Chromosome Numbers and Reproductive Life Cycles in Green Plants: A Genomic Perspective

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Abstract

Green plants, as the primary producers in the biosphere, play a crucial role in both the biosphere and human activities. Despite their importance, many aspects of their evolutionary history remain mysterious. One such mystery is the association between chromosome number and reproductive life cycle. Heterosporous plants, which include all seed plants, generally have smaller chromosome numbers compared to homosporous plants, which typically have larger chromosome numbers.

Previous hypotheses, such as the gametophytic selfing hypothesis, have tried to explain this pattern but have been rejected. Recent studies suggest that heterosporous lineages may have undergone higher rates of genome downsizing via chromosome number reduction, relative to homosporous lineages. However, the exact mechanisms underlying this process remain unclear.

In this study, we investigate the association between chromosome numbers and reproductive life cycles using genomic data from a wide range of green plant lineages. We focus on two main questions: (1) Have any gene families undergone significant expansion or contraction in heterosporous lineages? (2) Have any gene families undergone distinct selection patterns in heterosporous lineages? By exploring these questions, we aim to shed light on the genomic factors associated with the evolution of heterospory and its link to reduced chromosome numbers.

We conducted comparative genomic analyses across a broad sampling of green plant lineages, including 112 species spanning over 500 million years of evolutionary history. Using gene family evolution models and selection analyses, we identified gene families that have undergone significant expansion, contraction, or selection in heterosporous lineages. Our results reveal both shared and lineage-specific genomic changes associated with the evolution of heterospory. We found expansions in gene families related to developmental regulation, signaling pathways, and stress responses across heterosporous groups. Notably, the MATE efflux family showed consistent expansion and evidence of selection in heterosporous lineages, suggesting a potentially conserved role in heterospory evolution. Further research is needed to explore how such genes might be involved in chromosome behavior and spore production. Meanwhile, these findings highlight novel genomic insights that may underpin the association between heterospory and reduced chromosome numbers.

Introduction

The evolution of land plants (Embryophyta) has been marked by several key innovations, one of which is heterospory - the production of two distinct types of spores. This trait is strongly associated with seed-based reproduction and has evolved independently multiple times across plant lineages. Intriguingly, heterosporous plants, including all seed plants, generally have fewer chromosomes than their homosporous counterparts.

This pattern of reduced chromosome numbers in heterosporous lineages was first noted by (Klekowski and Baker 1966) , who showed that homosporous plants have, on average, about four times as many chromosomes as heterosporous plants. However, the underlying mechanisms driving this association remain poorly understood.Previous hypotheses attempting to explain the higher chromosome numbers in homosporous lineages have been largely unsuccessful. For instance, the gametophytic selfing hypothesis proposed by (Hickok and Klekowski 1973) suggested that homosporous plants primarily reproduce by self-fertilization using gametes from a hermaphroditic gametophyte. This process would produce completely homozygous zygotes, leading to a complete loss of allelic diversity. The resulting loss of variation was hypothesized to create selective pressure favoring polyploidy-based redundancy to avoid genetic load, thereby leading to selection for larger genomes.

However, this hypothesis was later rejected due to the tendency of polyploids to act as genetic diploids (C. H. Haufler and Soltis 1986). This diploidization process, where polyploid genomes undergo fractionation and gene loss to functionally resemble diploid genomes, would negate the proposed advantage of maintaining genetic diversity through polyploidy. The rejection of the gametophytic selfing hypothesis underscored the complex interplay between reproductive strategies, genome dynamics, and chromosome numbers in plants.

Recent genomic studies have provided new insights into this longstanding question. Contrary to earlier assumptions, homosporous plants appear to have lower rates of paleopolyploidy than heterosporous plants, despite their larger chromosome numbers (Clark et al. 2016; Carta, Bedini, and Peruzzi 2020; Mayrose and Lysak 2021). Instead, the high chromosome numbers in homosporous plants seem to result from higher retention of chromosomes from fewer rounds of polyploidy (Barker 2009; Christopher H. Haufler 2014; Marchant et al. 2021) . This retention is likely due to lower rates of fractionation, i.e., the

potentially permissive loss of duplicate genes and regulatory elements resulting from the relaxed effect of purifying selection on duplicated genomic elements.

These findings suggest a shift in perspective: rather than asking why homosporous lineages have larger chromosome numbers, we should investigate why heterosporous lineages have undergone greater genome downsizing (Kinosian, Rowe, and Wolf 2022). This reframing is particularly relevant given that heterospory is the derived condition within Embryophyta, having evolved independently at least 11 times across the plant evolutionary tree, including the three extant lineages.

The relationship between chromosome numbers and reproductive life cycles is not fully understood, despite its significance. Every plant lineage that evolved into seed-bearing species, extant and extinct, did so after the evolution of heterospory (Bateman and DiMichele 1994). Despite the crucial role of heterospory in the evolution of terrestrial plant life and its strong link to seed-based reproduction, the genomic mechanisms underlying transitions to heterospory and its association with reduced chromosome numbers remain unclear (Kinosian, Rowe, and Wolf 2022). Are there undiscovered patterns of neofunctionalization and/or subfunctionalization that affect reproduction and meiosis (and by extension chromosome numbers), shared among the extant heterosporous lineages?

The advent of large-scale genomic data and advanced computational methods offers an unprecedented opportunity to re-examine this longstanding question from a new perspective. Recent initiatives like the One Thousand Plant Transcriptomes Project (One Thousand Plant Transcriptomes Initiative 2019) have dramatically expanded the availability of genomic data across diverse plant lineages. Coupled with sophisticated phylogenetic and comparative genomic methods, such as gene family evolution models and selection analyses, we now have the tools to explore patterns of genomic change associated with heterospory at a resolution and scale previously impossible. This study leverages these modern data sources and methodologies to provide a fresh, data-driven exploration of the genomic underpinnings of heterospory and its association with chromosome number reduction.

In this study, we take an exploratory approach to investigate potential genomic factors associated with the evolution of heterospory and its co-occurrence with reduced chromosome numbers. Working under the hypothesis that similar genomic factors might be associated with

both a reduction in chromosome number and the evolution of heterosporous lineages, we focus on two main questions:

1. Have any gene families undergone significant expansion or contraction in copy number on lineages leading to heterosporous clades? If so, is there anything about putative gene function that provides a clue to the association of heterospory and chromosome number?

2. Have any gene families undergone distinct and parallel patterns of selection on lineages leading to heterosporous clades? If so, is there anything about putative gene function that provides a clue to the association of heterospory and chromosome number?

By addressing these questions, we aim to provide new insights into the genomic basis of heterospory evolution and its potential link to chromosome number reduction. This investigation not only contributes to our understanding of plant evolution but also has potential implications for agriculture and biotechnology, given the importance of seed plants in the human food supply chain.

To answer these questions, we employed a comprehensive genomic analysis approach, combining broad-scale examination with focused investigation of specific gene families. Our analysis pipeline involved sequence alignment, phylogenetic reconstruction, and statistical tests for gene family evolution and selection patterns. We applied this methodological framework in two ways:

An untargeted approach : This novel approach aims to identify broad patterns of genomic change associated with heterospory across the plant phylogeny. While this method offers the potential for unbiased discovery, we anticipate significant computational and algorithmic challenges due to the scale and depth of the data.

Targeted Gene Family Analysis : While our genome-wide approach offers the potential for broad insights, we anticipate significant computational and algorithmic challenges in analyzing such a vast dataset across diverse plant lineages. The sheer scale of the data, combined with the evolutionary distance between species, may lead to difficulties in sequence alignment, homology detection, and phylogenetic inference. Moreover, the computational resources required for such a comprehensive analysis may prove prohibitive.

To address these potential limitations, we propose a complementary targeted analysis focusing on specific gene families. This approach aims to examine a subset of genes involved in

cell cycling, spindle fiber formation, and chromatin dynamics – processes that may be particularly relevant to chromosomal profiles in a species.

By narrowing our focus to these select gene families, we can mitigate several challenges. A reduced dataset allows for more intensive computational analysis, enabling the use of more sophisticated algorithms that might be impractical at a genome-wide scale. Focusing on specific gene families may provide better phylogenetic resolution, especially for rapidly evolving genes that might be problematic in genome-wide analyses. Additionally, by selecting genes with potential functional links to chromosome numbers, we increase the likelihood of detecting biologically meaningful patterns that might be obscured in a broader analysis.

This targeted approach serves as a valuable complement to our untargeted analysis, allowing us to delve deeper into specific aspects of heterospory evolution while circumventing some of the technical challenges inherent in large-scale genomic comparisons across diverse plant lineages. It offers a way to balance the broad perspective of the untargeted analysis with a more focused examination of genes potentially crucial to the chromosomal profile of heterospory.

Both approaches utilize a common set of bioinformatic tools and statistical methods, including sequence alignment, phylogenetic reconstruction, and tests for gene family evolution and selection patterns. By combining these complementary strategies, we aim to balance the potential for novel insights from the untargeted approach with the more directed examination of potential candidate genes.

Materials and Methods

Sample selection

Our study aims to sample widely across land plants, including all major clades of vascular plants and bryophyte outgroups. This comprehensive sampling is crucial for addressing our research questions about gene family evolution and selection patterns across heterosporous and homosporous lineages.

Our interest in the evolution of heterospory led us to sample widely across land plants, including all major clades of vascular plants and bryophyte outgroups. We balanced two competing factors to determine the number and types of taxa to include: the need for a list large

enough to yield phylogenetic insights versus the need for a list small enough to remain computationally feasible.

Preliminary testing suggested that phylogenetic methodologies, including homology inference, gene family reconstruction in a phylogenetic context, heuristics-based gene-tree inference, and selection analysis, remain manageable in an untargeted, holistic fashion for up to approximately 100 species or transcriptomes when using current software and hardware with realistic price tags.

In line with our aim to investigate specific alterations on heterosporous lineages, we included representatives from all three extant heterosporous lineages. We sampled homosporous taxa evenly throughout lycophytes and ferns for evolutionary context.
Finally, we included outgroup samples from the three main bryophyte lineages.Our final dataset included 112 species, spanning at least 500 million years of evolutionary history, (Wang et al. 2022).

Data sources

To investigate gene family evolution and selection patterns across plant lineages, we used genomic data from three different sources:

1. (Pelosi et al. 2022): 4 species
2. Phytozome (Goodstein et al. 2012): 5 species
3. One Thousand Plant Transcriptomes Project (One Thousand Plant Transcriptomes Initiative 2019): 103 species

Data from Pelosi et al. (2022) were accessed from a public data repository (https://github.com/jessiepelosi/ferntxms) . Phytozome data was accessed via their public website (https://phytozome-next.jgi.doe.gov/). One Thousand Plant Transcriptomes Project data was retrieved using an R package (https://github.com/ropensci/onekp) that enables programmatic retrieval of OneKP data.

All sequence data were quality-checked and filtered for low-quality reads prior to analysis. Transcriptome assemblies were used as provided by the respective data sources.

This diverse dataset allows us to explore gene family expansions, contractions, and selection patterns across a wide range of plant lineages, directly addressing our research

questions about the genomic basis of heterospory and its association with chromosome number reduction.

Phylogenetic methodologies

Homology inference

To identify gene families shared among the studied species, a critical first step in addressing both of our research questions, we performed homology inference using OrthoFinder (v2.5.5) (David M. Emms and Kelly 2019) with default options. OrthoFinder was chosen for its ability to handle large datasets efficiently and its robust algorithm for identifying orthogroups. The bundled version of BLAST (McGinnis and Madden 2004) within the OrthoFinder installation was used as the sequence alignment tool. This generated a list of gene families (referred to as "orthogroups") shared among the studied species.This homology inference step provides the foundation for our subsequent analyses of gene family evolution and selection patterns across the phylogeny.

De novo Species-tree generation

To provide a robust phylogenetic framework for our analyses of gene family evolution and selection patterns, we generated a species tree using STAG and STRIDE (D. M. Emms and Kelly 2018; David M. Emms and Kelly 2017) . STAG constructs an unrooted species tree to account for multi-copy gene families, which STRIDE then roots. The final tree in Newick format is provided in the supplemental materials.This species tree is crucial for contextualizing the gene family expansions, contractions, and selection patterns we investigate in our study.

Gene family expansion and contraction inference

To address our first research question regarding gene family expansions and contractions in heterosporous lineages, we employed CAFE5 (Mendes et al. 2020), which employs a "birth-death" maximum likelihood model to reconstruct copy number variations at ancestral nodes for gene families. CAFE5 first estimates a global rate of change of evolution, or "lambda," and then employs that lambda in its implementation of maximum likelihood estimation.

Initially, we used CAFE5's default settings to test the species tree generated by STAG and STRIDE, as well as all gene families identified by OrthoFinder. However, CAFE5's inference model failed to initialize the exhaustive table of counts for all orthogroups. Through testing, we found that CAFE5's statistical model fails when the difference between the largest and smallest copy number in a gene family exceeds 68. To overcome this limitation, we filtered out gene

families exceeding this threshold, allowing us to proceed with the analysis while retaining the majority of gene families.

By reconstructing the history of gene family size changes across the phylogeny, this analysis allows us to identify gene families that have undergone significant expansion or contraction in heterosporous lineages, directly addressing our first research question.

Selection analysis

To address our second research question regarding distinct selection patterns in heterosporous lineages, we employed a multi-step approach:

1. Multiple sequence alignment: We performed multiple sequence alignment using both MUSCLE (Edgar 2004)and MAFFT(Katoh et al. 2002) . These tools were chosen for their accuracy in handling large datasets and their ability to produce alignments suitable for downstream phylogenetic analyses. However, we encountered challenges with some of the largest gene families (in terms of sequence length and species diversity). For these families, the alignments produced had an excessive number of gaps. In fact, the gaps in these families were so prevalent that downstream codon alignment resulted in non-uniform length DNA sequences, which are incompatible with gene-tree inference methodologies. Consequently, the families that produced non-uniform sequence alignments were excluded from further analysis.
2. Translation to codons: We used PAL2NAL (Suyama, Torrents, and Bork 2006) to convert the aligned protein sequences to codon alignments. This step is crucial for maintaining the correct reading frame and for enabling subsequent analyses of selection at the codon level.
3. Gene tree generation: We generated gene trees for each gene family using IQ-tree2 (Minh et al. 2020) with the GTR+F+I model and 100 non-parametric bootstrap replicates. The GTR+F+I model was chosen as it allows for rate heterogeneity across sites and empirical base frequencies, which is appropriate for coding sequences and provides a robust framework for subsequent selection analyses.
4. Selection analysis: We employed the BUSTED-PH (BUSTED-PHenotype) method, an extension of the BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification) approach (Murrell et al. 2015). BUSTED-PH is specifically designed to test whether a particular feature, phenotype, or trait is associated with positive selection

by comparing the selective pressures acting on gene families in lineages with and without the trait of interest.

The BUSTED-PH analysis requires a coding sequence alignment and a phylogenetic tree with the test and background branch sets clearly defined. We designated the branches leading to heterosporous lineages as the "test" set and the branches leading to homosporous lineages as the "background" set. Any remaining branches were designated as "nuisance" and excluded from the comparisons.

The analysis proceeds by fitting several random effects branch-site models to the data:

a) An "unrestricted" model that allows both the test and background branch sets to have their own independent ω (dN/dS) distributions.

b) A "constrained" model where $\omega \leq 1$ is enforced on the test branches. A likelihood ratio test between this model and the unrestricted model is used to determine if the test branches are subject to episodic diversifying selection.

c) A "constrained" model where $\omega \leq 1$ is enforced on the background branches. A likelihood ratio test between this model and the unrestricted model is used to determine if the background branches are subject to episodic diversifying selection.

d) A "shared" model where the ω distribution is constrained to be the same for test and background branches. A likelihood ratio test between this model and the unrestricted model is used to determine if the selective regimes differ between test and background branches.

The results of these likelihood ratio tests are then interpreted using a decision tree to provide guidance on whether episodic selection is associated with the presence of heterospory.By applying BUSTED-PH to our dataset, we aim to identify gene families that may have undergone distinct selection pressures in heterosporous lineages. This analysis directly addresses our second research question by revealing potential genomic signatures associated with the evolution of heterospory and its link to reduced chromosome numbers.

Data processing and visualization

Data processing and reshaping were performed using custom R and Python scripts (see supplemental section for code repository). Visualization of phylogenetic trees and gene family

evolution patterns was accomplished with the GGtree package in R (Yu 2020) . Additional visualizations were created using ggplot2 in R and matplotlib in Python.

Targeted subset

Given the scale of the data and the depth of the phylogeny at hand, the untargeted approach was likely to encounter computational and analytical challenges. In anticipation of these challenges, we implemented a targeted analysis as a subset of our broader study. The targeted study is simply an implementation of the same methods and algorithms on the whole dataset, with the following distinctions:

1. Gene selection: We focused on a curated list of genes involved in cell-cycling, spindle fiber formation, and chromatin dynamics, derived from The Arabidopsis Information Resource (TAIR)(Rhee et al. 2003).
2. CAFE5 analysis: While most analytical steps remained identical for both approaches, we specifically re-ran CAFE5 for the targeted gene set to allow for a more focused examination of gene family evolution in these genes of interest.
3. Computational resources: The reduced gene set in the targeted approach allowed for more intensive analysis and manual curation where necessary, without the computational constraints of the full dataset.

This targeted approach complements our untargeted analysis, providing a more detailed examination of specific gene families hypothesized to play key roles in heterospory evolution and chromosome number reduction.

Results

Species Tree Topology

The species tree generated using STAG and STRIDE is presented in Figure 1. This de novo generated tree provides the phylogenetic context for all subsequent analyses of gene family evolution and selection patterns. Importantly, the topology of our tree is largely consistent with current understanding of plant phylogenetic relationships as established in recent literature (Wickett et al. 2014; Ruhfel et al. 2014; One Thousand Plant Transcriptomes Initiative 2019).

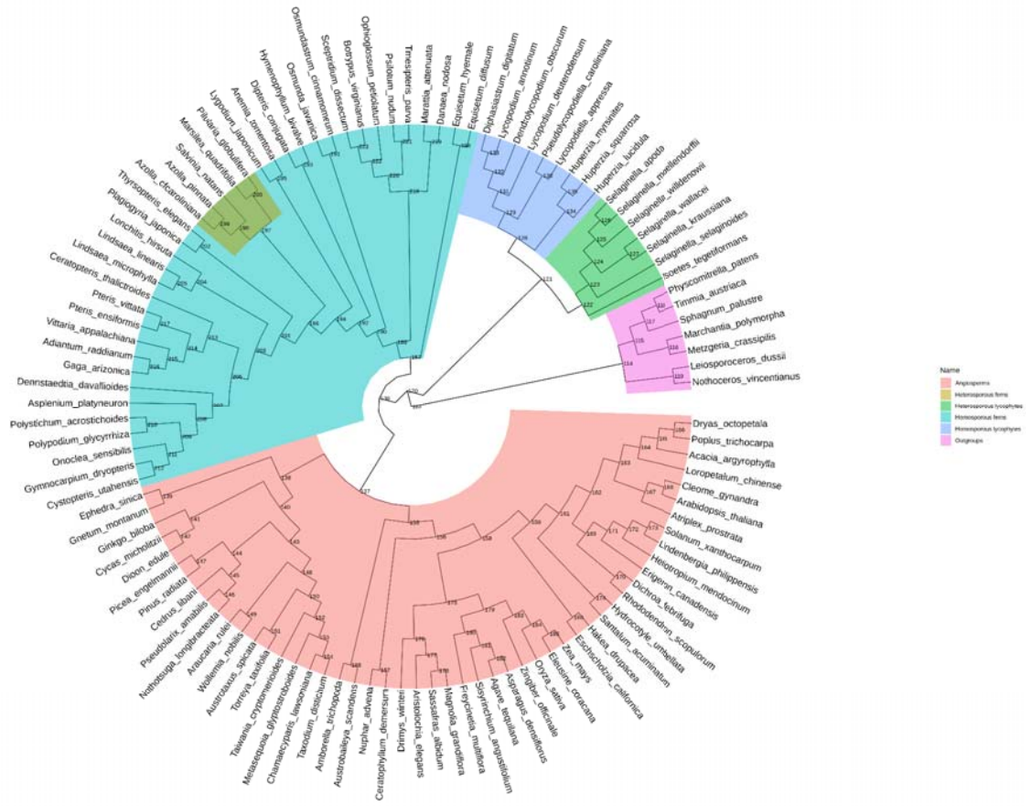

Fig 1: An illustration of the species tree of the project, with the various clades highlighted using a color scheme.

The Newick format of this tree is provided in the supplemental materials for reproducibility and further analysis.

Untargeted analysis

Gene Family Expansions and Contractions

Our analysis using CAFE5 revealed significant changes in gene family sizes across the phylogeny, particularly in lineages leading to heterosporous plants. In total, we detected 54 gene families with significant expansion or contraction leading to the three heterosporous nodes in our phylogeny. The distribution of these changes varied across the heterosporous lineages:

1. Seed plants (angiosperm ancestral node): 12 families with significant changes
2. Heterosporous ferns ancestral node: 11 families with significant changes
3. Heterosporous lycophytes ancestral node: 4 families with significant changes

To understand the functional implications of these changes, we examined the *Arabidopsis thaliana* (Rhee et al. 2003)orthologs within these gene families and their associated functions. Tables 1-3 present the full list of gene families with significant changes for each heterosporous lineage.

Families expanding on Angiosperm ancestral node

Orthogroup name	**Common functions in *A. thaliana* orthologous genes**
OG0000050	Encodes galactose oxidase; F-box protein involved in regulation; Involved in the morphological stability of the floral meristem.
OG0000104	Synthesis and deposition of secondary cell-wall.
OG0000108	Involved in the terpenoid synthesis pathways.
OG0000126	MATE efflux family protein; Involved in flavonoid metabolism, affecting root growth, seed development, pollen development and germination.
OG0000145	Leucine-rich receptor-like protein kinase family protein.
OG0000163	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family.
OG0000183	Encodes a plasmodesmata protein that may be involved with intercellular movement through plasmodesmata.
OG0000233	N/A (Arabidopsis genes missing)
OG0000304	Encodes protein kinase AME3; A member of a CDC2-related kinase subfamily; A LAMMER-type protein kinase that co-precipitates with serine/arginine rich (SR) proteins.
OG0000308	Seed imbibition.
OG0000451	0-acyltransferase (WSD1-like) family protein.
OG0000634	Non-mendelian inheritance model gene which when mutated leads to instability in floral structure.

Families expanding on Heterosporous ferns ancestral node

Orthogroup name	**Common functions in A. thaliana orthologous genes**
OG0000126	MATE efflux family protein; Involved in flavonoid metabolism, affecting root growth, seed development, pollen development and germination.
OG0000287	Adenine nucleotide alpha hydrolases-like superfamily protein; Universal stress protein.
OG0000368	CH2-like zinc finger protein.
OG0000399	Member of ATH subfamily; ABC2 homolog 5.
OG0000717	Homeobox protein; Filamentous hemagglutinin transporter.
OG0000857	EXS family protein.
OG0000860	Ovate family protein.
OG0001114	Pol-like polyprotein/retrotransposon.
OG0001616	Class I knotted-like homeodomain/homeobox gene family.

OG0002439	DNA polymerase catalytic subunit.
OG0002601	Copia-like polyprotein/retrotransposon.

Families expanding on Heterosporous lycophyte ancestral node:-

Orthogroup name	**Common functions in A. thaliana orthologous genes**
OG0000022	Family of genes that encode Beta glucosidase.
OG0000171	Transcription factor jmjc domain-containing protein. Encodes a probable H3K9me2 demethylase.
OG0000598	Oxidoreductase activity gene family.
OG0000634	Non-mendelian inheritance model gene which when mutated leads to instability in floral structure.

While all identified changes are potentially significant, several gene families stand out due to their known roles in plant development, reproduction, and stress responses:

Seed Plant parent node:

The ancestral node of seed plants exhibited expansions in gene families associated with reproductive adaptations. The MATE efflux family (OG0000126) showed significant growth, potentially enhancing control over flavonoid metabolism crucial for seed and pollen development. Concurrent expansions in LEA proteins (OG0000163) and seed imbibition-related genes (OG0000308) suggest a coordinated evolution of mechanisms supporting seed viability and germination efficiency.

Heterosporous parent node:

For the parent of heterosporous ferns, we observed a parallel expansion of the MATE efflux family (OG0000126), mirroring the pattern seen in seed plants. This convergence hints at a possible shared mechanism in heterospory evolution. The expansion of universal stress proteins (OG0000287) indicates enhanced stress resilience, while growth in the homeobox gene family (OG0000717) suggests changes in the developmental regulation pathways.

Heterosporous Lycophyte parent node:

For the parent of heterosporous Lycophyte, the most prominent change was the expansion of homeobox genes (OG0000717). This growth implies an increased sophistication in gene regulation networks, potentially facilitating the complex life cycle transitions characteristic of heterosporous plants.

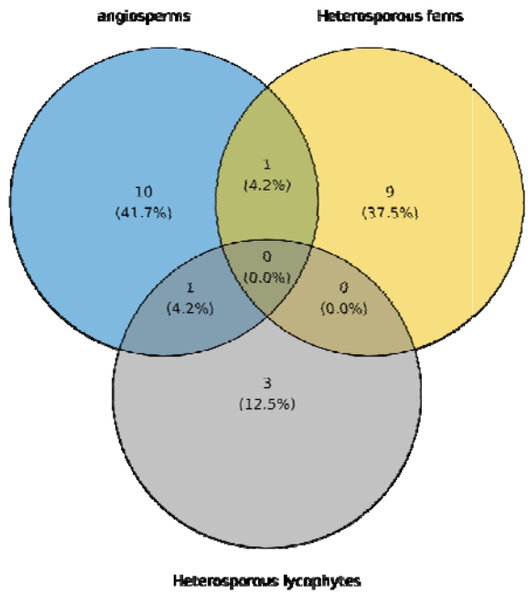

Fig 2: Distribution of Gene families expanding significantly on nodes leading to heterosporous lineages.

The dynamics of quantitative expansions/contractions across heterosporous lineages reveal a complex interplay of shared and lineage-specific genomic changes. We observed a consistent trend of expansions in gene families related to developmental regulation, signaling pathways, and stress responses across all three heterosporous groups. The recurrent expansion of certain families, such as MATE efflux and homeobox genes, across multiple lineages suggests potential common adaptive strategies in the evolution of heterospory. This pattern indicates that the transition to heterospory may be associated with increased genomic complexity in these functional categories, likely reflecting the need for more sophisticated regulation of reproductive processes and adaptations to new ecological niches. However, the unique expansion patterns in each group also highlight the diverse evolutionary paths to heterospory, underscoring the flexibility of genomic solutions to this reproductive innovation.

It's important to note that our analysis was limited to gene families with moderate levels of copy number variation due to computational constraints. Rapidly evolving gene families (CNV > 68) were excluded from this analysis, which may have led to the omission of some relevant genomic changes.

Selection Analysis Results

We employed the BUSTED-PH (Branch-Site Unrestricted Statistical Test for Episodic Diversification - Phenotype) method to investigate potential selection pressures on gene families in heterosporous lineages. This analysis complements our gene family expansion and contraction results, providing insights into the evolutionary forces acting on these genes.

Of the 54 gene families identified in our CAFE analysis, 51 were successfully analyzed using BUSTED-PH. Three families were excluded from this analysis due to challenges in producing uniform length sequence alignments, which are necessary for the BUSTED-PH method. This limitation highlights the computational and methodological challenges in analyzing rapidly evolving or highly divergent gene families.

Our analysis revealed diverse patterns of selection across the three heterosporous lineages: angiosperms (representing seed plants), heterosporous lycophytes, and heterosporous ferns. Tables 4-6 summarize the BUSTED-PH results for each lineage.

Type of Selection	Gene Families	Counts
Strong evidence of selection in the heterosporous phenotype	OG0000104, OG0000108, OG0000126	3
Selection in both heterosporous/homosporous, but with different regimes	OG0000050, OG0000163, OG0000183, OG0000233, OG0000304, OG0000308	6
Selection in both heterosporous/homosporous but with similar regimes	OG0000145, OG0000451	2
N/A (Misaligned codon translation)	OG0000634	1

Table 4: BUSTED-PH results for angiosperms

Type of Selection	Gene Families	Counts
Selection in both heterospory/homospory, but with different regimes	OG0000022, OG0000171, OG0000598	3
N/A (Misaligned codon translation)	OG0000634	1

Table 5: BUSTED-PH results for heterosporous lycophytes

Type of Selection	Gene Families	Counts
Strong evidence of selection in the heterosporous lineages	OG0000126, OG0000717, OG0000860, OG0001114, OG0002601	5
Selection in both	OG0000287, OG0000368,	3

heterosporous/homosporous, but with different regimes	OG0000399	
Evidence of selection with heterosporous but without a difference in selection regimes against homosporous lineages	OG0000857, OG0001616	2

Table 6: BUSTED-PH results for heterosporous ferns.

Selection Analysis Across Heterosporous Lineages

Our BUSTED-PH analysis revealed diverse patterns of selection across the three heterosporous lineages: angiosperms (representing seed plants), heterosporous ferns, and heterosporous lycophytes. In angiosperms, all 11 gene families with proper codon alignment showed some form of selection related to the heterospory phenotype. Specifically, three families (OG0000126, OG0000108, OG0000104) exhibited strong evidence of selection in the heterosporous lineage. Six families displayed different selection regimes between heterosporous and homosporous lineages, while two families (OG0000145, OG0000451) showed similar selection regimes across both lineage types. Notably, the MATE efflux family protein (OG0000126) demonstrated significant expansion and strong heterospory-specific selection, underscoring its potential crucial role in seed plant evolution.

In heterosporous ferns, analysis of eight gene families revealed similarly diverse selection patterns. Five families (OG0000126, OG0000717, OG0001114, OG0002601, OG0000860) showed strong evidence of heterospory-specific selection. Two families exhibited distinct selection regimes between heterosporous and homosporous lineages, while one family (OG0000857) showed selection in the heterosporous lineage without significant difference from homospory. Remarkably, the MATE efflux family protein (OG0000126) again exhibited both expansion and strong heterospory-specific selection, mirroring its pattern in angiosperms and suggesting a conserved role in heterospory evolution across plant lineages.

For heterosporous lycophytes, analysis of four gene families revealed a distinct selection pattern. Three families (OG0000022, OG0000171, OG0000598) exhibited selection in both heterosporous and homosporous lineages, but with different regimes. This suggests that while these gene families are under selection pressure in both lineage types, the nature of the selection differs between heterosporous and homosporous lycophytes. One family (OG0000634) could not be analyzed due to misaligned codon translation, an issue arising from significant evolutionary

divergence. Interestingly, the gene family OG0000171, encoding transcription factors with JmjC domains (likely involved in histone demethylation), showed significant expansion in our CAFE analysis and evidence of differential selection between heterosporous and homosporous lineages.

Comparing selection patterns across the three heterosporous lineages reveals several intriguing trends. The MATE efflux family protein (OG0000126) consistently showed strong evidence of heterospory-specific selection in both angiosperms and ferns, indicating a potentially conserved role in heterospory evolution. However, many gene families exhibited different selection patterns across lineages, suggesting that the genomic basis of heterospory may have evolved independently in each group. While most gene families in all lineages showed evidence of selection, the regimes often differed between heterosporous and homosporous lineages. This suggests that the nature of selection pressure may have shifted with the evolution of heterospory.

These findings provide evidence for both shared and lineage-specific genomic changes associated with the evolution of heterospory. The combination of gene family expansion and distinct selection pressures in heterosporous lineages suggests that the transition to heterospory may have involved significant genomic restructuring and adaptation, although the specific mechanisms vary across plant groups.

Targeted analysis results

Gene family expansion contractions

When CAFE5 was applied to our targeted subset of genes, only one gene family showed significant expansion on ancestral nodes shared by certain heterosporous lineages. Specifically, the gene family OG0000171 exhibited expansion on all ancestral heterosporous nodes, except the one common to the angiosperms. In other words, it saw significant expansion on nodes ancestral to both heterosporous ferns and lycophytes.

Functionally, this gene family encodes transcription factors with JmjC domains. These proteins are known to function as histone demethylases, playing crucial roles in epigenetic regulation. Notably, OG0000171 was also identified in our untargeted CAFE analysis as showing significant expansion in the heterosporous lycophyte lineage. Furthermore, our untargeted selection analysis revealed evidence of differential selection between heterosporous and homosporous lineages for this gene family.

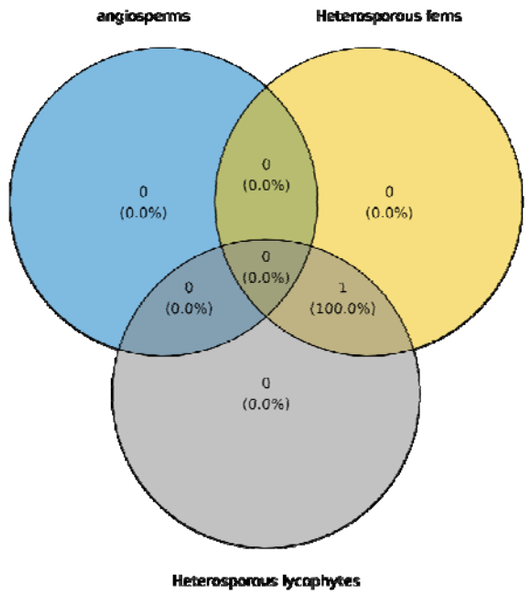

Figure 3: Distribution of targeted gene families expanding significantly on the ancestral nodes of heterosporous lineages.

Selection analysis

Classification	Families	Counts
Strong evidence of selection in the heterospory	OG0006212, OG0001910, OG0009354	3
Selection in both heterospory/homospory, but with different regimes	OG0001483, OG0004368	2
No evidence of selection with the heterospory	OG0002033, OG0006125, OG0004185	3
Evidence of selection with heterospory but without a difference in selection regimes against heterospory	OG0001960, OG0005057	2

Table7: Busted-PH results Cell cycling genes

Classification	Families	Counts
Strong evidence of selection in the heterospory	OG0006117, OG0003363, OG0002667, OG0003527, OG0003842, OG0003895, OG0002816, OG0001815, OG0004857, OG0003216, OG0004094, OG0003237, OG0003353, OG0001356, OG0003623	15
No evidence of selection with the heterospory	OG0003014, OG0007081, OG0003910, OG0004126, OG0003511, OG0001703, OG0000786, OG0004995, OG0006573, OG0001931	10
Selection in both heterospory/homospory, but with different regimes	OG0007348, OG0003283, OG0002641, OG0003831, OG0003952, OG0004130, OG0002000, OG0003152, OG0008222, OG0005403, OG0000662, OG0002436, OG0002660, OG0010423, OG0004443, OG0003796	16
Selection in both heterospory/homospory but with similar regimes	OG0007557, OG0013591, OG0002305, OG0003149, OG0001556, OG0006843, OG0003124	7
Evidence of selection with heterospory but without a difference in selection regimes against heterospory	OG0009805, OG0000295, OG0003886, OG0006752, OG0007124	5

Table7: Busted-PH results Chromatin genes

Classification	Families	Counts
Strong evidence of selection in the heterospory	OG0006665, OG0003365, OG0003350, OG0001741, OG0002005, OG0006506, OG0006866, OG0008219, OG0006588, OG0002021	10
No evidence of selection with the heterospory	OG0002639, OG0005946, OG0009490, OG0008479, OG0005865, OG0003510, OG0005125, OG0006812	8
Selection in both heterospory/homospory, but with different regimes	OG0006947, OG0003578, OG0001871	3
Evidence of selection with heterospory but without a difference in selection regimes against heterospory	OG0003738, OG0007082, OG0006944, OG0008868	4
Selection in both heterospory/homospory but with similar regimes	OG0006530	1

Table 8: Busted-PH results for Spindle fiber genes

Selection Analysis Results for Targeted Gene Families

Our targeted analysis focused on gene families involved in cell cycling, chromatin dynamics, and spindle fiber formation - processes we suspect are potentially crucial to chromosome behavior and reproductive mechanisms. The BUSTED-PH analysis revealed a complex landscape of selection pressures acting on these genes in heterosporous lineages, providing insights into the genomic underpinnings of this major evolutionary transition.

Given the complex nature of heterospory as a reproductive strategy, we hypothesized that its evolution might involve myriad changes across multiple cellular processes. Our results indeed reveal a diverse array of selection patterns across the analyzed gene families, consistent with this hypothesis.

Key findings from our analysis include:

- Heterospory-Specific Selection: A substantial number of gene families across all three categories showed strong evidence of selection specifically in heterosporous lineages. This pattern was particularly pronounced in chromatin-related genes, with nearly 30% of families in this category exhibiting heterospory-specific selection.
- Differential Selection Regimes: Many gene families showed evidence of selection in both heterosporous and homosporous lineages, but with different regimes. This pattern was most common in chromatin-related genes.
- Conservation: A notable proportion of gene families across all categories showed no evidence of selection associated with heterospory.
- Subtle Shifts: Some gene families showed evidence of selection in heterosporous lineages, but without significant differences from homosporous lineages.

Among the three categories, chromatin-related genes showed the highest proportion of families under heterospory-specific selection, followed by spindle fiber genes. Cell cycling genes exhibited the most diverse range of selection patterns.The observed patterns suggest a complex interplay of genomic changes associated with this reproductive transition, involving both conservation of core functions and adaptive changes in others.

Discussion

Our study explored the genomic underpinnings of heterospory evolution in plants, focusing on gene family dynamics and selection pressures. We identified significant expansions,

contractions, and selection pressures in gene families on branches leading to angiosperms, heterosporous ferns, and lycophytes. However, without functional verification, we cannot be certain about the connection between these computationally generated insights and their in vivo biological impact on meiosis or chromosome numbers.

Gene Family Dynamics

Our analysis revealed expansions in gene families related to developmental regulation, signaling pathways, and stress responses across all three heterosporous lineages. Many of these expanded families, particularly those involved in reproductive processes and hormone signaling, also showed evidence of selection. The specific families affected and the nature of selection pressures varied among lineages, indicating both shared and lineage-specific evolutionary paths to heterospory.

Selection Pressures and Chromatin Dynamics

The untargeted analysis highlighted a high proportion of chromatin and spindle fiber genes under heterospory-specific selection. This suggests significant changes in gene regulation and cell division mechanisms during heterospory evolution. Such changes could be critical for controlling gene expression during the development of distinct spore types, altering meiotic processes for megaspore and microspore production, and modifying chromosome behavior in gametophyte differentiation. The diverse selection patterns in cell cycling genes suggest a balance between innovation in reproductive strategies and the conservation of fundamental cellular processes.

Targeted Gene Family Insights

Our targeted approach identified one gene family (OG0000171), encoding JmjC domain-containing proteins involved in histone demethylation, showing significant expansion in heterosporous ferns and lycophytes. This family was also identified in our untargeted analysis, emphasizing the potential importance of epigenetic regulation in heterospory evolution.

The expansion and strong selection pressure on the MATE efflux family protein (OG0000126) in both seed plants and heterosporous ferns suggest a potentially conserved role in heterospory evolution. The expansion of stress-response-related gene families, particularly in heterosporous lycophytes, might indicate adaptations to new ecological niches, potentially facilitating the colonization of diverse terrestrial environments.

Implications for Chromosome Number Evolution

While we didn't directly observe genomic signals for genes involved in chromosome number regulation, the observed changes in developmental regulation, cellular signaling, and epigenetic modification genes could potentially influence meiotic processes or genome stability. This might relate to the tendency for reduced chromosome numbers in heterosporous plants, though this connection requires further investigation.

The complex pattern of selection across gene families suggests that the transition to heterospory was a multifaceted evolutionary process involving changes in various cellular systems. This aligns with the multiple independent origins of heterospory in plant evolution, indicating both common genomic changes and flexibility in achieving heterospory.

Study Limitations

Input

Our study has limitations. One of the most significant limitations is the nature of the data itself. Illustrated in Figure 4 and Figure 5 are BUSCO (Benchmarking Universal Single-Copy Orthologs) scores (Simão et al. 2015), a metric used to assess the completeness of genome assemblies by evaluating the presence of a set of highly conserved genes expected to be found in nearly all eukaryotes.

These illustrations show that our data has a wide range of completeness and areas needing improvement. Strictly speaking, for the data used here, absence of evidence is not evidence of absence. As seen in Figure 4 (density plot of "Missing percentage" BUSCO scores) and Figure 5 (density plot of "Complete percentage" BUSCO scores), while the availability of non-model plant genomic data has significantly improved in recent years, there is still room for further enhancement. The distribution of these scores highlights the progress made but also the challenges that remain in obtaining complete genomic data for diverse plant lineages.

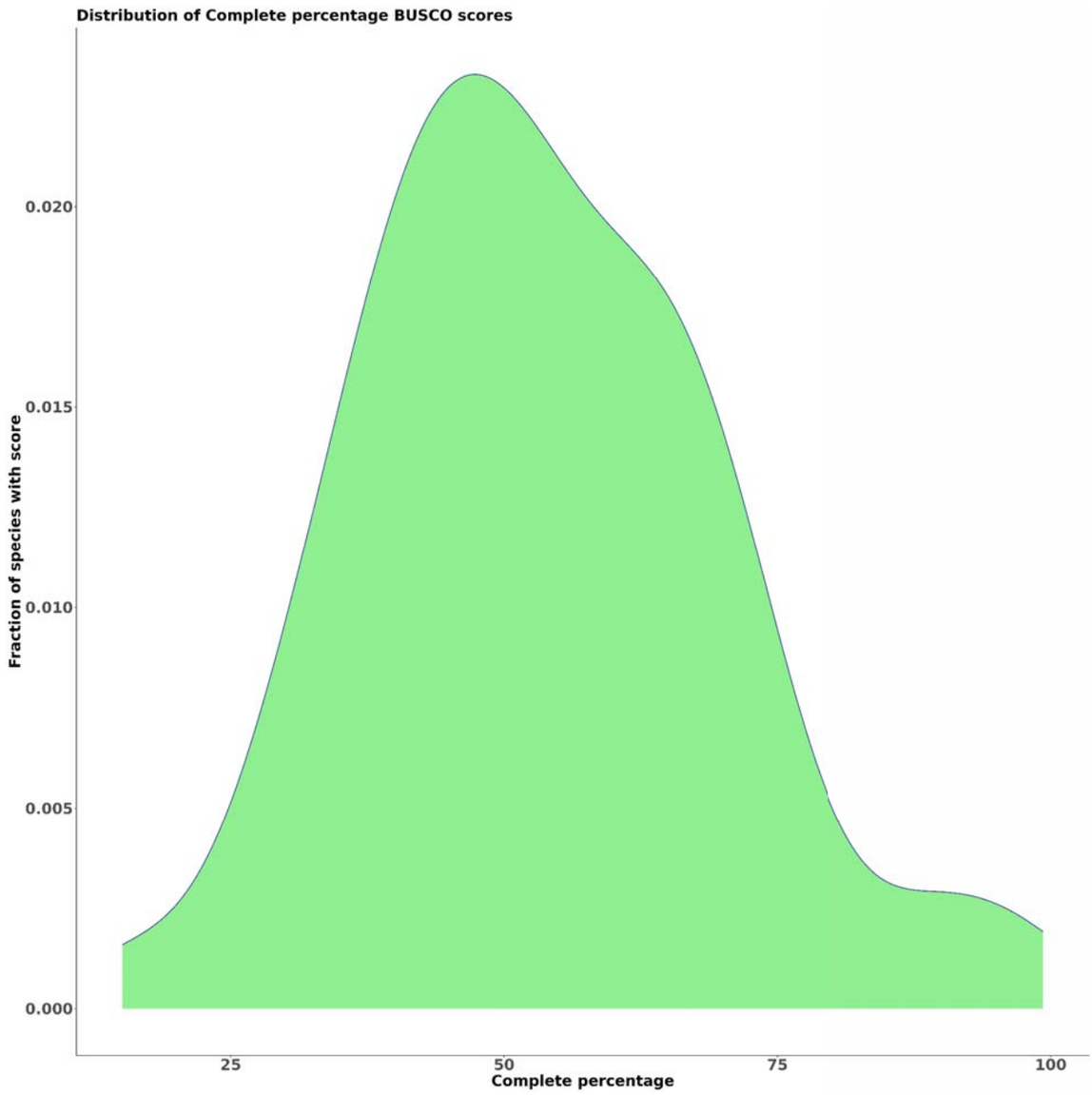

Figure 4: A density of the “Complete percentage” of BUSCO scores of the peptide data of our list of species.

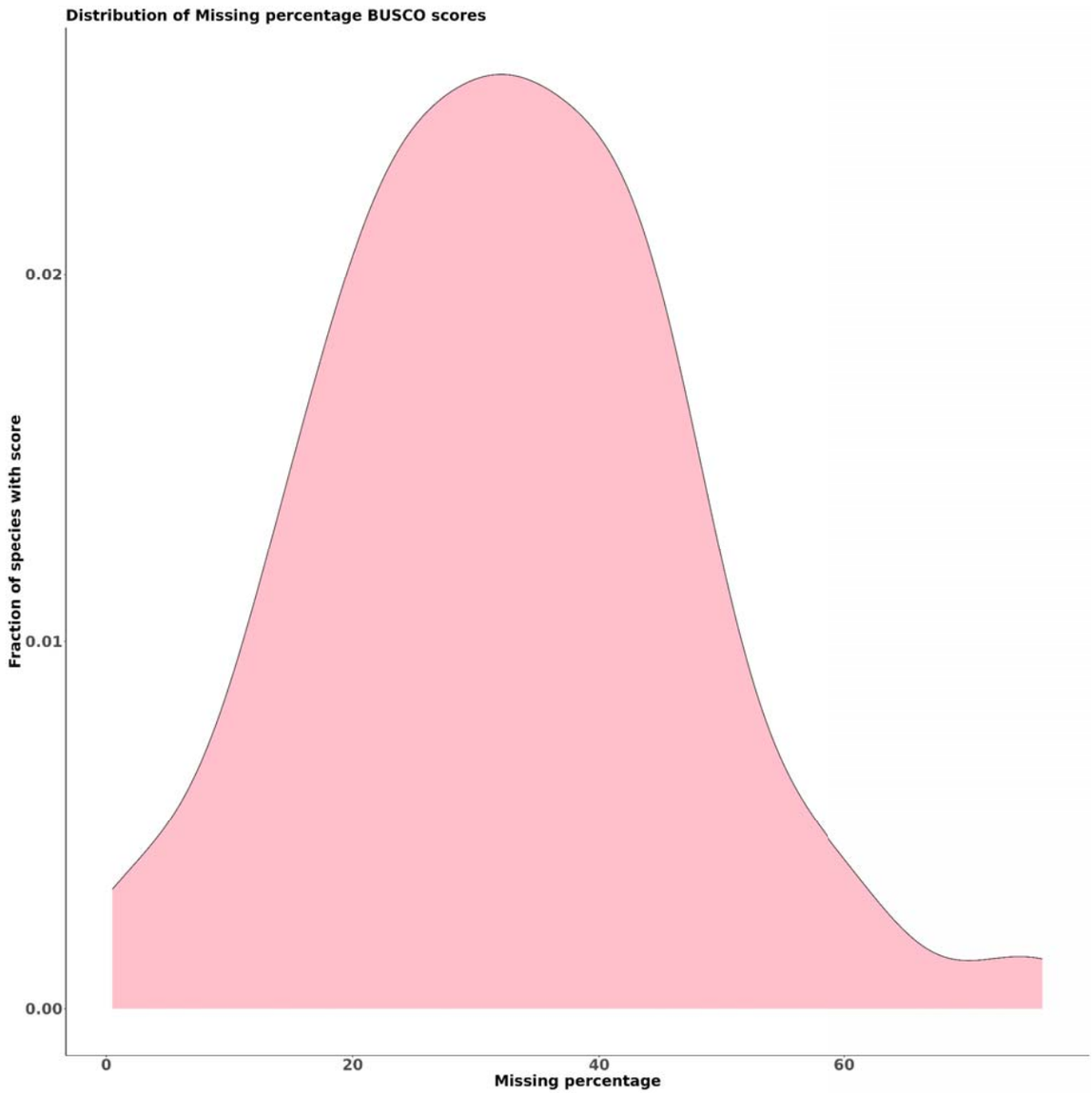

Figure 4: A density of the “Missing percentage” of BUSCO scores of the peptide data of our list of species.

Compute and algorithms

Computational constraints led to the exclusion of rapidly evolving gene families (CNV > 68) from the CAFE5 analysis. Reliance on *Arabidopsis thaliana* for functional annotation may have biased interpretations towards angiosperm-centric functions. The targeted approach, while focused, may have missed important gene families not included in our initial selection criteria.

Between our targeted and untargeted data processing, we observed a discrepancy in the quantitative outcome for OG0000171. In the untargeted CAFE5 analysis, OG0000171 was expanding only in heterosporous lycophytes, while in the targeted analysis, it significantly expanded in both heterosporous lycophytes and heterosporous ferns. This discrepancy highlights the differences between our targeted and untargeted approaches.The targeted approach allows for focused analysis of specific gene families, potentially increasing sensitivity to subtle changes. However, it depends heavily on existing functional annotations and prior hypotheses about

relevant genes, which can be a limitation, especially when studying non-model organisms or evolutionary innovations with poorly understood genetics. Conversely, the untargeted approach offers a broader view of genomic changes, capable of identifying patterns across the genome, but its comprehensive scope can sometimes overwhelm the algorithm in CAFE5. While both approaches found similar overall patterns, the untargeted approach showed attenuated results for certain gene families. This difference underscores the importance of cautious interpretation and suggests that more refined algorithms, better suited for this scale and depth of phylogenetics, could be beneficial in future studies.

Conclusions

We observed a not entirely unexpected outcome: a myriad interplay of quantitative and selective changes in various points across various lineages,generating novel insights about the mechanisms that underpin the evolution of heterospory at the genomic level. The consistent expansion and selection of certain gene families across heterosporous lineages, particularly those involved in developmental regulation, signaling pathways, and stress responses, suggest common adaptive strategies in the evolution of heterospory. The MATE efflux family, showing both expansion and strong selection pressure in multiple heterosporous lineages, emerges as a particularly interesting candidate for further study. This is in line with established theories,such as the Haig and Westoby hypothesis (Haig and Westoby 1988) of the evolution of heterospory. This model posits that heterospory evolved due to increasing vegetative complexity and competitive pressures in early terrestrial environments. As the conditions for the establishment of young sporophytes became more challenging, selection favored larger spores with greater nutrient reserves. This led to the evolution of a size threshold at which the production of small, male-only spores (microspores) alongside larger female spores (megaspores) became advantageous.Our genomic findings offer new molecular insights into this model. The observed expansions in gene families associated with hormone signaling and stress responses across heterosporous lineages align with the ecological shifts proposed by Haig and Westoby. These genetic changes may have facilitated the adaptive responses necessary for survival in more complex and competitive environments. For instance, the expansion of the MATE efflux family, which is involved in hormone transport and stress tolerance, may have played a crucial role in regulating the growth and development of both gametophytes and young sporophytes under challenging conditions.

Ecological studies investigating the relationship between habitat characteristics—such as vegetative complexity and environmental stressors—and the degree of heterospory in extant species could further empirically support the Haig and Westoby model. These studies can be complemented by functional genomic experiments targeting MATE gene families, focusing on the impact of mutations in these families on in vivo biology. Such experimentation would also enable a test for possible connections between MATE gene families and meiosis or chromosome numbers.

The expansion and selection of chromatin-related genes in relation to the phenotype of heterospory underscore the potential role of epigenetic regulation in plant evolution and adaptation. As immobile organisms, plants require a high degree of plasticity to cope with their surrounding environmental factors. This finding is consistent with the increasing recognition of epigenetic mechanisms as crucial players in significant evolutionary and adaptive transitions, especially the non-random nature of the inheritance of epigenetic adaptations (Miryeganeh and Saze 2020).

Future research should aim to bridge the gap between these genomic insights and the ecological and developmental aspects of heterospory evolution. Investigating the expression patterns and functions of the expanded gene families during spore and gametophyte development, particularly in relation to nutrient allocation and stress responses, would be beneficial. Additionally, comparative studies of spore development and early sporophyte establishment across species with varying degrees of heterospory could help elucidate how these genetic changes translate into adaptive advantages in different environments. By integrating our genomic findings with ecological and developmental approaches, we can advance our understanding of heterospory evolution. This comprehensive approach has the potential to clarify how genetic innovations facilitated the major life cycle transitions proposed by Haig and Westoby, thereby addressing longstanding questions about this pivotal innovation in plant evolutionary history.

While we didn't observe direct genomic signals for chromosome number regulation, the changes in developmental and epigenetic genes could potentially influence meiotic processes or genome stability. This indirect link provides a new perspective on the longstanding question of reduced chromosome numbers in heterosporous plants.

The differences in genomic changes observed among angiosperms, ferns, and lycophytes, despite their shared emergence of heterospory, reflect the flexibility in genomic pathways that can lead to this reproductive innovation.

Our study highlights both the power and limitations of large-scale comparative genomic approaches. While we identified broad patterns across diverse plant lineages, computational constraints and reliance on model organism annotations posed significant challenges. These limitations underscore the need for more powerful computational tools and more comprehensive functional annotations across non-model organisms.

Despite methodological constraints, our study has yielded insights into the genomic underpinnings of heterospory evolution. It demonstrates the potential of large-scale comparative genomics to address fundamental questions in plant evolution, while also revealing areas where methodological improvements are needed. Future studies will benefit from an interplay between more targeted questions and more powerful algorithms, as well as the increased computational power and expanded genomic data that will inevitably become available. This synergy between biological insight and methodological advancement will be crucial for deepening our understanding of major evolutionary transitions in plants and their implications for plant biology.

Future Directions

The objective of this study was to leverage contemporary data, algorithms, and methodologies to explore novel insights into a longstanding enigma in plant biology. Accordingly, we have identified a list of genes that represent promising candidates for future experimental validation. Subsequent investigations could focus on the functional characterization of the identified gene families, especially those exhibiting both expansion and strong selection pressure. These studies may yield deeper insights into their specific roles in the development and maintenance of heterospory.

Future research would benefit from the development of more sophisticated algorithms capable of handling rapidly evolving gene families without computational bottlenecks. This could include improvements to existing tools like CAFE5 or the creation of new tools better suited to handle extreme variations in gene family size across evolutionary time.

Advancements in sequence alignment techniques, especially those more sensitive to gene families evolving over longer time scales, could significantly enhance our ability to detect and analyze genomic changes associated with major evolutionary transitions like heterospory.

While integrating other types of data such as metabolomics or detailed phenotypic information could theoretically provide a more comprehensive picture of heterospory evolution, the practical challenges of obtaining such data across diverse plant lineages are considerable. In the interim, comparative studies focusing on the genomic regions surrounding these gene families could investigate potential regulatory changes that might be driving the observed patterns of gene family evolution and selection.

Ongoing advancements in the efficiency, accuracy, and cost-effectiveness of next-generation sequencing (NGS) and computational technologies are likely to enhance the results of the experimental design used in this study in future iterations. Improvements in NGS technology will increase the availability of genomic data for non-model plants, while advancements in computational tools should enable the implementation of more sophisticated phylogenetic algorithms.

References

Barker, Michael S. 2009. “Evolutionary Genomic Analyses of Ferns Reveal That High Chromosome Numbers Are a Product of High Retention and Fewer Rounds of Polyploidy Relative to Angiosperms.” *American Fern Journal* 99 (2): 136–41.

Bateman, Richard M., and William A. DiMichele. 1994. “Heterospory: The Most Iterative Key Innovation in the Evolutionary History of the Plant Kingdom.” *Biological Reviews of the Cambridge Philosophical Society* 69 (3): 345–417.

Carta, Angelino, Gianni Bedini, and Lorenzo Peruzzi. 2020. “A Deep Dive into the Ancestral Chromosome Number and Genome Size of Flowering Plants.” *The New Phytologist* 228 (3): 1097–1106.

Clark, James, Oriane Hidalgo, Jaume Pellicer, Hongmei Liu, Jeannine Marquardt, Yannis Robert, Maarten Christenhusz, et al. 2016. “Genome Evolution of Ferns: Evidence for Relative Stasis of Genome Size across the Fern Phylogeny.” *The New Phytologist* 210 (3): 1072–82.

Edgar, Robert C. 2004. “MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput.” *Nucleic Acids Research* 32 (5): 1792–97.

Emms, David M., and Steven Kelly. 2017. “STRIDE: Species Tree Root Inference from Gene Duplication Events.” *Molecular Biology and Evolution* 34 (12): 3267–78.

———. 2019. “OrthoFinder: Phylogenetic Orthology Inference for Comparative Genomics.” *Genome Biology* 20 (1): 238.

Emms, D. M., and S. Kelly. 2018. “STAG: Species Tree Inference from All Genes.” *bioRxiv*. https://doi.org/10.1101/267914.

Goodstein, David M., Shengqiang Shu, Russell Howson, Rochak Neupane, Richard D. Hayes, Joni Fazo, Therese Mitros, et al. 2012. “Phytozome: A Comparative Platform for Green Plant Genomics.” *Nucleic Acids Research* 40 (Database issue): D1178–86.

Haig, David, and Mark Westoby. 1988. “A Model for the Origin of Heterospory.” *Journal of Theoretical Biology* 134 (2): 257–72.

Haufler, Christopher H. 2014. “Ever since Klekowski: Testing a Set of Radical Hypotheses Revives the Genetics of Ferns and Lycophytes.” *American Journal of Botany* 101 (12): 2036–42.

Haufler, C. H., and D. E. Soltis. 1986. “Genetic Evidence Suggests That Homosporous Ferns with High Chromosome Numbers Are Diploid.” *Proceedings of the National Academy of Sciences of the United States of America* 83 (12): 4389–93.

Hickok, Leslie G., and Edward J. Klekowski. 1973. “Abnormal Reductional and Nonreductional Meiosis in Ceratopteris: Alternatives to Homozygosity and Hybrid Sterility in Homosporous Ferns.” *American Journal of Botany* 60 (10): 1010–22.

Katoh, Kazutaka, Kazuharu Misawa, Kei-Ichi Kuma, and Takashi Miyata. 2002. “MAFFT: A Novel Method for Rapid Multiple Sequence Alignment Based on Fast Fourier Transform.” *Nucleic Acids Research* 30 (14): 3059–66.

Kinosian, Sylvia P., Carol A. Rowe, and Paul G. Wolf. 2022. “Why Do Heterosporous Plants Have So Few Chromosomes?” *Frontiers in Plant Science* 13. https://doi.org/10.3389/fpls.2022.807302.

Klekowski, E. J., Jr, and H. G. Baker. 1966. “Evolutionary Significance of Polyploidy in the Pteridophyta.” *Science* 153 (3733): 305–7.

Marchant, D. Blaine, Guang Chen, Shengguan Cai, Fei Chen, Peter Schafran, Jerry Jenkins, Shengqiang Shu, et al. 2021. “Dynamic Genome Evolution in a Model Fern.” *Nature Plants* 8 (9): 1038–51.

Mayrose, Itay, and Martin A. Lysak. 2021. “The Evolution of Chromosome Numbers: Mechanistic Models and Experimental Approaches.” *Genome Biology and Evolution* 13 (2). https://doi.org/10.1093/gbe/evaa220.

McGinnis, Scott, and Thomas L. Madden. 2004. “BLAST: At the Core of a Powerful and Diverse Set of Sequence Analysis Tools.” *Nucleic Acids Research* 32 (Web Server issue): W20–25.

Mendes, Fábio K., Dan Vanderpool, Ben Fulton, and Matthew W. Hahn. 2020. “CAFE 5 Models Variation in Evolutionary Rates among Gene Families.” *Bioinformatics* , December. https://doi.org/10.1093/bioinformatics/btaa1022.

Minh, Bui Quang, Heiko A. Schmidt, Olga Chernomor, Dominik Schrempf, Michael D. Woodhams, Arndt von Haeseler, and Robert Lanfear. 2020. “IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era.” *Molecular Biology and Evolution* 37 (5): 1530–34.

Miryeganeh, Matin, and Hidetoshi Saze. 2020. “Epigenetic Inheritance and Plant Evolution.” *Population Ecology* 62 (1): 17–27.

Murrell, Ben, Steven Weaver, Martin D. Smith, Joel O. Wertheim, Sasha Murrell, Anthony Aylward, Kemal Eren, et al. 2015. “Gene-Wide Identification of Episodic Selection.” *Molecular Biology and Evolution* 32 (5): 1365–71.

One Thousand Plant Transcriptomes Initiative. 2019. “One Thousand Plant Transcriptomes and the Phylogenomics of Green Plants.” *Nature* 574 (7780): 679–85.

Pelosi, Jessie A., Emily H. Kim, W. Brad Barbazuk, and Emily B. Sessa. 2022. “Phylotranscriptomics Illuminates the Placement of Whole Genome Duplications and Gene Retention in Ferns.” *Frontiers in Plant Science* 13 (July): 882441.

Rhee, Seung Yon, William Beavis, Tanya Z. Berardini, Guanghong Chen, David Dixon, Aisling Doyle, Margarita Garcia-Hernandez, et al. 2003. “The Arabidopsis Information Resource (TAIR): A Model

Organism Database Providing a Centralized, Curated Gateway to Arabidopsis Biology, Research Materials and Community." *Nucleic Acids Research* 31 (1): 224–28.

Ruhfel, Brad R., Matthew A. Gitzendanner, Pamela S. Soltis, Douglas E. Soltis, and J. Gordon Burleigh. 2014. "From Algae to Angiosperms-Inferring the Phylogeny of Green Plants (Viridiplantae) from 360 Plastid Genomes." *BMC Evolutionary Biology* 14 (February): 23.

Simão, Felipe A., Robert M. Waterhouse, Panagiotis Ioannidis, Evgenia V. Kriventseva, and Evgeny M. Zdobnov. 2015. "BUSCO: Assessing Genome Assembly and Annotation Completeness with Single-Copy Orthologs." *Bioinformatics* 31 (19): 3210–12.

Suyama, Mikita, David Torrents, and Peer Bork. 2006. "PAL2NAL: Robust Conversion of Protein Sequence Alignments into the Corresponding Codon Alignments." *Nucleic Acids Research* 34 (Web Server issue): W609–12.

Wang, Qing-Hua, Jian Zhang, Yang Liu, Yu Jia, Yuan-Nian Jiao, Bo Xu, and Zhi-Duan Chen. 2022. "Diversity, Phylogeny, and Adaptation of Bryophytes: Insights from Genomic and Transcriptomic Data." *Journal of Experimental Botany* 73 (13): 4306–22.

Wickett, Norman J., Siavash Mirarab, Nam Nguyen, Tandy Warnow, Eric Carpenter, Naim Matasci, Saravanaraj Ayyampalayam, et al. 2014. "Phylotranscriptomic Analysis of the Origin and Early Diversification of Land Plants." *Proceedings of the National Academy of Sciences of the United States of America* 111 (45): E4859–68.

Yu, Guangchuang. 2020. "Using Ggtree to Visualize Data on Tree-Like Structures." *Current Protocols in Bioinformatics / Editoral Board, Andreas D. Baxevanis ... [et Al.]* 69 (1): e96.