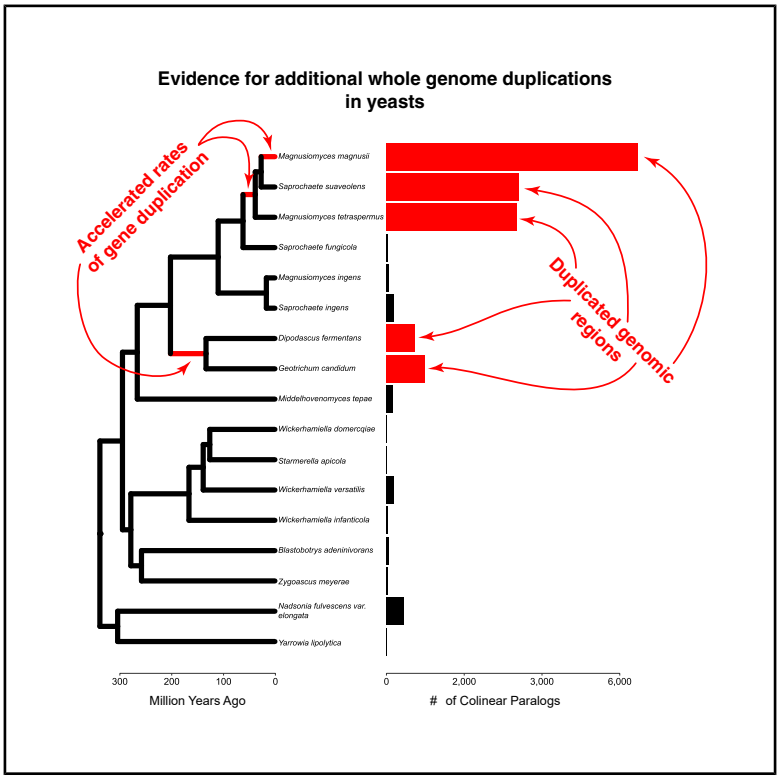
Report

Current Biology

Discovery of additional ancient genome duplications in yeasts

Graphical abstract



Highlights

- Evidence for three whole-genome duplications (WGDs) in Dipodascales yeasts
- Impacts are broad but share features with the known WGD in Saccharomycetales
- Duplications within metabolic and signaling networks are more likely to be retained
- Ancient WGD in fungi is likely underreported

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In brief

David et al. report evidence for at least two additional ancient whole-genome duplication events in Saccharomycotina yeasts, challenging the idea that such events are extremely rare in fungi.

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Report

Discovery of additional ancient genome duplications in yeasts

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SUMMARY

Whole-genome duplication (WGD) has had profound macroevolutionary impacts on diverse lineages,[1,2] preceding adaptive radiations in vertebrates,[3–5] teleost fish,[6,7] and angiosperms.[8–10] In contrast to the many known ancient WGDs in animals,[11,12] and especially plants,[13–15] we are aware of evidence for only four WGDs in fungi.[16,17] The oldest of these occurred ~100 million years ago (mya) and is shared by ~60 extant Saccharomycetales species,[18,19] including the baker's yeast *Saccharomyces cerevisiae*. Notably, this is the only known ancient WGD event in the yeast subphylum Saccharomycotina. The dearth of ancient WGD events in fungi remains a mystery.[16] Some studies have suggested that fungal lineages that experience chromosome[20] and genome[16] duplication quickly go extinct, leaving no trace in the genomic record, while others contend that the lack of known WGDs is due to an absence of data.[16,17] Under the second hypothesis, additional sampling and deeper sequencing of fungal genomes should lead to the discovery of more WGD events. Coupling hundreds of recently published genomes from nearly every described Saccharomycotina species, with three additional long-read assemblies, we discovered three novel WGD events. Although the functions of retained duplicate genes originating from these events are broad, they bear similarities to the well-known WGD that occurred in the Saccharomycetales.[18] Our results suggest that WGD may be a more common evolutionary force in fungi than previously believed.

RESULTS AND DISCUSSION

Evidence for three WGD events in the Dipodascales

To detect signatures of ancient whole-genome duplication (WGD), we first used the gene/species tree reconciliation algorithm implemented in OrthoFinder v3.0.1b1[21] to obtain a preliminary estimate of gene duplication across the Saccharomycotina phylogeny. High rates of gene duplication along specific lineages of the species tree can be indicative of WGD events and have been used to identify ancient WGDs in plants,[22–24] animals,[25] and fungi.[26] An initial analysis using a 400-species backbone phylogeny (Figure S1) successfully recovered the known ancient WGD near the base of Saccharomycetales (labeled WGD1). This lineage possessed the second-highest rate of duplications, over 10× higher than the average internal lineage. However, the lineages with the first and third highest rates of duplication occurred in the Dipodascales, a clade separated from the Saccharomycetales by ~300 million years (Ma) of evolution, prompting further investigation.

To improve the resolution of gene family evolution within both clades, we increased our sampling in Saccharomycetales (135 genomes) and Dipodascales (184 genomes) and re-performed the gene/species tree reconciliation analysis. The lineage containing WGD1 was again successfully identified by a spike in duplication rate, experiencing 53.1 gene duplications/Ma (Figure 1). Two internal spikes were also evident in Dipodascales. The most recent ancestral lineage to the genera *Dipodascus* and *Geotrichum* (spanning from 201–132 mya) had a duplication rate of 21.8 duplications/Ma, leading us to hypothesize that a WGD may have occurred along this lineage (labeled WGD2). Similarly, the most recent ancestral lineage to the species *Magnusiomyces tetraspermus* and *Saprochaete suaveolens* (spanning from 62–38 mya) had a duplication rate of 23.62 duplications/Ma (labeled WGD3) (Figure 1). Additionally, the highest species-specific duplication rate by far belonged to *M. magnusii*, with 130 gene duplications/Ma, 2.4× higher than any other Dipodascales species. *M. magnusii* also possesses the largest known

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Figure 1. Three additional whole-genome duplications in Saccharomycotina yeasts

Rates of gene duplication are mapped onto Saccharomycetales (left) and Dipodascales (right) phylogenies. Whole-genome duplications are indicated along the lineages in which they are predicted to occur. Trees have been pruned for visualization purposes. See Figure S1 for rates of gene duplication across the full backbone phylogeny and Figure S3 for a comparison between OrthoFinder and AleRax.

Saccharomycotina genome (43.2 Mb, 3.4× larger than *S. cerevisiae*), leading us to hypothesize another WGD (labeled WGD4, spanning from 27 to 0 mya) specific to this species alone.

Although gene/species tree reconciliation approaches are useful in identifying putative WGDs, they are limited in their ability to distinguish true WGD from bouts of single or segmental gene duplication events.[27] Additionally, reconciliation approaches are generally not sensitive to biological processes, such as gene conversion or unbalanced paralog retention (e.g., via paralog loss in outgroup taxa) that can cause duplication events to appear younger.[28] Such is likely the case in our dataset, where both WGD2 and WGD3 are directly ancestral to lineages with similarly elevated rates of duplication (Figure 1). High duplication rates are therefore necessary, but not sufficient, for WGD inference or for the confident identification of the timing of WGD on a phylogeny.

A complementary approach to identify WGD involves the detection of colinear segments of paralogs (called multiplicons).[29,30] Many multiplicons distributed throughout a genome are difficult to explain without invoking WGDs and provide compelling evidence.[1,2] However, such analysis requires highly contiguous assemblies. Assemblies with fewer than 100 contigs were available for one post-WGD2 (*G. candidum*: 28 contigs) and one post-WGD3 (*Sap suaveolens*: 12 contigs) species. To generate additional evidence for hypothesized WGDs and their placements on the yeast phylogeny, we re-sequenced three additional species: *D. fermentans* for WGD2 (19 contigs), *M. tetraspermus* for WGD3 (33 contigs), and *M. magnusii* for WGD4 (71 contigs). These five genomes, along with 81 other highly contiguous Saccharomycotina genome assemblies (Data S1), were then searched for multiplicons (Figure 2A).

Of the 86 highly contiguous genomes, every species predicted to have experienced a WGD had more colinear paralogs than those that did not (Figure 2A). As expected, cumulative multiplicon size negatively scaled with age. The genome predicted to have experienced the most recent WGD4 had the largest sum of total multiplicons (6,459 paralogs), followed by the two post-WGD3 genomes (3,409 and 3,352 paralogs; Figure 2A). Between the two oldest WGD events, post-WGD2 genomes retained more duplicates on average (865.5 paralogs) than post-WGD1 genomes (737.8 paralogs). By contrast, the average non-WGD genome possessed 46.5 colinear paralogs. Interestingly, whereas multiplicons for most post-WGD species occurred in duplicates, in *M. magnusii*, they appear in tetraplicate (Figure 2B), providing further evidence for WGD4 as an event specific to this species. WGD4 is also supported by examination of multiplicon size in *M. magnusii*, which is nearly twice (189.5%) that of sister species *Sap suaveolens*.

Multiplicons between *D. fermentans* and *G. candidum* had many orthogroups in common, as did *Sap suaveolens*, *M. tetraspermus*, and *M. magnusii* (Figure 2B). However, multiplicons between post-WGD2 and post-WGD3 species (*D. fermentans* and *Sap suaveolens*, for example) contained largely distinct orthogroups (Data S2). Of all possible cross-species multiplicon pairs that shared a Jaccard similarity index of at least 0.25 with respect to orthogroup membership, only 1.7% were between species descended from different WGD events, consistent with our inference that WGD2 and WGD3 represent two distinct events (Figure 1).

Another common hallmark of ancient WGD is large increases in the number of chromosomes across lineages.[31] However, fungi possess small, loosely packed chromosomes, which make karyotyping difficult.[32] Furthermore, Saccharomycotina yeasts have extremely diverse telomeres[33,34] and

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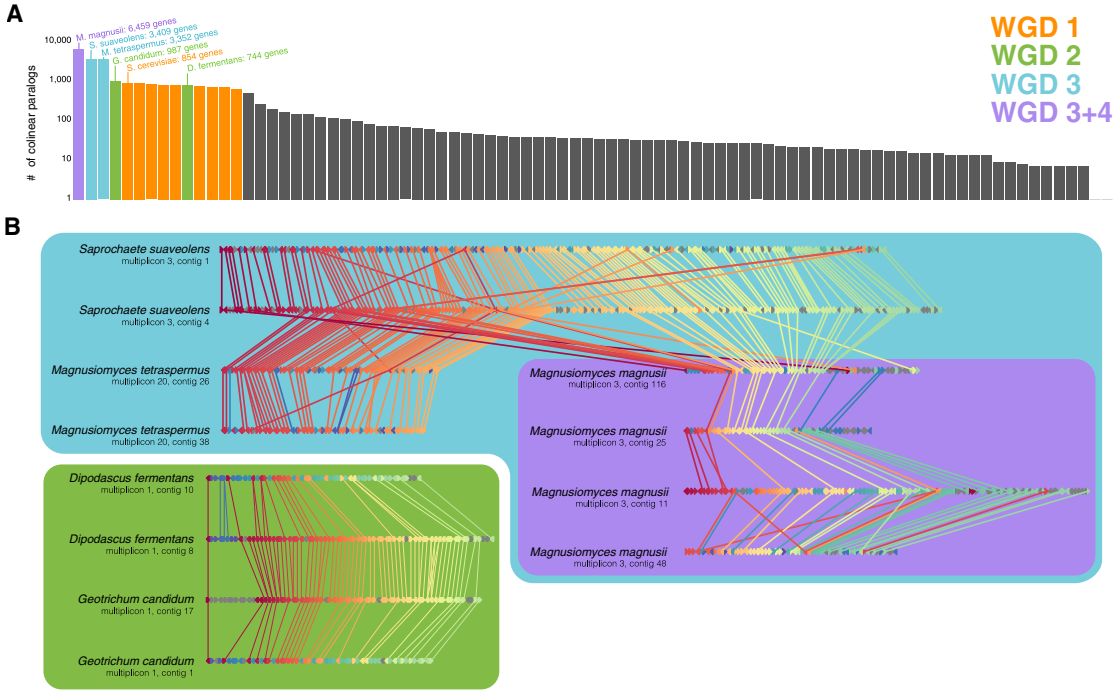

Figure 2. Conserved gene order between duplicated segments (multiplicons) within a genome provides compelling evidence for WGD

(A) Cumulative multiplicon size of each highly contiguous Saccharomycotina assembly, as measured by the number of unique paralogs. Genomes predicted to have undergone ancient WGD are highlighted.

(B) Gene order of select multiplicons. Gene order within contigs is represented by triangles, colored by orthogroup. Homology is indicated by lines drawn between genes. Orthogroups that only appear once are depicted in gray. For a full comparison of shared orthogroups between multiplicons, see Data S2. For comparison of individual ohnolog pairs, see Data S3.

centromeres,[35,36] which makes chromosome number estimation challenging even for highly contiguous assemblies. Despite these obstacles, chromosome count estimates from several Dipodascales species exist, including one representative from each proposed WGD. For species not inferred to have undergone WGD, count estimates range from four[37] to five[38,39] chromosomes. This estimate increases to seven chromosomes for the post-WGD3 species *Sap suaveolens*[40] and eight[41] or nine[42] for the post-WGD2 species *G. candidum*. Finally, 13 chromosomes have been inferred from pulsed field gel electrophoresis for *M. magnusii*,[43] which is predicted to have undergone two rounds of WGD (both WGD3 and WGD4). Therefore, although limited chromosome count estimates in Dipodascales preclude a formal analysis, we interpret the available data to be consistent with our conclusions (Table 1).

WGD can occur either within lineages (autopolyploidization) or between lineages via hybridization[26,44] (allopolyploidization). WGD1 was originally hypothesized to be an autopolyploidization event due to the highly conserved gene order within descendant genomes.[19] However, a gene/species tree reconciliation analysis later recovered a lineage pre-dating WGD1 with a higher rate of gene duplications than the WGD1 lineage itself.[26] The authors posit that this unusual finding could be explained by gene tree discordance produced through hybridization,[26] which suggests allopolyploidization. As our reconciliation analysis recovered no such peak in gene duplication rates preceding any of the novel WGD events (Figure 1), we hypothesize that these events occurred either through autopolyploidization or through allopolyploidization between closely related parent species.

In contrast to ancient WGD, which was presumed to be extremely rare in Saccharomycotina, recent allopolyploidization

Table 1. Chromosome count estimates

Species	Chromosome count	Method	Reference
Magnusiomyces capitatus	4	PFGE	Brejová et al.[37]
Magnusiomyces ingens	4	PFGE	Brejová et al.[37]
Saprochaete fungicola	5	long-read sequencing	Brejová et al.[38]
Saprochaete ingens	5	long-read sequencing	Hodorová et al.[39]
Saprochaete suaveolens	7	long-read sequencing	Lichancová et al.[40]
Geotrichum candidum	8	PFGE	Gente et al.[41]
Galactomyces reessii	9	PFGE	Naumova et al.[42]
Magnusiomyces magnusii	13	PAGE	Filipp et al.[43]

Chromosome counts for available species within the Dipodascales.

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events are quite common. Polyploidy is a frequent outcome of hybridization between extant taxa, including several industrial strains.[45–50] To prevent these events from being mistaken for ancient WGD, no known hybrids were included in the dataset. However, as a relatively young event, WGD4 bears many similarities to recent allopolyploidization events. WGD4 is specific to just one species, and sequence identity between ohnologs is much greater (82%) than older WGDs in the Dipodascales (WGD2, 46%; WGD3, 69%) (Data S3). Therefore, further investigation is warranted to better elucidate the timing and mechanism of WGD4 to determine whether it represents a recent allopolyploidization of a cryptic hybrid or a true "ancient" WGD.

Functional consequences of ancient genome duplication in yeasts

WGD is often referred to as an engine of evolutionary innovation,[2,4] preceding jumps in phenotypic complexity in plants[51] and animals.[5] In fungi, it has been hypothesized that WGD1 facilitated aerobic glycolysis in several yeast species.[52,53] Also known as the "Crabtree/Warburg effect," this biochemical process empowers baking, brewing, and winemaking. However, others have noted that some species that have not undergone WGD1 are still capable of aerobic glycolysis to a lesser extent.[54,55] As this example illustrates, identifying cause-effect relationships from rare evolutionary events, such as WGD, is exceedingly difficult.[56,57] The discovery of additional WGD events in Saccharomycotina affords new opportunities for understanding how WGD events contribute to evolutionary innovation.

To test whether certain functional gene classes were more likely to be retained in duplicate following WGD, and if these classes were convergently shared across events, we ran enrichment analysis on retained ohnologs for each event. Notably, WGD4 ohnologs were significantly enriched (adjusted $p < 0.05$) for only five InterPro Gene Ontology[58] categories (Data S4) and for none of the Kyoto Encyclopedia of Genes and Genomes[59] (KEGG) pathways (Data S5). As 76.6% of all *M. magnusii* genes are ohnologs, we hypothesize that not enough time has passed since WGD4 for genes to become lost and patterns within retained copies to emerge. As discussed above, it is not yet known whether WGD4 represents a truly ancient WGD or a recent allopolyploidization. Therefore, we turn our attention to the three older WGD1, WGD2, and WGD3 (WGD1,2,3 for short) for the remainder of this discussion.

The functional effects of WGD appeared widespread. Ohnologs were significantly enriched (adjusted $p < 0.01$) in at least six of the seven main KEGG pathway categories across WGD1,2,3 (Data S5). Despite the diversity of affected pathways, commonalities remain. Four related metabolic pathways were significantly enriched (adjusted $p < 0.01$) in one of the three WGD events (Data S5). For example, WGD2 ohnologs were enriched in the propanoate metabolic pathway. Propanoate esters and other related compounds are responsible for the characteristic fruity scent of *G. candidum*,[60] a popular yeast in cheesemaking,[61] as well as one of the major natural flavorants of cocoa.[62,63] Many other *Geotrichum*/*Dipodascus* species are similarly known for their flavor-enhancing properties.[64]

As mentioned above, the metabolic effects of WGD1 have been studied previously.[52–54,65] Enzymes and hexose transporters involved in glycolysis have been preferentially retained in post-WGD1 species, in particular those genes whose dosage most greatly increases glycolytic flux.[65] We find that all ten of these genes have been retained in duplicate from at least one of the three newly reported WGD events, even those not retained from WGD1. Three gene families, *HXT*, *ENO*, and *PYK*, were retained across all four events. The enzyme products of *ENO* and *PYK* catalyze the last two steps of glycolysis, whose overexpression greatly increases glycolytic rates.[55,66] *HXT* is also notable, as dosage of these hexose transporter-encoding genes has the single biggest positive impact on glycolytic flux by a wide margin.[65]

It has also been hypothesized that WGD1 coincided with the rise of angiosperms and the increased availability of simple sugars found in fruit and nectar.[18] Our discovery of multiple WGDs reveals that these events are not tied to specific time periods. However, we do find that WGD events are associated with metabolic specialism in yeasts. Post-WGD species metabolize significantly fewer substrates than species that have not undergone WGD (phylogenetic ANOVA $p < 2.2e{-}16$), a result that persists even if WGD1 taxa are excluded (phylogenetic ANOVA $p = 1e{-}3$). We hypothesized that additional gene copies in pre-existing metabolic pathways produced by WGD events facilitate higher-throughput metabolic output on glucose, reducing evolutionary pressure to metabolize alternative substrates. However, we found that post-WGD species have significantly *reduced* growth rates on the simple sugars mannose (phylogenetic ANOVA $p = 4.6e{-}2$) and fructose (phylogenetic ANOVA $p = 1.6e{-}3$), while differences on glucose were insignificant (phylogenetic ANOVA $p = 0.9$). Clearly, more work is required to disentangle the effects of WGD on metabolic rate, growth rate, and niche breadth, an effort we hope will be aided by the discovery of these additional events.

Although metabolic pathways were differentially enriched across WGD events, other pathways exhibited stronger signatures of convergence. In particular, several signaling pathways were consistent beneficiaries of WGD. The mitogen-activated protein kinase (MAPK), glucagon, and insulin signaling pathways were all significantly enriched (adjusted $p < 0.01$) across WGD1,2,3 (Data S5; Figures 3 and S2). MAPK is an ancient family of kinases that drive phosphorylation cascades, which trigger a variety of cellular mechanisms of *S. cerevisiae* in response to stressful conditions, such as high osmolarity,[67] damage to the cell wall,[68] or starvation.[69] Although fungi do not naturally produce glucagon or insulin, these general pathways are deeply conserved across eukaryotes.[70,71] These pathways contain protein kinases such as AMPK and PKA, which serve important nutrient-sensing functions and trigger cellular responses accordingly.[72,73] Both AMPK and PKA contain duplicate genes from all four WGD events (Figure S2).

Conclusion

We report the presence of three previously unknown WGD events, denoted WGD2, WGD3, and WGD4 in the Dipodascales clade. Despite ~300 Ma of evolution separating them, WGD2 and WGD3 share similar outcomes with the previously known WGD1 that occurred in Saccharomycetales, as well as other WGDs across eukaryotes. In particular, ohnologs involved in signal transduction were significantly retained across all confirmed ancient WGDs. Genes with many protein-protein

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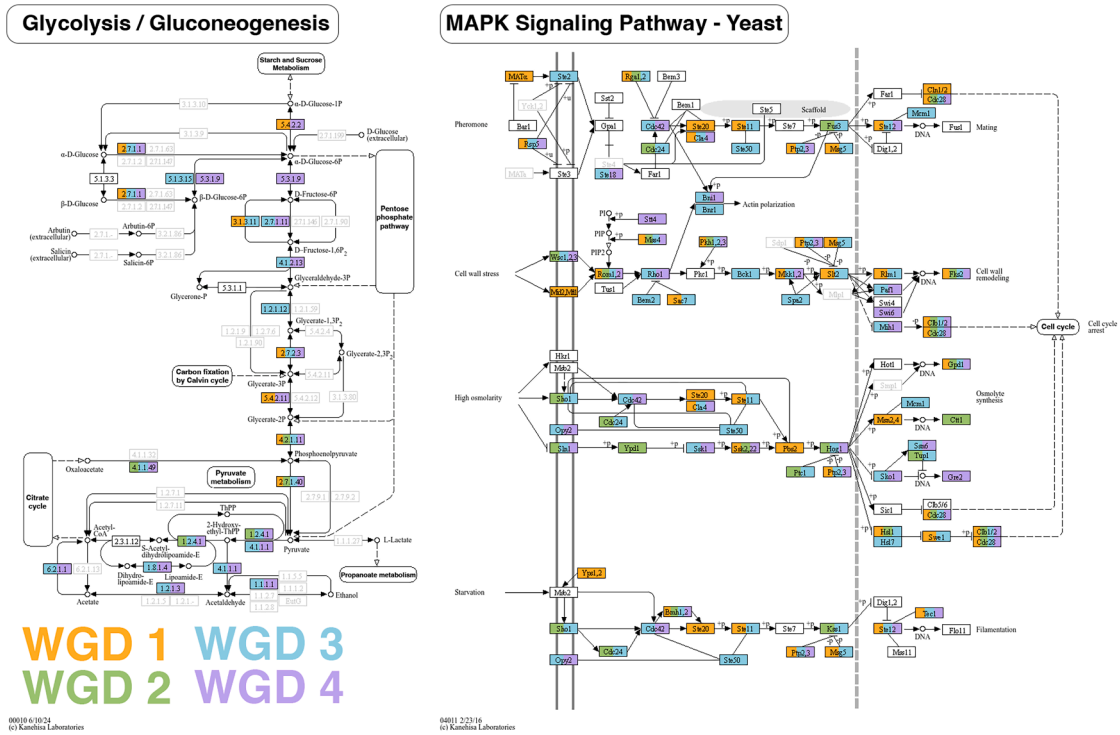

Figure 3. Impacts of whole-genome duplication in two exemplary KEGG pathways: Glycolysis (left) and MAPK (right)

Genes are colored by which event(s) ohnologs were retained. Transparent genes are those that were not identified in any genome in any amount. See Figure S2 for additional signaling pathways.

interactions, such as those embedded in dense signaling networks, are expected to be more sensitive to dosage effects and therefore more likely to be retained following WGD.[7,74] Indeed, genes coding for signal transducers have been preferentially retained following WGD in both plants[75] and animals.[76] Genes that are highly sensitive to dosage are also more likely to be associated with genetic disorders[77] and ohnologs in humans,[78] and other vertebrates[79] are known to be more closely associated with disease. Our findings support this connection because many pathways significantly enriched (adjusted $p < 0.01$) with ohnologs from WGD1,2,3 are related to human disease. Proteoglycans in cancer, insulin resistance, and COVID-19 were each enriched by all three events (Data S5). The observed functional similarities between ohnologs from events separated across kingdoms suggest that the effects of WGD and other major evolutionary events may be predictable.[80]

Another feature common to WGD-enriched pathways is their role in adaptation under diverse environments. Previous studies in yeasts have shown how metabolic[65] and signaling[69,71] pathways provide heterogeneous responses to hostile conditions and to the quantity and quality of available nutrients. The enrichment of these gene families following WGD may explain why modern polyploid *S. cerevisiae* strains are more fit in challenging environments, such as non-optimal carbon sources,[81] human hosts,[82] or brewing vats.[83] This result may further help to address the more widely observed trend of polyploid plant[84] and animal[85] species occurring in extreme, rapidly changing environments.

The nonrandom distribution of retained ohnologs in environmental information and resource processing networks indicates WGD as a potential driver of evolutionary innovation. Gene duplication is often associated with the gain of new function (neofunctionalization).[4,86,87] WGD provides perhaps the only opportunity for *trans*-regulated networks to neofunctionalize wholesale by making redundant copies of the entire network. However, more work is required to identify whether various modes of functional divergence are involved, such as neofunctionalization or escape from adaptive conflict,[88] or if simply increasing dosage of conserved copies is sufficient to precipitate major evolutionary change.[65] Previously thought to be largely absent from fungi,[16] these results underscore the importance of WGD in all eukaryotic kingdoms. We anticipate that more fungal WGDs will be discovered as sampling and sequencing continue to improve and that these events will yield a fuller portrait of eukaryotic genome evolution.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to, and will be fulfilled by, the lead contact, Antonis Rokas (antonis.rokas@vanderbilt.edu).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- These data are available at Figshare: 10.6084/m9.figshare.29852876.
- All original code is available at Zenodo: https://doi.org/10.5281/zenodo.18303192.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.T.D.; data curation, K.T.D.; formal analysis, K.T.D.; investigation, L.H., C.G., J.L.S., A.P., and P.G.; resources, L.H., C.G., C.T.H., and A.R.; software, K.L.D. and J.L.S.; visualization, K.T.D.; writing – original draft, K.T.D.; writing – review and editing, K.T.D., C.T.H., M.P., and A.R.

DECLARATION OF INTERESTS

J.L.S. is an advisor for ForensisGroup Inc. and a scientific consultant for FutureHouse Inc. A.R. is on the advisory board of *Current Biology* and is a scientific consultant for LifeMine Therapeutics, Inc.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Dipodascus fermentans	Portuguese Culture Collection	PYCC 3480
Magnusiomyces magnusii	ARS Culture Collection	NRRL Y-17563
Magnusiomyces tetraspermus	ARS Culture Collection	NRRL Y-7288
Chemicals, peptides, and recombinant proteins		
Zymolyase Ultra	Zymo Research	E1007-2
Critical commercial assays		
Quick-DNA HMW MagBead Kit	Zymo Research	D6060
Quick-DNA Fungal/Bacterial Miniprep Kit	Zymo Research	D6005
Deposited data		
Genomic and gene annotation data	Figshare	https://doi.org/10.6084/m9.figshare.29852876
Software and algorithms		
Canu v2.2	Koren et al.[89]	https://github.com/marbl/canu
Racon v1.5.0	Vaser et al.[90]	https://github.com/isovic/racon
Pilon v1.24	Walker et al.[91]	https://github.com/broadinstitute/pilon/wiki
LINKS v1.8.7	Warren et al.[92]	https://github.com/bcgsc/LINKS
flye v2.9.6	Kolmogorov et al.[93]	https://github.com/mikolmogorov/Flye
funannotate v1.8.16	Palmer and Stajich[94]	https://github.com/nextgenusfs/funannotate/tree/master
tantan v40	Frith[95]	https://gitlab.com/mcfrith/tantan
Augustus v3.3.2	Stanke et al.[96]	https://github.com/Gaius-Augustus/Augustus
SNAP v2006-07-28	Korf[97]	https://hpc.nih.gov/apps/snap.html
GlimmerHMM	Majoros et al.[98]	https://github.com/kblin/glimmerhmm
BUSCO v2.0	Waterhouse et al.[99]	https://gitlab.com/ezlab/busco
OrthoDB v9	Zdobnov et al.[100]	https://www.ezlab.org/orthodb_v12_userguide.html
CD-HIT v4.8.1	Fu et al.[101]	https://github.com/weizhongli/cdhit
EvidenceModeler v1.1.1	Haas et al.[102]	https://github.com/EVidenceModeler/EVidenceModeler
DIAMOND v2.1.8	Buchfink et al.[103]	https://github.com/bbuchfink/diamond

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
KofamKOALA	Aramaki et al.[104]	https://www.genome.jp/tools/kofamkoala/
InterProScan v5.74_105.0	Jones et al.[105]	https://github.com/ebi-pf-team/interproscan
Orthofinder v3.0.1b1	Emms and Kelly[21]	https://github.com/davidemms/OrthoFinder
AleRax v1.2.0	Morel et al.[106]	https://github.com/BenoitMorel/AleRax
wgd v2	Chen et al.[107]	https://github.com/heche-psb/wgd
ADHoRe v3.0	Proost et al.[108]	https://github.com/VIB-PSB/i-ADHoRe
R v4.3.2	R Core Team[109]	https://www.r-project.org/
clusterProfiler	Wu et al.[110]	https://guangchuangyu.github.io/software/clusterProfiler/
geiger	Pennell et al.[111]	https://cran.r-project.org/web/packages/geiger/index.html

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Dipodascus fermentans PYCC 3480^T (NRRL Y-1492) was obtained from the Portuguese Yeast Culture Collection (PYCC) and was routinely grown on solid yeast peptone dextrose (YPD) media at 25°C. *M. magnusii* (NRRL Y-17563) and *M. tetraspermus* (NRRL Y-7288) were routinely grown on solid yeast peptone dextrose (YPD) media at room temperature (22°C). Liquid cultures were inoculated from a single colony and grown in 25 ml YPD at room temperature in a 125 ml baffled flask shaking at 225 rpm.

METHOD DETAILS

D. fermentans extraction and assembly

Genomic DNA from overnight grown cultures of *D. fermentans* was obtained using the Quick-DNA Fungal/Bacterial Miniprep Kit from Zymoresearch (cat no. D6005), following the manufacturer's protocol. Long-read data was obtained using Oxford Nanopore Technology, with a MinION flowcell. For de novo assembly, Canu v2.2[89] was used with default parameters, only adjusting the genome size flag to 25 m. The resulting contigs were corrected with two rounds of Racon v1.5.0,[90] one with the Nanopore reads and the other with publicly available Illumina reads[112,113] (SRR16988715). Afterwards, several rounds of Pilon v1.24[91] were performed using Illumina reads until no changes were seen on the change file. To further increase the contiguity of the assembly, LINKS v1.8.7[92] was implemented.

M. magnusii and *M. tetraspermus* extraction and assembly

High molecular weight DNA from *M. magnusii* and *M. tetraspermus* was extracted using the Zymo Quick-DNA HMW MagBead Kit (cat no. D6060). The manufacturer's protocol was followed, but lysis was optimized for non-conventional yeast species. The yeast cells were pelleted by centrifugation at 5000 x *g* and resuspended in 1 ml of 1 M sorbitol with 50 mM dithiothreitol (DTT) and incubated at 30°C for 10 min. The cells were then washed in fresh 1 M sorbitol and resuspended in 200 ul 1 M sorbitol with 5 U of Zymolyase Ultra (Zymo Research, cat no. E1007-2) and incubated at 37°C for 2 hrs. Once cells were spheroplasted, 205 ul phosphate buffered saline, 20 ul of 10% SDS, and 10 ul proteinase K were added, and the spheroplasts were lysed at 55°C for 10 min with occasional inversion. The DNA in the lysate was then bound to the beads following the manufacturer's protocol. After extraction, the DNA was enriched for high molecular weight DNA using a bead cleanup with a custom buffer (10 mM Tris-HCl, 1 mM EDTA pH 8, 1.6 M NaCl, 11% PEG) as previously described.[114] Genome sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology. Both genomes were assembled with flye v2.9.6[93] using the 'nano-hq' option.

Gene annotation

To infer gene boundaries for the newly generated genomes of *D. fermentans*, *M. magnussi*, and *M. tetraspermus* we used funannotate v1.8.16.[94] To do so, each genome was first masked using tantan v40[95] using the funannotate mask function. Next, each genome was annotated using the funannotate predict function, which is a wrapper function to use multiple gene calling algorithms and creates a

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consensus set of gene boundaries. Prediction algorithms implemented include Augustus v3.3.2,[96] SNAP v2006-07-28,[97] and GlimmerHMM[98] each algorithm was trained on gene models predicted using BUSCO v2.0[99] gene models from the OrthoDB v9[100] database of near-universally single-copy orthologs from fungi. For Augustus, the 'optimize_augustus' argument was used. Additional gene boundaries were predicted by mapping gene annotations from a clustered set of proteins from 332 Saccharomycotina proteomes[112]; clustering was done using CD-HIT v4.8.1[101] using default settings. The results from each gene prediction algorithm were used to create a consensus set of gene boundaries using EVidenceModeler v1.1.1[102] with the "repeats2evm" argument. All approaches were given the same weight, except high-quality gene annotations (defined as >90% exon evidence) predicted by Augustus, which were given twice the weight of other algorithms. Gene models less than 50 amino acids in length and putatively transposable elements were subsequently removed. Putative transposable elements were identified using sequence similarity searches conducted using DIAMOND v2.1.8[103] and the funannotate database of repeat sequences. The resulting gene models were functionally with both KEGG v114.0[59] and InterPro v106.0[58] databases. KEGG orthologs were identified through the KofamKOALA[104] web server. InterPro Gene Ontology annotations were assigned using InterProScan v5.74_105.0.[105] Each gene model was annotated locally using the 'disable-precalc' option.

Gene tree inference

Three comparative genomic datasets were used by this study. The first was based on the recently published Y1000+ Project dataset.[113] The full 1,154 genomes of this dataset proved computationally intractable and was subsampled down to 400 using the following procedure: a genome with the shortest terminal branch in the species tree was pruned at random, unless that genome had $\leq$50 contigs. This process was repeated iteratively until 400 genomes remained in the tree. This sampling strategy maximized phylogenetic breadth and depth while retaining highly-contiguous genomes that could be used for synteny analysis. Gene trees were inferred with OrthoFinder v3.0.1b1[21] using the Y1000+ species tree as a reference. OrthoFinder identified large spikes in duplication rate within the class Dipodascales, warranting further investigation. Therefore, a second dataset was assembled using all 184 available Dipodascales genomes, as well as a third dataset of 135 Saccharomycetales genomes where WGD1 was known to occur. OrthoFinder was run on both of these datasets as before. A full record of all genomes used in this study can be found at Data S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Gene/species tree reconciliation analysis was performed using Orthofinder's hybrid species overlap/duplication-loss-coalescent algorithm. This approach has been shown to maximize accuracy while remaining tractable for large comparative analyses.[21] As validation, we also performed a fully probabilistic analysis using AleRax v1.2.0[106] on a small subset of 30 genomes. Despite recovering far fewer duplication events due to the restricted sample size, relative peaks in duplication rate were largely congruent between the two approaches (Figure S3).

Synteny analysis was performed using the wgd v2 software package.[107] First, paralogous gene sets were identified within each of 83 Saccharomycotina genome assemblies with $\leq$50 contigs, in addition to the 2 new genomes sequenced by this study, using the 'wgd dmd' command with default parameters. Next, colinear segments (multiplicons) were called with the 'wgd syn' command, also under default parameters. 'wgd syn' is a wrapper function for i-ADHoRe v3.0, which detects statistically significant colinear regions from gene homology matrices constructed between each pair of contigs to identify and assemble multiplicons.[108]

Enrichment analysis was performed using the clusterProfiler v4.10[110] package in R v4.3.2.[109] The function 'enrichKEGG' was used for KEGG pathway enrichment (Data S5), while the function 'enricher' was used for gene ontology enrichment (Data S4). False discovery rate[115] was used to control for multiple testing in both cases. Target genes were ohnologs that occurred in duplicate across colinear segments within genomes from species predicted to have experienced a given WGD event, against a background of all genes in the genome(s). We acknowledge this working definition of ohnologs is conservative, as not all ohnologs are expected to remain in order over long timescales. As a result, our analysis like underestimates the total number of ohnologs in each genome. For example, this approach recovers only 882 ohnologs in *S. cerevisiae*, which is thought to have at least 1,102.[116] As *M. magnusii* experienced two rounds of WGD, target genes in this species were further filtered to those estimated to have duplicated prior to the *M. magnusii* + *Sap*. *Suaveolens* split (for WGD3) and those duplicates specific to *M. magnusii* (for WGD4).

To test whether niche breadth or growth curves were significantly different in post-WGD species, we performed phylogenetic ANOVA using the 'aov.phylo' function as implemented in the eiger v2.0.11[111] package. Niche breadth data was taken from David et al.,[80] and growth curves were obtained from Opulente et al.[113]