (*Trifolium repens*) as a host in our initial experiments, though further experiments have revealed other legumes such as *Lotus corniculatus* are even better hosts (Brown, Moore, and Twyford 2021). *Plantago lanceolata* is a good choice of

forb, and *Cynosorus cristatus* a suitable grass that confers vigorous *Euphrasia* growth. Seed provenance is also important, with commercial seed stocks more likely to be genetically uniform and would be expected to produce more even *Euphrasia* growth, while genetic diversity in wild-collected seeds may produce more uneven but more representative growth. In our Fair Isle *Euphrasia* experiment, we used wild-collected seeds as well as cuttings of wild-collected heather (*Calluna vulgaris*) and juniper (*Juniperus communis*). The use of more diverse hosts, particularly those with different soil requirements (such as acid-loving heathers), is likely to require a different potting media for optimal host growth.

Our experimental studies have tested a range of growing conditions. Our first two experiments used an older Hartley wooden-framed glasshouse, where we experienced relatively high mortality, and issues with flower buds aborting due to high glasshouse temperatures on warm summer days. Our second two experiments then used a new Venlo Glasshouse, which is a controlled multi-span growing house that is better-ventilated and climatically controlled. Finally, for the fifth experiment the whole study was conducted in an outside frame (Fig. 4), which is a metal sided frame that protects from damage by animals or wind. While there were benefits to growing plants outside (less-vigorous growth more similar to that seen in the wild), these plants grew relatively poorly, due to the partial shade caused by the sides of the frame. We also found the pots tended to become waterlogged, which created problems after extended wet periods encountered in the spring of 2019. This was less of a problem in the glasshouse where watering could be more easily controlled. In our future work we plan to conduct experiments outside in dedicated frames with minimal shading, and adapt the potting substrate to be freer draining.

Regardless of the growing conditions, watering has proven 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 proved necessary for vigorous *Euphrasia* growth in small pots of nutrient-poor RBGE1. Our feeding regime began in May. When *Euphrasia* flowered, feeding was increased from fortnightly to weekly. Liquid feed was diluted at 1.5% by a Dosatron and applied when watering with a fine rose. The vigour of some of the hosts visibly improved after feeding, while feeding also promoted *Euphrasia* growth and prevented chlorosis. While feeding is necessary for optimal growth, we plan to test a reduced feeding regime in the future to better mimic natural soil conditions.

Pot experiments, particularly those conducted outside, require regular weeding. We have paid special attention to weeding at the start of the season, before *Euphrasia* germinates, to avoid plants parasitising weeds. We also remove mosses and liverworts when they threaten to smother *Euphrasia*. To avoid disturbing the roots of either the *Euphrasia* or host all weeding is done with tweezers.

Field trials

The effect that parasitic plants have in reducing the vigour of surrounding vegetation is often exploited commercially. Parasitic yellow rattle (*Rhinanthus minor*) is a common species in many natural and semi-natural plant communities and is widely grown in meadows, reducing the need for mowing and maintenance (Westbury and Dunnett 2007; Ameloot, Verheyen, and Hermy 2005). *Euphrasia*, as a related hemiparasite, could be used for similar purposes, with the wide habitat range of *Euphrasia* species making it potentially useful and appropriate in habitats unsuitable for *Rhinanthus*.

To produce seeds for large-scale planting, *Euphrasia* has to be cultivated on a field scale. In a collaboration with Scotia Seeds (www.scotiaseeds.co.uk), we have set up field plots with a view to understand the feasibility of cultivating *Euphrasia* at scale. To do this a well-established protocol for cultivating *Rhinanthus minor* was adapted for *Euphrasia*. Previous attempts to produce crops by introducing *Euphrasia* seeds into established grass following scarification (the *Rhinanthus* approach) had failed, possibly due to competition from grasses.

Therefore, we attempted field-trials of *Euphrasia* in the season of 2018/2019 using a modified protocol (Brown, Twyford & Laverack, Unpublished Results). We planted two 24 metre cleared bare soil plots with *E. arctica* seeds of two different provenances at 500 seeds per meter square (0.625g), and with four different host treatments at 2g per meter square. Our hosts included: *Lotus corniculatus* (Bird's Foot Trefoil), *Cynosurus cristatus* (Crested Dog's Tail), *Plantago lanceolata* (Ribwort Plantain) and Mavisbank Meadow Mix (a mix of over 17 species of herbs and 6 species of grass, see here: www.scotiaseeds.co.uk/shop/mavisbank-mix). Plants were maintained with minimal maintenance—plots were not watered, and spot weeding was used to remove any vigorous weeds that may have competed for resources.

Preliminary results indicate that cultivation of *Euphrasia arctica* on a field scale yields vigorous plants, at least 20cm tall (Fig. 5). Plants of this height typically produce ~300 flowers and therefore around 1800 seeds (assuming six viable seeds per capsule). For harvesting efficiency, it is recommended that either *L. corniculatus* or *P. lanceolata* is used as a host, as grass species cause *Euphrasia* to form flexuous stem bases, which are difficult to harvest. *P. lanceolata* seeds in particular are easily separated from *Euphrasia* seeds, making this pairing a practical combination to produce pure *Euphrasia* seeds without contamination of other species, an important consideration in seed production. Control of annual weeds will be an important part of any field production protocol and sowing in rows may be helpful. Using this approach, *E. arctica* could be managed on a field scale and give suitable yields for seed production.

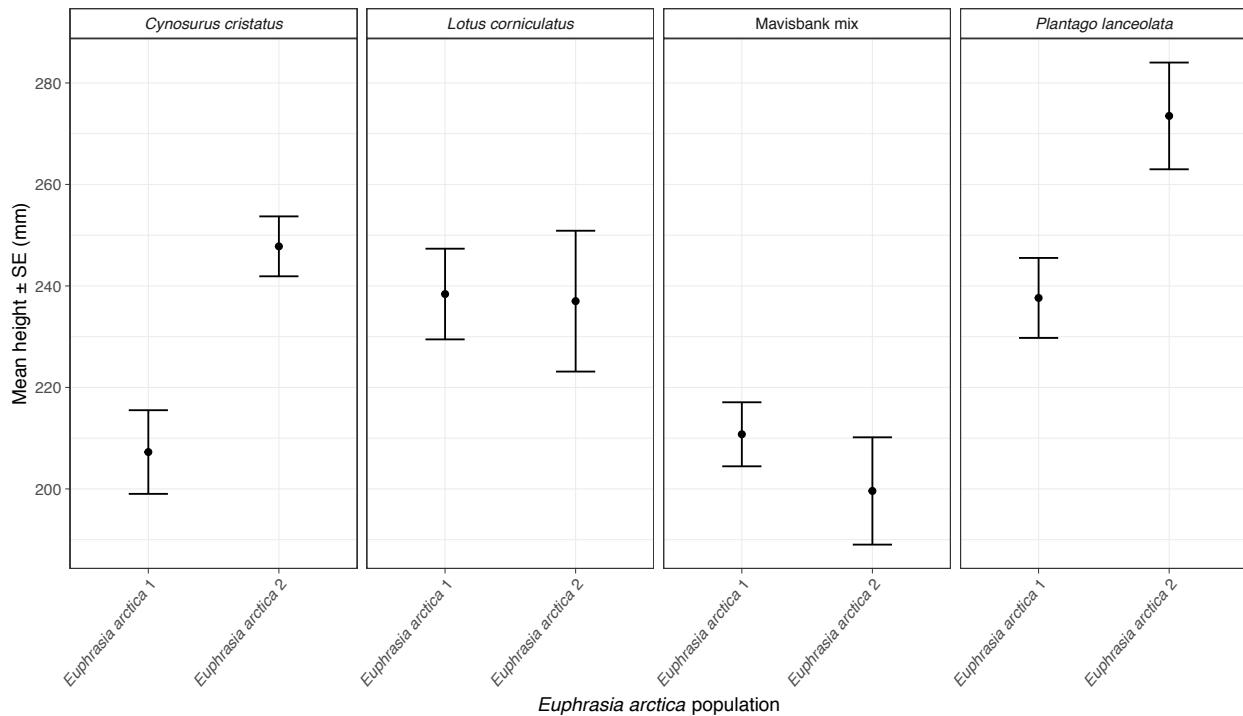


Figure 5: Mean heights and standard errors of *Euphrasia arctica* populations grown in experimental field plots at Scotia Seeds, Angus (Scotland). Each panel shows the host plant(s). Measurements were pooled from three replicate plots for *E. arctica* genotype 1 and one plot for *E. arctica* genotype 2 (from North Berwick and Inverkeithing, respectively).

Reciprocal transplant experiments

While the protocols outlined above emulate aspects of natural conditions, some circumstances may require plants to be grown in the wild, such as when conducting evolutionary and ecological studies of local adaptation. Local adaptation is the situation where plants from different origins perform best at their ‘home’ source site (Blanquart et al. 2013). The gold standard for testing for local adaptation is the reciprocal transplant experiment, which assesses the performance of plant populations from two or more different sites (Kawecki and Ebert 2004). Plants from each population are simultaneously grown at both their home, and the ‘away’ site. Reciprocal transplant experiments have recently been conducted with hemiparasitic *Rhinanthus minor*, focusing on adaptation to elevation differences between sites (Hargreaves and Eckert 2019), showing the

feasibility of this approach for hemiparasitic plant research. We have conducted a reciprocal transplant experiment to assess local adaptation to site conditions and hosts in Fair Isle *Euphrasia*, in parallel with the pot experiment described above.

Conducting a transplant experiment in the wild with hemiparasitic plants brings unique challenges. Unlike other transplant experiments, clearing all local vegetation is not usually an option, as local hosts are required. However, precautions must be taken to minimise contamination with any local *Euphrasia* in the soil seedbank. As such, we have filled small planting holes with a planting medium known to be free of other *Euphrasia* seeds, but where root growth is not impeded, so *Euphrasia* can attach to surrounding hosts. A pilot experiment conducted near Inverkeithing, Fife, with inflated ‘Jiffy’ peat pellets sunk into tight fitting holes (Fig. 6) proved unsuccessful, with the outer mesh of the pellets not decomposing within the field season, and many peat pellets being disturbed or displaced (Becher, Personal Observations). Instead, in our final experiment on Fair Isle, we filled small holes in the ground with John Innes No. 1 compost and subsequently added a *Euphrasia* seed to each hole.



Figure 6: Growing *Euphrasia* inside inside expandable “Jiffy” pellets planted in a field site at Inverkeithing. (A) Shows a planting array of pellets in the autumn, (B) shows an establishing seedling in the spring. After four months in the ground, the pellet’s outer mesh showed no sign of decomposition.

Euphrasia seeds from two populations of each of the three species, were used both for a standardised common

garden study at the RBGE (see above, and Becher et al. 2020) and in a reciprocal transplant experiment (Becher, Brown & Twyford, Unpublished results). The transplant experiment aimed to test whether species germinate better in their home site rather than in a novel environment. We set up four transplant sites, one in grassland, one in heathland and two at the coast. Each site comprised eight blocks, with each block comprising 72 planting holes. Each block was split into six sub-blocks (of 12 planting holes), one for each provenance, with assignment being done at random. Germination success the following May differed considerably between habitats and genotypes (Table 2). Overall, there was no significant effect of home or away sites in generalised linear models ($p = 0.497$) and thus no general sign of local adaptation for germination across the experiment. There were, however, different results for individual species. Grassland *E. arctica* germinated better on average in non-home sites than in home sites (difference 10%, $p = 0.003$), while coastal *E. foulensis* germinated better at home sites (difference 12%, $p < 0.001$). For *E. micrantha*, the difference in germination between sites was not significant ($p = 0.468$). As such, *Euphrasia* species differ in their germination responses, but in a complex manner. The lack of a simple signal of local adaptation (e.g. home-site superiority) is not entirely surprising, especially given the small spatial scale of our experiment, as well as the nature of site differences (e.g. sites differ in multiple aspects such as soil, exposure and vegetation).

While in many respects this type of experiment in the wild provides the most natural settings, it comes with extensive challenges. For example, some substrates (such as heathland) are hard to dig, re-finding individual *Euphrasia* plants can be difficult, and germination in natural conditions is very patchy. In our Fair Isle experiment, attrition at each stage of establishment, coupled with surrounding vegetation growth and local germination of *Euphrasia* (not from the experimental setup), prevented us following long-term plant survival and growth. Despite these issues, it is still possible to carry out these kinds of experiments in natural settings, with trial-and-error to optimise methods, large sample sizes for plant recovery and statistical robustness, and careful monitoring required for success.

Table 2. Germination of *Euphrasia* species in a reciprocal transplant experiment on Fair Isle. The numbers indicate successful germination from 72 planting holes per genotype and site. Asterisks (*) indicate where a genotype was grown in its home habitat. Statistical significance was calculated using Generalised linear models with a binomial error distribution in R (R Core Team 2019), and with marginal differences calculated using the emmeans package (Lenth 2020).

Transplant site					
Species	Population origin	Wirrvie Brecks	Buness	Bird Observatory	North lighthouse
<i>E. arctica</i>	FI Chapel	34	30	17*	35
	School	24	18	21*	32
<i>E. foulensis</i>	South lighthouse	29	45*	19	22*
	Buness	17	24*	15	34*
<i>E. micrantha</i>	Wirrvie Brecks	24*	25	16	29
	Airstrip	16*	4	18	14

Conclusions and future directions

The genus *Euphrasia* represents an excellent study system to investigate the evolution of parasitism, the importance of natural hybridisation, and the role of mating system variation. *Euphrasia* can easily be brought into cultivation in petri dishes in the lab, and in pots both in the glasshouse and outside. *Euphrasia* can be grown on many different species of host plant, making it an ideal system to investigate parasite-host interactions. Field trials have been met with success on a commercial scale, however reciprocal transplant experiments in the wild are difficult and these protocols require further optimisation. In summary, key considerations when growing *Euphrasia*, are: - Seed stratification is essential for germination. This can be

simulated with artificial cold or plants can be overwintered outside. - Unsorted wild-collected seeds have a low germination rate of around 50%. The best germination rates are achieved from late summer collections made prior to seed dehiscence, followed by seed sorting and cold storage. - Relatively few plant species are good hosts that confer substantial growth benefits to *Euphrasia*. Good hosts include many legumes, as well as *Plantago lanceolata* and *Cynosorus cristatus*. - *Euphrasia* kept in pots outside should not be shaded, while those under glass require ventilation to prevent plants overheating and aborting developing buds. - *Euphrasia* is less competitive than *Rhinanthus* in dense grassland swards. As such, established vegetation may need to be cleared for successful establishment.

There are many possibilities for future research building on these protocols. The cultivation of plants under laboratory conditions will allow developmental studies to investigate haustoria formation and the attachment of *Euphrasia* to different host plants (as has been done in *Rhinanthus*, see Rümer et al. 2007), and to generate contaminant-free tissue samples for genomic sequencing to understand the genes underlying parasitism (Yang et al. 2015). Common garden experiments will be used to investigate hemiparasite-host below ground interactions, and to test whether *Euphrasia* has host preferences. Further large-scale reciprocal transplant experiments should be performed to understand the extent and nature of local adaptation in the genus. Trials to establish the best methods for establishing *Euphrasia* in ecological restoration and seed quality and storage work would be useful for seed production and use in restoration.

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