# Ecological divergence is marked by specific plastic gene expression and distinct rhizosphere among sibling allopolyploid marsh orchids (*Dactylorhiza*)

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**Abstract**

In addition to demographic and dispersal constraints, a complex interplay of biotic and abiotic factors shapes the distribution of taxa across landscapes, with each species adapting uniquely to the challenges and opportunities presented by its habitat. Plasticity, the capacity to dynamically adjust one's phenotype in response to environmental shifts, is a pivotal trait enabling species to exploit a wider niche space and withstand suboptimal conditions. This adaptability is particularly crucial for newly formed allopolyploid lineages seeking to establish themselves in diverse environments. The amalgamation of different parental genomes in such hybrids can enhance their plastic responses to varying habitats, yet the long term impacts of plasticity on allopolyploid establishment success remain elusive. Here we use ecologically divergent sibling allopolyploid marsh orchids (*Dactylorhiza*, Orchidaceae) in reciprocal transplantation experiments at two localities to gain insights into the drivers of ecological divergence and species persistence in the face of gene flow. We show that while consistent abiotic differences characterise the alternative environments, the great majority of gene expression differences between the sibling allopolyploids is plastic. Few genes exhibit fixed expression differences, suggesting that despite ecological divergence, plasticity may mediate gene flow between the sibling allopolyploids, thereby preventing the accumulation of genetically encoded expression differences. Extending our investigations to the rhizosphere, we uncover distinct fungal communities between the roots of the two sibling allopolyploids, consistent across the two localities. This finding suggests that both biotic and abiotic factors contribute to the distribution and delimitation of *Dactylorhiza* sibling allopolyploids. Altogether, our results suggest that plasticity can exert both diversifying and homogenising influences on the establishment of recurrently-formed allopolyploid species.

**Keywords:** constitutive gene expression divergence, *Dactylorhiza majalis*, *Dactylorhiza traunsteineri*, fungal community, gene expression plasticity, polyploidy.

**Running title:** Expression plasticity in marsh orchids

## Introduction

Polyploidy, or the duplication of the whole genome, results in a large influx of genetic redundancy which has been variously recognised as a diversifying force, potentially promoting an increased capacity to express different gene copies under varying environmental conditions (de Jong & Adams, 2023). An elevated genetic redundancy is expected to result in phenotypic plasticity (Mattingly & Hovick, 2023), or the ability for an individual to adjust to environmental fluctuations and thus track a dynamically changing trait optimum beyond that allowed by its fixed genotype (Schlichting & Pigliucci, 1998). However, increased levels of phenotypic plasticity are regarded as inherently costly in relatively stable or extreme environments. The latter type of environments are often occupied by polyploids and hybrids, in particular as compared to habitats of progenitors, which allows them to escape direct competition with diploid relatives. Founding populations of nascent polyploid species are often necessarily small and if sympatric with a majority of diploid cytotypes, this poses additional challenges associated with low genetic diversity and minority cytotype disadvantage (Husband, 2000; Levin, 2002). Phenotypic plasticity can allow polyploids to overcome a depauperate gene pool by extending their habitable niche beyond their fixed genotypic capacity (Castillo et al., 2018), at the same time enabling colonisation of environments unoccupied by diploid relatives, thus representing an immediate source of adaptive potential (Hahn et al., 2012). Conversely, an increased plasticity may be able to facilitate gene flow between establishing polyploids and their relatives, alleviating in the long term some of the effects of low genetic diversity. Studies addressing the role of plasticity in polyploid evolution often focus on the immediate implications of polyploid plasticity, such as whether plasticity is heightened in polyploids (Drunen & Johnson, 2022). The role of plasticity in the demographic dynamics of polyploid species is still poorly understood (Mattingly & Hovick, 2023).

To understand the interplay between plasticity, gene flow and ecological delimitation in polyploids, we use the marsh orchids *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. and *D. traunsteineri* (Saut. ex Rchb.) Soó (Fig. 1a), sibling allopolyploids ($2n = 80$) resulting from two independent and unidirectional polyploidisation events between *D. incarnata* and *D. fuschii* (the maternal parent) (Brandrud et al., 2020; Pillon et al., 2007). Typically, *D. majalis* is larger than *D. traunsteineri*, has more flowers per inflorescence and broader leaves (Fig. 1a). It is generally accepted that *D. traunsteineri* is the younger of the two sibling allopolyploids, and estimates of formation place both origins at different times in the recent half of the Quaternary (Brandrud et al., 2020; Hawranek, 2021; Nordström & Hedrén, 2009). Both sibling allopolyploids have a wide European distribution, *D. majalis* tends towards southern locations from the Pyrenees to southern Scandinavia, while *D. traunsteineri* towards northerly locations in Scandinavia and the British Isles, with their distributions overlapping in the Alps. The two orchids have generalist pollination syndromes, corresponding to a food-deception strategy (i.e., no reward is offered to pollinators). Gene flow between the two allopolyploids has been documented in areas of sympatry, with a tendency for more intense gene flow from *D. majalis* to *D. traunsteneri* (Balao et al., 2016). However, the sibling allopolyploids maintain distinct ecologies differentiated by soil chemistry, nutrient content, and water availability (Paun et al., 2011; Wolfe et al., 2023), and both species are found in their respective ecologies even at local scales in regions of sympatry.

We investigate *Dactylorhiza majalis* and *D. traunsteineri* in two localities in the Austrian Alps, where the sibling allopolyploids exist in sympatry and experience substantial gene flow (Balao et al., 2016; Brandrud et al., 2020). Using a reciprocal transplantation design, we grow individuals from each species in the environment of the other to understand how much the ecological preference of each species is genetically encoded, and how much is underpinned by plasticity. Moreover, using this design enables us to detect genes which have fixed expression differences between the two species, representing genes which have become canalised (are no longer plastic and have adapted to the environment of each respective species), and avoid the homogenising effects of gene flow in sympatric populations. Comparing the proportion and function of plastic and fixed genes in admixed populations can enable us to understand the basis upon which *D. majalis* and *D. traunsteineri* are delimited in their ecological ranges, and how much of the gene space is involved in this delimitation.

We also investigate the potential for fungal community profiles to reinforce species boundaries, utilising the populations of *Dactylorhiza majalis* and *D. traunsteineri* at St. Ulrich and Kitzbuhel to test the consistency of profiles across independent localities. Mycorrhizal fungi provide vital support during the developmental cycle of orchids, and can ameliorate effects of nutrient deficiencies in plant partners (Bhantana et al., 2021; Saboor et al., 2021), however the broader role of mycorrhizal fungi in determining host plant success and, in general, species boundaries is poorly understood. Leveraging the capture of contaminants during RNA-seq experiments, we perform fungal taxonomic profiling of root RNA-seq data from native and transplanted samples to test whether the environments of *D. traunsteineri* and *D. majalis* differed in fungal community profiles. Differences in soil fungal communities may represent an ecologically relevant difference between sibling allopolyploid habitats, and could imply a role for symbiotic organisms in delimitation of species boundaries.

This work uses *in situ* measurements of gene expression, functional response to different environments, and fungal community abundance to investigate the consequences of transgressing ecological optima between two sibling allopolyploid species. In doing so, we hope to provide a greater understanding of how resilience against environmental heterogeneity interplays with ecological adaptation to maintain species boundaries between sibling allopolyploid marsh orchids in the face of gene flow.

## Methods

### Study sites

Two localities have been selected for transplantation experiments. The first locality is close to lake Schwarzsee north-west of Kitzbühel (Tyrol, Austria) which is the type locality of *D. traunsteineri* (Foley, 1990). The populations of *D. majalis* and *D. traunsteineri* are separated by an area lacking *Dactylorhiza* orchids (shortest air distance ca 100m). Here, *D. majalis* grows in shady places under trees. *Dactylorhiza traunsteineri* plants grow in the open marshland closer to the lake, mostly on elevated and thus dryer locations (hummocks), nevertheless soil humidity is higher than in the *D. majalis* habitat. Whereas in general the soil specific for *D. majalis* stands is significantly richer in most macro- and micronutrients as compared to *D. traunsteineri* (Wolfe et al., 2023), the specific site at Kitzbuhel shows the opposite trend for the content in Phosphorus (Fig. 1b).

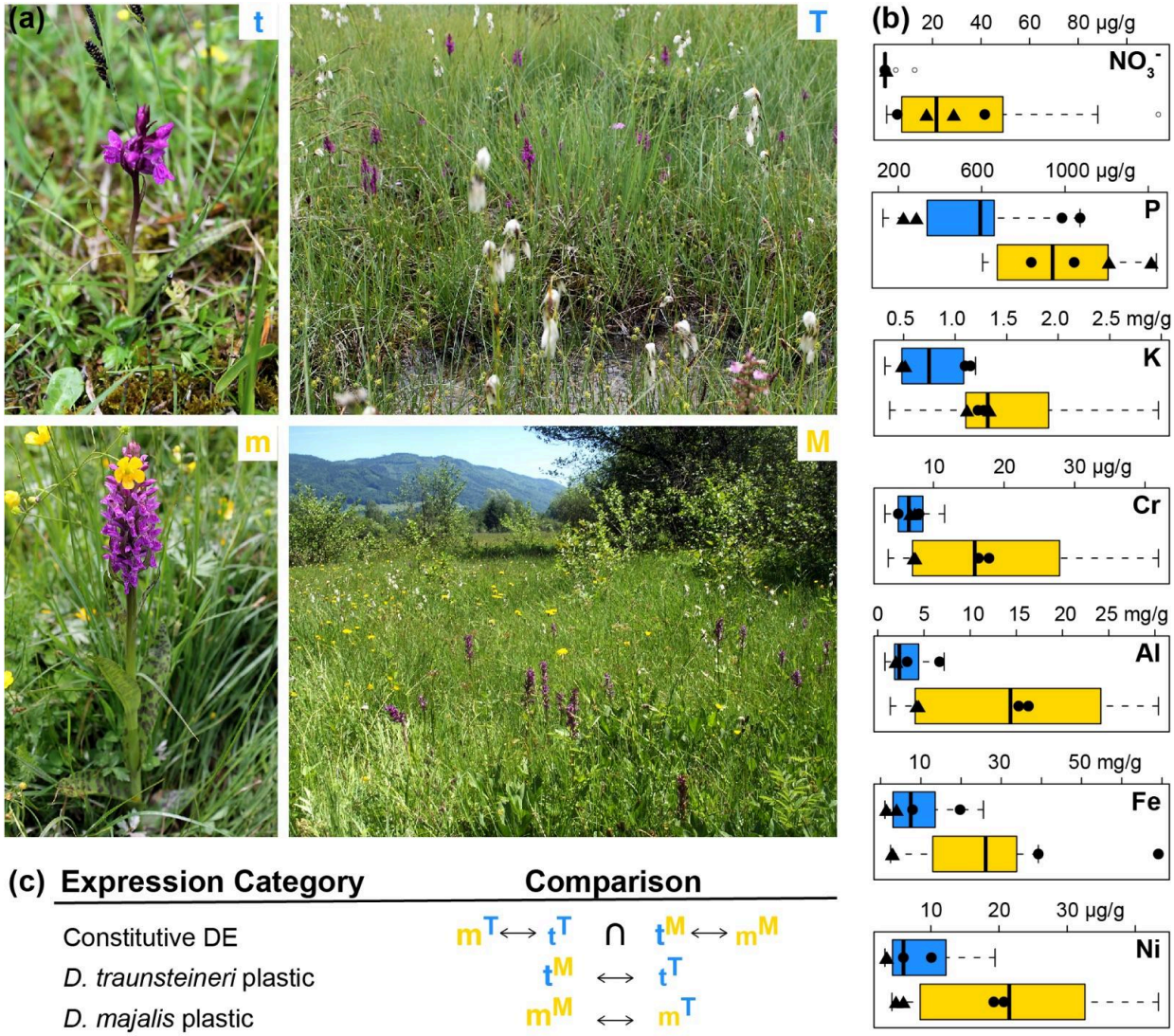


**Figure 1. The study system and experiment design.** (a) The study species: t, *Dactylorhiza traunsteineri* and m, *D. majalis* growing at one of the transplantation sites at St. Ulrich am Pillersee, Austria. T and M show impressions of the two environments at the same locality. (b) Divergent preference for selected soil characteristics between *D. traunsteineri* (blue) and *D. majalis* (yellow) across multiple European populations (redrawn from Wolfe et al., 2023). With filled circles two measurements per species are indicated at the experimental site at Kitzbuhel and with triangles at the site at St. Ulrich am Pillersee, both Austria. (c) Scheme of main comparisons made in differential expression (DE) testing. As above, lower case letters indicate sample species, superscript capital letters indicate the environment in which the sample was experimentally grown. ↔ indicates test of differential expression between sample groups, ∩ indicates intersection of genes found to be differentially expressed between sample groups.

The second locality is situated south of Pilersee, northwest of St. Ulrich am Pillersee (Tyrol, Austria). Here the two species are also separated by an area (shortest distance ca 200m) that lacks either species but it hosts rare individuals of the diploid *D. incarnata*. They are also separated by a ca 50m wide tree stand. Both species at St Ulrich am Pillersee occupy very humid soils. Previous genetic analyses have shown that gene flow between the two species is

more prominent at Kitzbuhel, whereas admixture is also present to some extent at St. Ulrich am Pillersee (Balao et al., 2016; Brandrud et al., 2020).

**Reciprocal transplantation and RNA sequencing**

Ten wild individuals each of *D. majalis* and *D. traunsteineri* were randomly selected at each of the two localities. For each locality and species, five individuals were transplanted and grown in the environment of the other species, and five individuals were transplanted and grown in their own environment to control for transplantation effects. *Dactylorhiza* plants are perennials and grow a new tuber each growing season to support growth in the following season (Wolfe et al., 2023), therefore plants were sampled after two growing seasons to eliminate as much as possible the effects of the original environment. Leaf and root tissues were collected in the same morning for each locality and stored in RNAlater (Sigma-Aldrich) overnight at 4°C, before transferring to -80°C.

Total RNA was extracted using the mirVana Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instructions, and quantitated using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), using the wavelength ratio of A260/280 to estimate purity. A Plant RNA Pico kit was used on a 2100 Bioanalyzer (Agilent Technologies) to confirm the quality of the RNA isolates. DNA digestion was performed with DNase I (Promega), to remove any DNA contamination. Ribosomal RNA was depleted using the RiboZero Plant Kit for RNA-Seq (Illumina) according to manufacturer's instructions. RNA was fragmented by hydrolysis for 2 minutes at 94° and cDNA synthesis was performed with random hexamers following the TruSeq Stranded RNA kit protocol (Illumina). To achieve strand specificity, the second strand was synthesised with dUTPs. The libraries were sequenced using directional sequencing on an Illumina NovaSeq instrument at the VBCF NGS Unit (www.vbcf.ac.at/ngs). Few accessions did not survive during the experiment, whereas some accessions used have been identified as heavily introgressed between the two species or backcrossed towards the diploid *D. incarnata* (the latter at the locality of St. Ulrich). Additional accessions have been excluded either because of insufficient quality of the RNA extraction or of insufficient reads obtained. Altogether 16/17 transplanted accessions from Kitzbuhel have been retained for the bioinformatic analyses for leaves and roots, respectively, and 13/14 accessions from St Ulrich.

**Genetic clustering**

Quality filtered and adapter trimmed reads were mapped to the *D. incarnata* v.1.0 genome (Wolfe et al., 2023) using its .gff annotations and STAR v2.7.11a (Dobin et al., 2013) in two-pass mode, filtering out any reads which mapped to more than ten positions. To evaluate the genetic structure of the randomly selected accessions, we used ANGSD v0.941 (Korneliussen et al., 2014) for estimating genotype likelihoods, as this approach is suitable for RNA-seq data (Szukala et al., 2023), which is expected to include highly variable sequencing depth among genes and exons. For this purpose the mapped bam files of the two tissues for each accession were merged with SAMtools v1.7 (Li et al., 2009). ANGSD was further run based on a SAMtools model (-GL 2), inferring the major/minor alleles (-doMajorMinor 1) and estimating allele frequencies (-doMaf 1). In the first round we retained SNPs that were identified outside annotated repeat regions, and which had a p-value lower than 2e-6 (-SNP_pval 2e-6), a minimum mapping quality of 20 (-minMapQ 20), a minimum base quality of 20 (-minQ 20), with

data for at least 80% of individuals (-minInd 26) and a minor allele frequency corresponding to two accessions (-minMaf 0.06). In a second run we used a 'sites' file to retain only one SNP per 2-kb window of the resulting variant positions, aiming to reduce linkage disequilibrium between the markers. Covariance matrices computed from the obtained genotype likelihoods were used for principal components analyses (PCA) using pcANGSD v0.99 (Meisner & Albrechtsen 2018) and were plotted in R.

**Differential expression analysis**

Uniquely mapped reads were summarised per tissue type across the coding sequence, excluding introns, for each sample using featureCounts from Rsubread v2.0.4 (Liao et al., 2019). Prior to differential expression analysis, the counts matrix was filtered to exclude low expression genes, retaining only genes expressed at greater than one count per million in at least three samples, corresponding to the number of retained biological replicates in the smallest group in the analyses. DESeq2 v1.36.0 (Love et al., 2014) was used to identify differentially expressed genes (DEGs), normalising counts using median of ratios. To avoid incorporating signals of population structure and history, DEGs were detected for each locality (St Ulrich or Kitzbuhel) separately.

First, we identified constitutive gene expression differences, defined as DEGs between the sibling allopolyploids consistently identified in either environment, by finding genes which are significantly ($p < 0.05$, LogFC > 1.5) up- or downregulated between species in both environments i.e. the intersection of DEGs found in comparisons $m^M$ vs $t^M$ and $m^T$ vs $t^T$ (where m stands for *D. majalis*, t for *D. traunsteneri*, superscript M for the *D. majalis* environment and superscript T for that of *D. traunsteineri*; see also "constitutive DE" comparison in Fig. 1c).

To determine whether there is a significant overlap of DEGs between each comparison (i.e. whether we find more constitutive DEG than we expect by chance) we use the super exact test implemented in the SuperExactTest package in R (Wang et al., 2015), using the number of genes included in the DESeq2 analyses as the background set from which constitutively DEGs are sampled.

Then we identified genes which showed plastic expression patterns (significantly differentially expressed, $p < 0.05$, LogFC > 1.5) in each species and locality when transplanted to the environment of the opposite species, by quantifying DEGs between $t^M$ and $t^T$ for *D. traunsteineri*, and between $m^T$ and $m^M$ for *D. majalis* (see "*D. traunsteineri* plastic" and "*D. majalis* plastic" comparisons respectively in Fig. 1c). Functional enrichment analyses were performed on the resultant groups of genes using the Bioconductor package topGO v2.48.0 (Alexa & Rahnenfuhrer, 2023) using the weight01 algorithm.

**Fungal taxonomic abundance analysis**

We performed community profiling of fungal taxa in RNA-seq samples from root samples of *D. majalis* and *D. traunsteineri* plants grown in the two environments using MiCoP (LaPierre et al., 2019), a mapping based approach for classification and abundance of viral and fungal sequences. MiCoP uses a rapid read mapping approach to achieve more granular classification estimates which are more sensitive to low-abundance species than comparable methods such as kmers or megablast (Altschul et al., 1990). Paired end reads from each root sample were mapped to the fungal RefSeq database accessed 7th November 2017 (Robbertse & Tatusova,

2011) included with the MiCoP release with bwa mem v0.7.11 (Li & Durbin, 2009), followed by computation of fungal abundances from the alignment file. Differential association of specific fungal taxa with the environment of either *D. majalis* or *D. traunsteineri* was analysed using LDA effect size (LEfSe, Segata et al., 2011). Features were scaled to sum to 10,000, using a p-value of 0.05 for both Kruskal-Wallis and Wilcoxon tests, and an effect size of discriminative features threshold of LDA > 2.

## Results

### Global effects of transplantation

Reciprocal transplantation experiments were performed between *Dactylorhiza majalis* and *D. traunsteineri* in two localities in Austria where the ranges of the two species came in close proximity to one another, sequencing total mRNA from leaf and root tissue of five biological replicates per species, treatment, and locality. Leaf tissue libraries, consisting of between 16 and 146 million reads, and root tissue libraries, of 56 and 148 million, were mapped to the *Dactylorhiza incarnata* genome, resulting in on average 25.7% and 45.4% uniquely mapped reads respectively (see Supplementary table 1 for further details). After excluding low confidence variants, those associated with repetitive regions, and keeping putatively unlinked loci, we retained 315,846 polymorphic positions for investigating the genetic structure of accessions with a PCA based on genotype likelihoods computed in ANGSD. The first axis of the PCA (Supplementary Figure 1) tends to separate the two species, whereas the second axis of the PCA generally clusters accessions by locality. In line with previous results (Balao et al. 2016, Brandrud et al. 2020) the accessions of *D. majalis* from both localities form a more cohesive genetic cluster, whereas the accessions of *D. traunsteneri* separate very clearly by locality. Finally, whereas the accessions of *D. traunsteineri* show no genetic separation by environment, the samples of *D. majalis* show for each locality a minimal ‘genetic’ separation by environment on the second PCA axis, that may track back to a somewhat different expression pattern between the two environments (i.e., different sequencing depth and presence absence of expression resulting in somewhat different genotype likelihoods).

Following variance stabilising transformation with DESeq2, we used expression-based PCA to visualise global transcriptional profiles of the accessions (Figure 2). Outlier samples were first identified and removed on the basis of number of features with zero counts, clustering with other samples, and the broader variability exhibited by the treatment group.

Per locality expression profiles of leaf and root tended to group samples by species, and then by environment (native or transplant). This effect was more evident in root tissue, which showed clear separation of species, whereas leaf tissue was not consistent in separating species by expression profile.

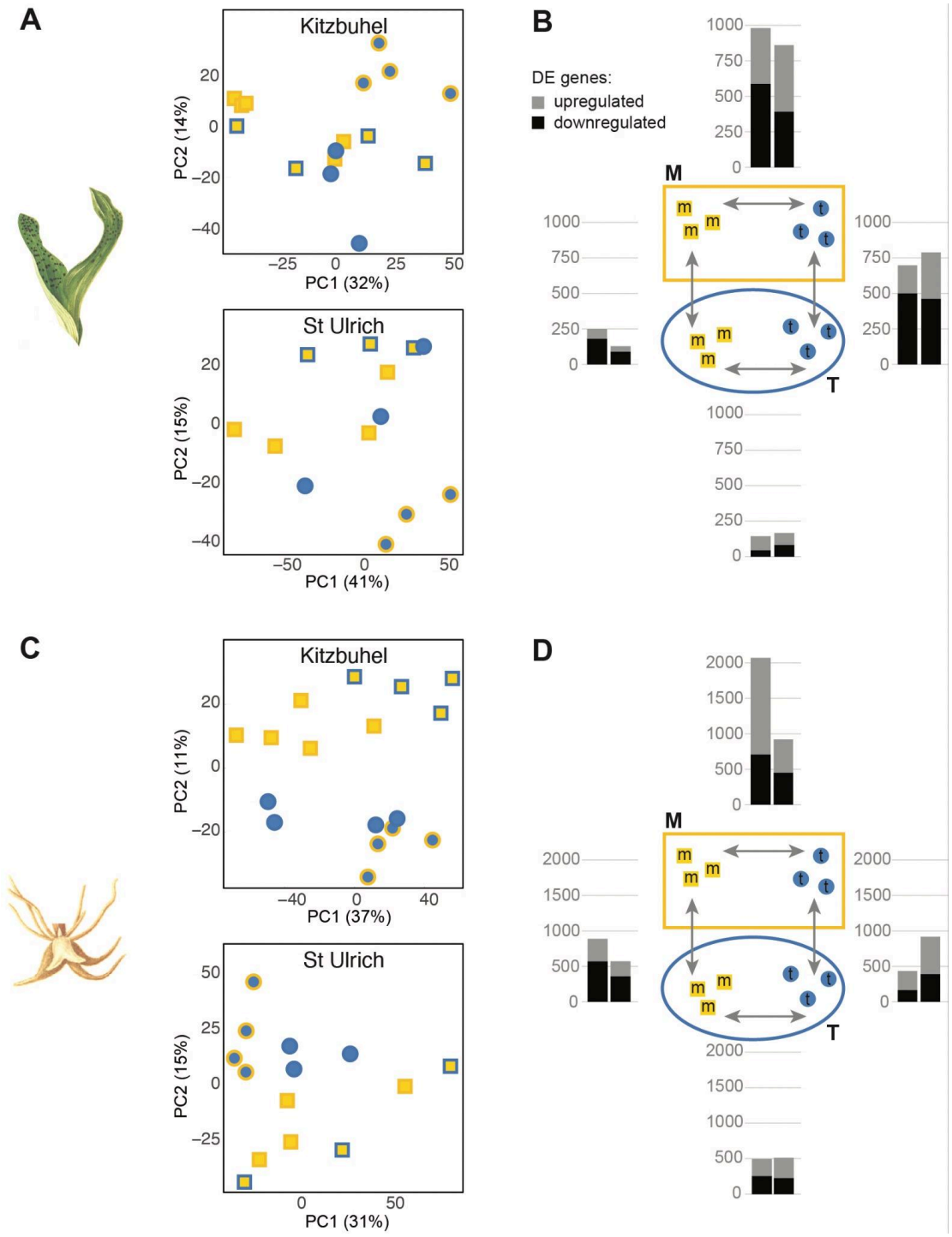


**Figure 2.** Variability of expression profiles in *Dactylorhiza majalis* and *D. traunsteineri* across locality, tissue and environment. **A, C** PCA plots for leaf and root data, respectively. Points represent replicates of *D. majalis* and *D. traunsteineri* (rectangles and circles respectively) grown in *D. majalis* or *D. traunsteineri* environments (symbols with blue or yellow lines, respectively). PCA analysis was performed on each locality separately. **B, D** Numbers of genes up- (grey bars) or down-regulated (black bars) between *Dactylorhiza majalis* (yellow symbols) and *D. traunsteineri* (blue symbols) and environments (yellow line, *D. majalis* environment; blue line, *D. traunsteineri* environment. The number of symbols in the experimental set up in the centre does not necessarily correspond to the actual analysed accessions per group which ranges between three and five.

**Constitutive and plastic expression**

We identified genes which were constitutively differentially expressed between *D. majalis* and *D. traunsteineri*, meaning they are differentially expressed ($p < 0.05$) in either direction in the environment of both species (see "constitutively differentially expressed" comparison in Figure 1), thus estimating the extent to which fixed differences had accumulated in the two species. Alongside constitutively differentially expressed genes, we identified genes which were differentially expressed between species in only one of the two environments, and therefore reflected differential plasticity linked to specific environmental characteristics (Figure 3).

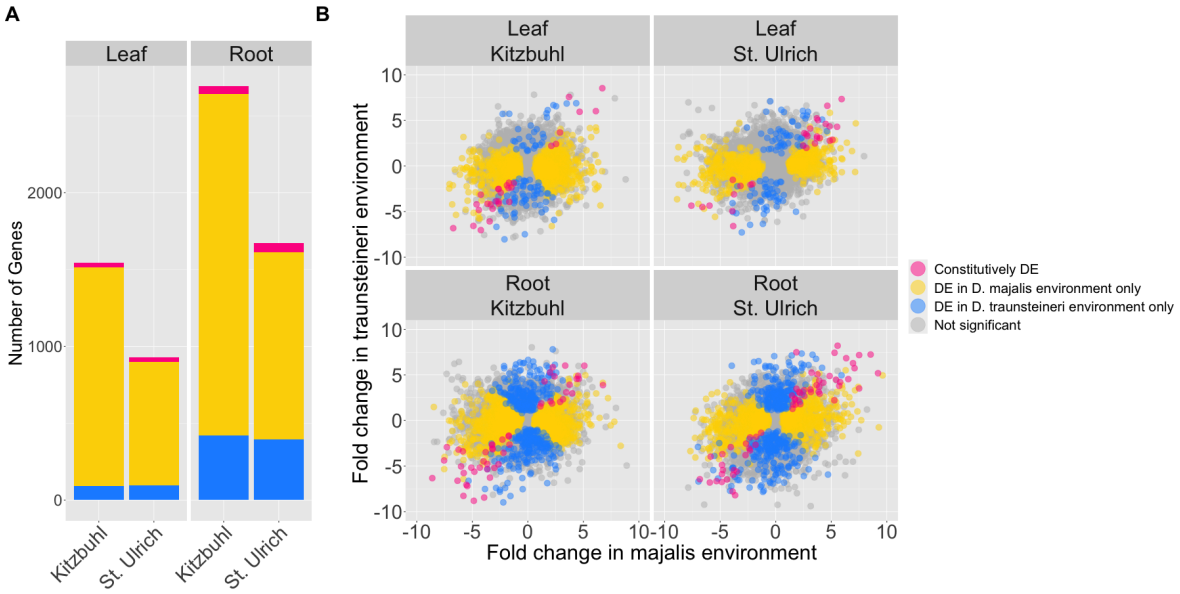


**Figure 3. A** Number of genes differentially expressed between *Dactylorhiza majalis* and *D. traunsteineri* in both environments (pink), in the *D. majalis* environment only (yellow), and in the *D. traunsteineri* environment only (blue). **B** Fold change in gene expression between *Dactylorhiza majalis* and *D. traunsteineri* in the *D. traunsteineri* environment (Y-axis) and between *D. majalis* and *D. traunsteineri* in the *D. majalis* environment (X-axis). *D. majalis* was arbitrarily set as the base condition, therefore positive values indicate upregulation in *D. traunsteineri* relative to *D. majalis* and negative values indicate upregulation of *D. majalis* relative to *D. traunsteineri*. Genes shown in colour are designated differentially expressed (FDR < 0.05).

Root tissue had more genes expressed (at least 1 count per million in at least three individuals) overall compared to leaf tissue, 35,251 and 34,844 genes were expressed in root tissue in Kitzbuhel and St. Ulrich respectively, compared to 30,808 and 28,872 genes in leaf tissue for the same localities. Of these, 60 and 49 genes in root tissue were constitutively differentially expressed in St. Ulrich and Kitzbuhel respectively, and 29 and 28 genes in leaf tissue for the same localities. All four comparisons showed a significantly greater number of constitutively expressed genes than expected by chance ($p < 0.001$, super exact test, supplementary table 2 and supplementary table 3). The differences between the native habitats of *Dactylorhiza majalis* and *D. traunsteineri* can elicit species specific responses during transplantation to the habitat of the opposite species, reflecting the characteristics specific to that environment. Far more DEGs were found between *Dactylorhiza majalis* and *D. traunsteineri* in the environment of *D. majalis* than that of *D. traunsteineri*, 3,044 and 1,780 genes were

differentially regulated between *D. traunsteineri* and *D. majalis* in Kitzbuhel and St Ulrich respectively in the *D. majalis* environment, and 644 and 675 genes for the same comparisons in the *D. traunsteineri* environment (supplementary table 4). Functional annotation of constitutively differentially expressed genes (Figure 4) showed antioxidant activity, sodium ion transport and nucleotide and nucleoside metabolism to be among the functions enriched in constitutively differentially expressed genes in both leaf and root tissues, and response to chitin being enriched in constitutively differentially expressed root genes. Carbohydrate metabolism, stress response and water transport were among the enriched terms in genes differentially expressed in an environment specific fashion, a full list of enriched terms is provided in supplementary table 5.

To compare transcriptional plasticity between *Dactylorhiza majalis* and *D. traunsteineri*, we identified differentially expressed genes between individuals of each species grown in their native environment versus those grown in the environment of the opposite species (see *D. traunsteineri* plastic and *D. majalis* plastic, respectively, in Figure 1), performing separate comparisons per locality (Figure 5). Root tissue showed more transcriptional plasticity than leaf tissue after transplantation of each species to the environment of the opposite species, and both tissues generally tended to upregulate more genes than downregulate. Most differentially regulated genes were unique to a species and locality (Figure 5A), *Dactylorhiza traunsteineri* leaf tissue showed the most overlap in plastic genes between Kitzbuhel and St. Ulrich of 14.6%, however most other comparisons showed fewer than 10% of plastic genes were replicated across both localities. For full counts of differentially expressed genes in each category, see supplementary table 4.

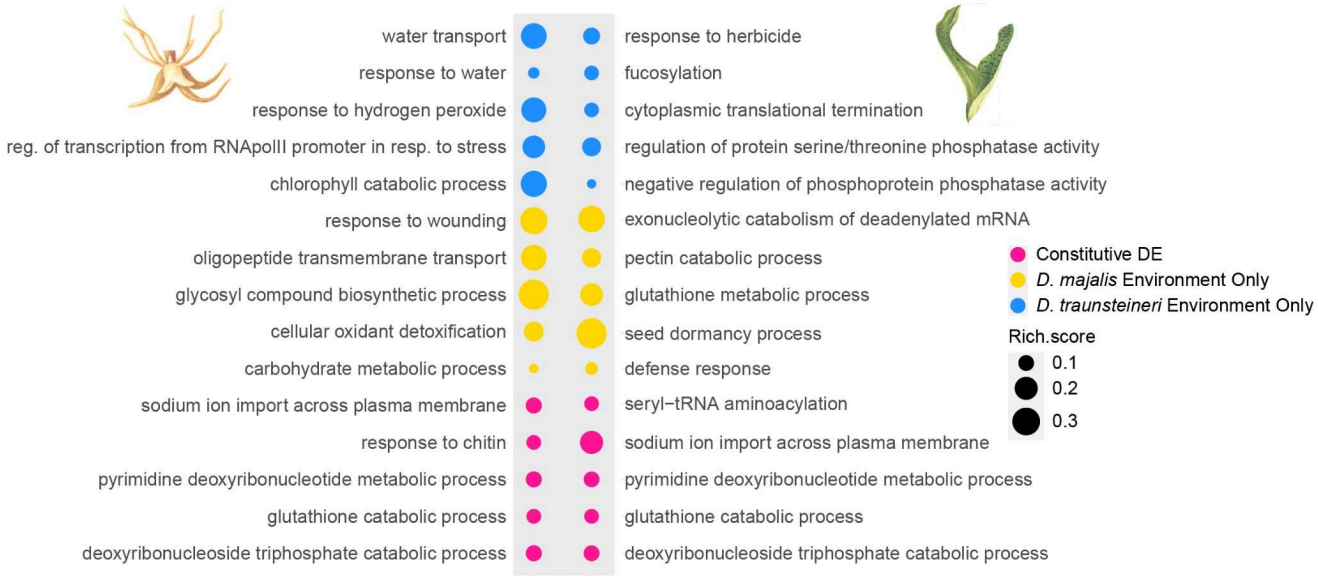


**Figure 4.** GO terms significantly enriched (Fishers exact test $p < 0.05$, rich factor > 0.07) in genes that are differentially expressed between *Dactylorhiza majalis* and *D. traunsteineri* in both species' environments (constitutive DEGs, blue), genes which are differentially expressed only in only the *D. majalis* environment (yellow) and genes which are differentially expressed only in the *D. traunsteineri* environment (pink). Differentially expressed genes for comparisons in Kitzbuhel and St. Ulrich are combined prior to functional enrichment analysis. Functional enrichment was performed on differentially expressed genes in each category (FDR < 0.05) using TopGO using the weight01 algorithm.

To better understand the functional context of plasticity in *Dactylorhiza majalis* and *D. traunsteineri*, we performed GO term enrichment analysis to identify significantly overrepresented functional categories (Fishers exact test $p < 0.05$, rich factor > 0.07) in genes exhibiting plasticity in their expression pattern (fold change > 1.5, FDR < 0.05 in a single species grown in native versus non-native environment). Rich factor represents the ratio of DEGs contributing to an enriched pathway compared to the number of genes annotated to that pathway in total, and thus gives an estimate of effect size.

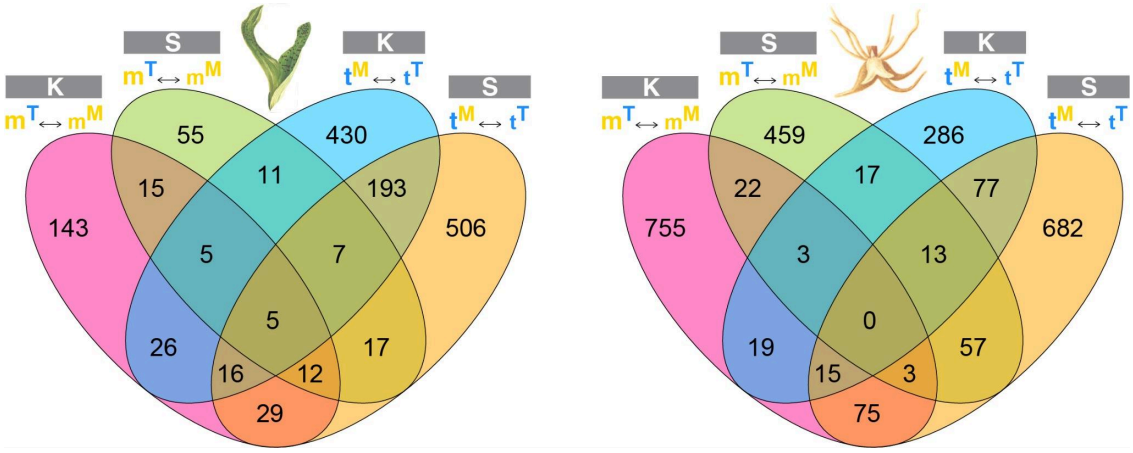


**Figure 5**. Venn diagram of plastic DE genes in each species (m, *Dactylorhiza majalis*; t, *D. traunsteineri*) when transplanted to the alternative environment (FDR < 0.05, FC < 1.5) within the same locality (K, Kitzbuhl; S, St. Ulrich). Labels follow Fig 1c.

Antioxidant processes such as L-ascorbic acid biosynthesis and phosphate starvation functions were upregulated in *D. majalis* leaf tissue, and antioxidant and nitrogen metabolism functions such as pyroxidal phosphate biosynthetic process was downregulated. In contrast, phosphate starvation and phosphate signalling functions involving inositol were downregulated in *D. traunsteineri* leaf tissue as well as antioxidant glutathione and L-ascorbic acid related terms, and nitrogen transport related functions such as L-amino acid transport. Plastic genes related hyperosmotic salinity response were upregulated in *D. traunsteineri* leaf tissue, as well as negative regulators of phosphate cycling such as negative regulation of phosphoprotein phosphatase activity. Functions upregulated in *D. traunsteineri* root tissue included photosynthesis, plant growth regulatory function such as gibberellin biosynthetic process, and systemic acquired resistance. Carotene catabolic process was found to be downregulated in *D. traunsteineri* root tissue, as was phosphate transmembrane transport, response to chitin, oxylipin biosynthetic process, and response to hydrogen peroxide. *D. majalis* roots showed upregulation of metal ion transport such as copper and cadmium, secondary shoot formation, and glucose metabolism. Root tissue in *D. majalis* had downregulated functions including response to reactive oxygen species such as hydrogen peroxide, oxylipin biosynthetic process, and carbohydrate metabolism.

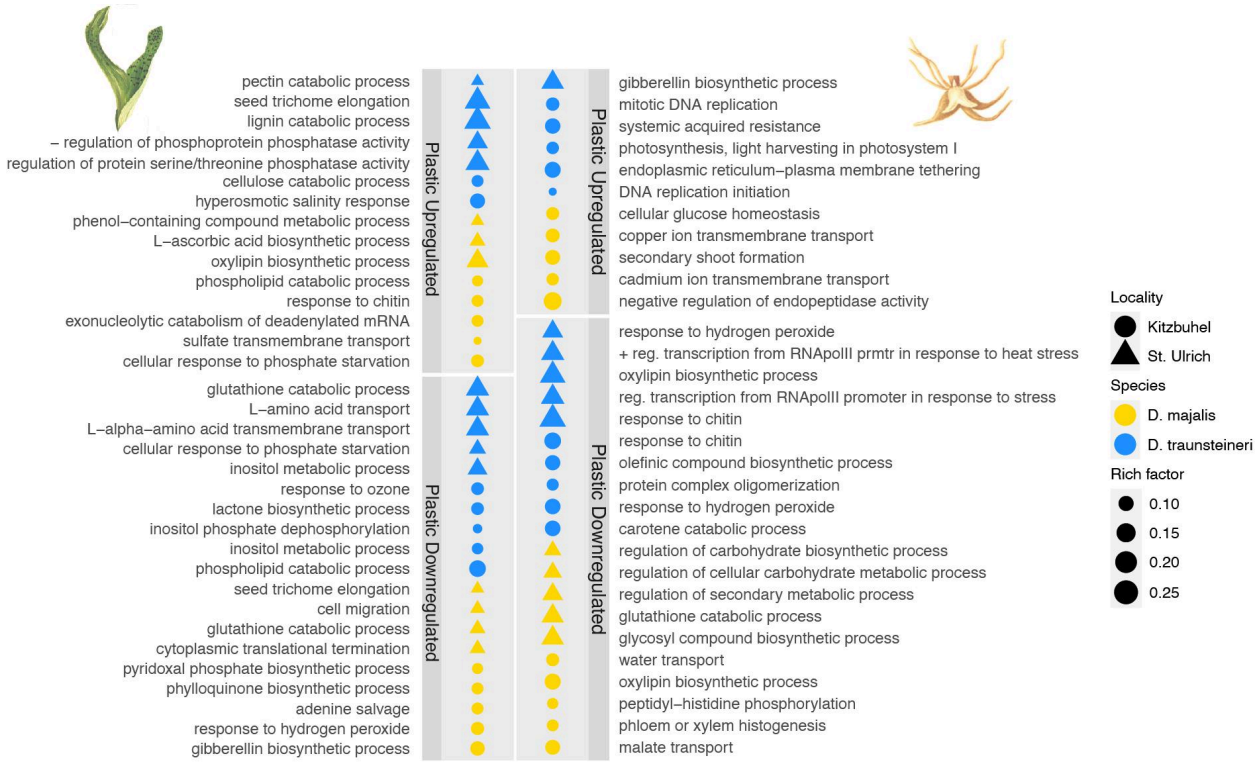


**Figure 6.** Dotplot showing GO terms significantly enriched (Fishers exact test $p < 0.05$, rich factor > 0.07) in genes differentially expressed in each species upon transplantation to the environment of the opposite species, by tissue and locality. GO terms enriched in upregulated genes are shown in red, and downregulated in blue, the size of points corresponds to the rich factor. Top 10 GO terms enriched in upregulated and downregulated genes each are presented only, where available. A full list of significantly enriched functional terms is available in supplementary table 6.

## Fungal taxonomic profiles of native and transplanted root samples

We sought to characterise the fungal species detected in root RNA-seq data in *Dactylorhiza majalis* and *D. traunsteineri* because mycorrhizal and other fungi play an important role as determinants of plant fitness through their life cycle. Mapping RNA-seq reads to the Fungal RefSeq database with MiCoP (LaPierre et al., 2019) identified fungal community profiles which differed the most between environment (that of *D. majalis* or *D. traunsteineri*) rather than species (Figure 7). Transplantation of *D. majalis* and *D. traunsteineri* to the environment of the opposite species had the effect of shifting the fungal community profile of root tissue to that of the surrounding environment, rather than root tissue reflecting native fungal community profiles. The *D. majalis* environment was characterised by the dominance of the order Wallemiales, a small group of xerophilic fungi in the Basidiomycota division of fungi (Zalar et al., 2005), and while this taxon was also often well represented in the *D. traunsteineri* environment, it's lower incidence was accompanied by a higher mean relative abundance of non-Wallemiales fungal taxa (46.8% and 19.7% mean relative abundance of non-Wallemiales fungal taxa in the *D. majalis* environment in *D. majalis* and *D. traunsteineri* respectively, compared to 74.4% and 55.7% in the *D. traunsteineri* environment for the same species).

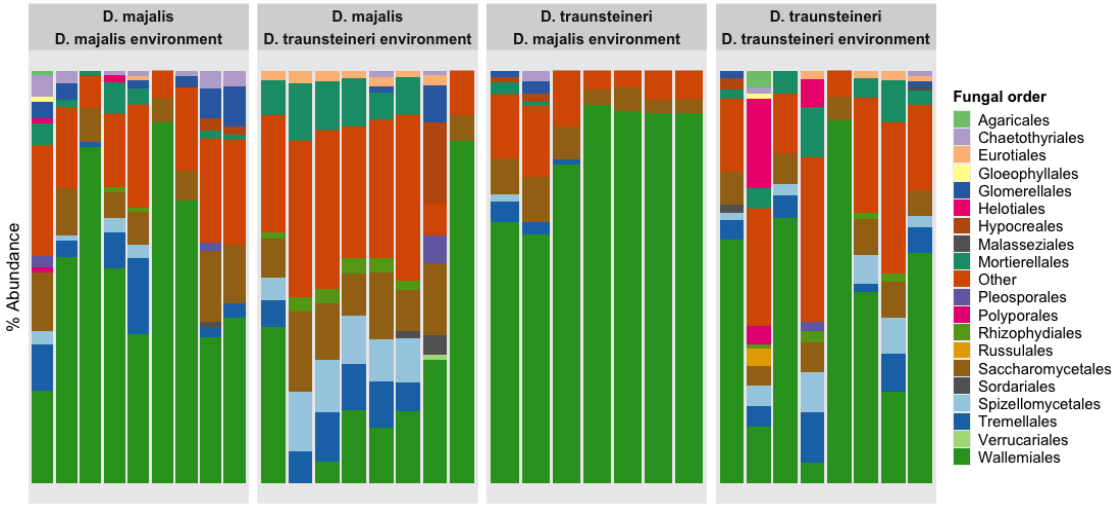


**Figure 7**. Percent abundance of fungal orders identified in root RNA-seq per sample. Fungal orders which were unclassified or present at a proportion of less than 1% are labelled “Other”.

The *D. majalis* environment was characterised by the dominance of the order Wallemiales, a small group of xerophilic fungi in the Basidiomycota division of fungi (Zalar et al., 2005), and while this taxon was also often well represented in the *D. traunsteineri* environment, it’s lower incidence was accompanied by a higher mean relative abundance of non-Wallemiales fungal taxa (46.8% and 19.7% mean relative abundance of non-Wallemiales fungal taxa in the *D. majalis* environment in *D. majalis* and *D. traunsteineri* respectively, compared to 74.4% and 55.7% in the *D. traunsteineri* environment for the same species).

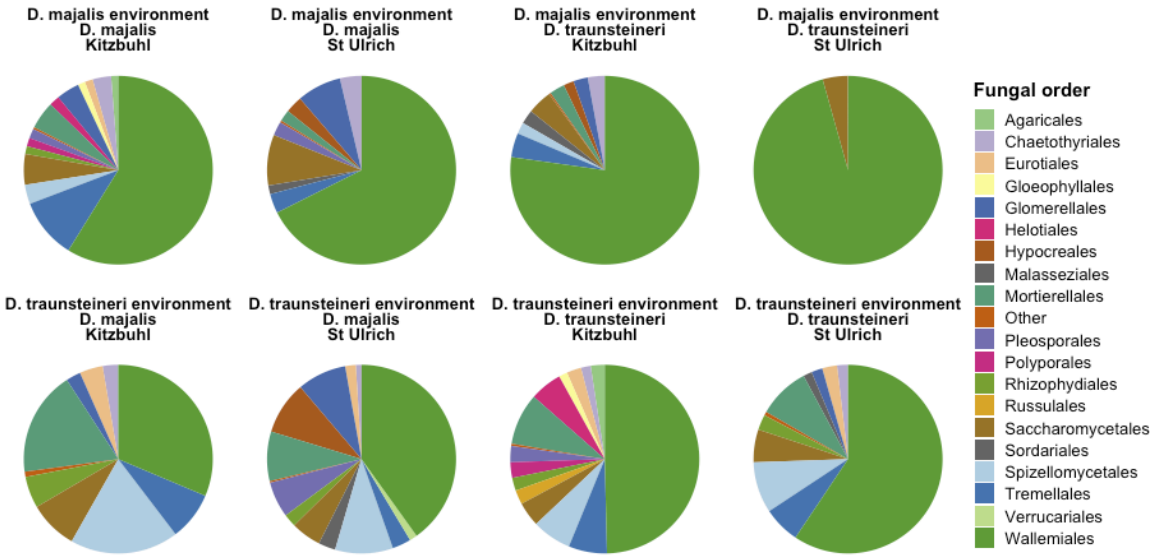


**Figure 8.** Mean relative abundance of fungal orders in transplanted and native individuals of *Dactylorhiza majalis* and *D. traunsteineri* disaggregated by locality. Fungal orders which were unclassified or present at a proportion of less than 1% are labelled “Other”.

The remaining taxonomic composition of the fungal community profiles included taxa which are known to be mycorrhizal partners of some members of the Orchid family, such as Russulales (Paduano et al., 2011), Mortierellales (Herrera-Rus et al., 2020), Polyporales (Downing et al., 2020), and Hypocreales (Jiang et al., 2019), as well as orders containing pathogenic fungal species such as Glomerellales (Baroncelli et al., 2018).

Disaggregating community profiles additionally by locality (Figure 8) recapitulated a pattern of dominance by Wallemiales in the environment of *D. majalis*, and a more taxonomically diverse profile in the *D. traunsteineri* environment. LEfSe analysis (Supplementary Figure 2) confirmed that Wallemiales was the only order significantly discriminative of the *D. majalis* environment (LEfSe LDA=3.14, p=0.0092). Wallemiales showed a higher mean relative abundance in the *D. majalis* environment, with between 49.4% and 90.3% mean relative abundance of Wallemiales regardless of species, compared to between 18.4% and 43.4% in the *D. traunsteineri* environment. In contrast, three orders, Mortierellales (LEfSe LDA=2.44, p=0.0011), Spizellomycetales (LEfSe LDA=2.48, p=0.0010) and Tremellales (LEfSe LDA=2.21, p=0.022), were significantly discriminative of the *D. traunsteineri* environment. The two most significantly discriminative orders of the *D. traunsteineri* environment, Mortierellales and Spizellomycetales, had a mean relative abundance of between ca. 5 and 10% in *D. majalis* transplants and *D. traunsteineri* native individuals in both St. Ulrich and Kitzbuhel. In contrast, these orders were found only in Kitzbuhel in the *D. majalis* environment at a mean relative abundance of over 2%, with a maximum mean relative abundance of ca. 4%. Only one order (Tremellales, LEfSe LDA=2.32, p=0.0022) was found to be discriminative of the St. Ulrich and Kitzbuhel localities when testing for differential abundance using locality as the main class and environment as a subclass, suggesting that the environments of *D. majalis* and *D. traunsteineri* were the predominant drivers of fungal community differences and that this effect is relatively stable across localities.

## Discussion

### Dominant plastic responses are punctuated by fixed transcriptional differences between species

Ecological plasticity is an important phenomenon which can allow species to move outside of their genetically encoded trait optima to withstand environmental fluctuations (Fox et al., 2019) or colonise new ranges (Richards et al., 2006).

Polyploid ecological plasticity is often viewed through the lens of neopolyploid establishment, its contribution to overcoming obstacles such as minority cytotype exclusion and competition with diploids (Anneberg et al., 2023; Husband, 2000) is an important consideration for understanding how polyploids escape the initial barriers set by their parental species. Polyploid speciation relies upon reinforcement of reproductive barriers to prevent genetic assimilation by more widespread cytotypes, however adaptive cross-ploidy introgression of genes can increase the fitness of establishing polyploid taxa (Chapman & Abbott, 2010; Han et al., 2015). In this context, ecological plasticity can allow polyploids to both colonise and establish themselves in novel environments, and tolerate environments inhabited by related taxa where gene flow can occur, thus potentially exposing establishing polypoids to opposing evolutionary forces.

To unravel some of these further implications of ecological plasticity for polyploid evolution, we used reciprocal transplantation of *Dactylorhiza majalis* and *D. traunsteineri* to quantify the plastic range of each species relative to the amount of fixed expression differences accumulated between the sibling species. We searched for genes which showed constitutively fixed expression differences between *D. majalis* and *D. traunsteineri*, and thus could represent genes which underpinned adaptation to their differing ecologies, however we found very few constitutive DEGs, most of which were in root tissue comparisons. That root tissue had the most constitutive DEGs between the sibling allopolyploids is consistent with findings of widespread differences in nutrient profiles between the two species' environments, suggesting that the below-ground environment exerts greater divergent selective pressure on *D. majalis* and *D. traunsteineri*. Rather than finding many constitutively DEGs between the sibling allopolyploids in all environments, most DEGs between *D. majalis* and *D. traunsteineri* were in response to a single environment (either that of one species or another, see Figure 3), and more such DEGs were found in the *D. majalis* environment than the *D. traunsteineri* environment (ca. 2000 DEGs versus ca. 600 genes respectively), suggesting that *D. traunsteineri* undergoes greater transcriptional remodelling in the *D. majalis* environment than vice versa. More widespread and competitive species like *Dactylorhiza majalis* (Paun et al., 2011) are thought to have higher levels of plasticity (Hiatt & Flory, 2020) than those with narrow distributions such as *D. traunsteineri*, and the transplantation of *D. majalis* to the *D. traunsteineri* environment may represent a smaller perturbation than vice versa, and may fall more within the tolerated range of *D. majalis*. The nutritionally depauperate environment of *Dactylorhiza traunsteineri* require it to modify key parts of its metabolism (Wolfe et al., 2023), however if the basis for this metabolic modification is plastic, the transplantation of *D. traunsteineri* to the *D. majalis* environment may allow it to return to a transcriptional baseline to make use of the newly available resources, which is reflected in the functional analysis of plastic genes (Figure 6 and discussed later).

Plasticity can reduce the incidence of maladaptive interspecific interactions (Otte et al., 2016), providing a buffer without which competitive exclusion or genetic swamping would not allow for coexistence of species (Hess et al., 2022). Polyploid plasticity, especially along environmental clines, is an important component for achieving the ecological speciation required for successful polyploid establishment (Shimizu-Inatsugi et al., 2017), and the heterogeneous soil types inhabited by *Dactylorhiza majalis* and *D. traunsteineri* may have provided an important substrate on which ecological divergence between the allopolyploid species began. Transcriptional plasticity allowing *Dactylorhiza traunsteineri* to survive in nutritionally poor environments may be rewarded by allowing it to successfully establish as a young allopolyploid, developing genetic isolation away from the swamping effects of hybridisation when grown in sympatry with sibling allopolyploids. While global transcriptional profiles do not tend to discriminate *D. majalis* and *D. traunsteineri* (Figure 2), the small number of constitutionally differentially expressed genes may represent evidence of nascent differentiation in response to habitat-specific conditions.

**Sibling allopolyploids exhibit plasticity in each other's habitats by altering root system architecture, redox state and mycorrhizal interaction**

Growing each allopolyploid sibling in the habitat of the other stimulated remodelling of functions reflecting their previously found habitat differences such as soil nutrient availability

(Wolfe et al., 2023); *D. traunsteineri* downregulated genes concerned with cellular response to phosphate starvation in leaves and phosphate ion transmembrane transport in roots, and upregulated genes enriched in L-amino acid transport, which mediates nitrogen distribution (Yao et al., 2020), suggesting an alleviation of pressure in nutrient availability when grown in the *D. majalis* environment. A concomitant increase in the same functional terms is not seen in *D. majalis*, rather a suite of processes reflecting specific responses to nutrient starvation such as root system architecture remodelling. Nitrogen and phosphate depletion has widespread consequences for plants including reduced photosynthetic rates and reduced capacity to respond to abiotic stress (Bechtaoui et al., 2021; Shao et al., 2020). In response to nutrient limitation, plants can undergo root architecture remodelling to increase root surface area and thus access to nutrients (Kiba & Krapp, 2016), achieving this through inhibition of primary root growth coupled with an increase in lateral roots formed through stimulation of secondary root formation (Sánchez-Calderón et al., 2005). Accordingly, genes annotated to secondary shoot formation and regulation are upregulated in *Dactylorhiza majalis* root tissue and downregulated in *D. traunsteineri* root tissue, and because secondary formation of both root and shoot are thought to stem from common regulatory mechanisms (Strock & Lynch, 2020) the annotation of root genes with shoot formation is expected to stem from similarity of function.

Furthermore, *Dactylorhiza majalis* grown in the nutrient poor environment of *D. traunsteineri* upregulates genes in functions known to participate in sensing and responding to nutrient starvation, including iron ion homeostasis, an important mediator of phosphate starvation response (Rai et al., 2015) and sucrose catabolic process, know to be involved with initiation of signalling cascades triggering phosphate limitation responses (Hammond & White, 2008).

*D. traunsteineri* showed downregulation of chitin responsive genes in roots, as well as downregulation of genes annotated to carotene catabolic process, a key component of mycorrhization with fungal partners (Fester et al., 2002). The consistently divergent fungal community profiles found between the *D. traunsteineri* and *D. majalis* environments (Figures 7 and 8), as well as chitin responsive genes also being constitutively differentially expressed in the roots of sibling allopolyploids (Figure 4) suggest that the rhizosphere may exert divergent selective pressure on *D. traunsteineri* and *D. majalis*. Nevertheless, the downregulation of mycorrhization related genes in *D. traunsteineri* root tissue grown in the *D. majalis* environment emphasises that *D. traunseineri* can plastically adjust to the surrounding rhizosphere if required.

(Wolfe et al., 2023) showed a lower energetic efficiency of photosynthesis in *D. traunsteineri* compared to *D. majalis*, and a higher rate of photooxidative regulation through quenching. Leaf tissue of *D. traunsteineri* grown in the *D. majalis* environment shows plastically reduced levels of pathways known to alleviate oxidative stress and maintain efficient photosynthesis such as glutathione (Müller-Schüssele et al., 2020) and L-ascorbate (Chen et al., 2021). Moreover, we identified upregulated functions related to phylloquinone, involved in electron transport in photosystem I (Basset et al., 2017), and polyprenol, a component of thylakoid membrane function (Hofmann, 2017), indicating a plastic increase in *D. traunsteineri* photosynthetic efficiency upon release from pressures in its native environment.

Interestingly, up- and downregulated genes in *Dactylorhiza majalis* root tissues were enriched in photosynthesis related terms. Photosynthesis tends to be inhibited under low nutrient availability (Farhat et al., 2016; Mu & Chen, 2021), however due to roots being non-photosynthetic tissue, study of photosynthesis regulation in root tissue is sparse. (Kang et al., 2014) identified

photosynthetic genes downregulated in the root tissue of *Arabidopsis thaliana* during phosphorous starvation, speculating that phosphorous depletion may stimulate aberrant photosynthesis to produce elevated levels of reactive oxygen species (ROS). Both nutrient deficiency (Ahanger et al., 2017; Kim et al., 2010) and photosystem I of the photosynthetic apparatus (Furutani et al., 2020) have been linked to increased levels of ROS, and response to ROS is decreased in *D. traunsteineri* when grown in the non-native *D. majalis* environment, suggested by the downregulation of genes enriched in L-ascorbic acid and glutathione pathways, noted for their ROS scavenging role (Akram et al., 2017). While we can only speculate on the basis for photosynthetic inhibition in *D. majalis* roots, the absence of up- or down-regulated photosynthetic functions in *D. majalis* leaf tissue suggests the pattern we see in root is a genuine response to the environment of *D. traunsteineri* rather than artifactual.

**Interspecific fungal community differences**

Fungal reads were extracted from *Dactylorhiza* root RNA-seq reads, and it is not clear what proportion of the sequence reads represent bystander fungi present in the surrounding rhizosphere compared to reads which originate from genuine orchid mycorrhizal partners. Nevertheless, the results reflect a difference in the fungal communities present in the environments of the sibling allopolyploids; the *D. traunsteineri* environment was more diverse than the *D. majalis* environment, three orders were discriminant of the *D. trausnteineri* environment (Mortierellales, Spizellomycetales and Tremellales) while Wallemiales was the only fungal order which was significantly discriminant of the *D. majalis* environment. This result is interesting given the limitation of resources such as nitrogen and phosphorus in the *D. traunsteineri* environment, which may prompt cultivation of more specialist mycorrhizal partners (Kohler et al., 2015; Wyatt et al., 2014).

Numerous environmental factors are important for successful symbiotic germination of orchid seeds with their mycorrhizal partners, and studies in *Dactylorhiza majalis* have found this process to be highly temperature dependent (Rasmussen et al., 1990). Interestingly, climatic niches for *D. traunsteineri* and *D. majalis* were found to differ in particular according to temperature seasonality and mean temperature of the driest quarter (Wolfe et al., 2023), suggesting key factors involved in germination success differ between *D. majalis* and *D. traunsteineri*. The study presented here used adult plants to perform reciprocal transplantations, therefore individuals tested had germinated and naive soil. We can therefore not conclusively say whether the fungal community profile in the native soil of one sibling allopolyploid would preclude germination of the other.

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