Discovery of giant viruses as past and present infections of zoosporic fungi

Keywords: giant virus, fungi, endogenous viral elements (EVEs), NCLDV, Nucleocytoviricota, mycovirus, zoosporic fungi

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Abstract

Giant viruses of the phylum *Nucleocytoviricota* have emerged as particularly notable due to their increasingly recognized impacts on eukaryotic genome evolution. Their origins are hypothesized to predate or coincide with the diversification of eukaryotes, and they have been detected in hosts that span the eukaryotic tree of life. But surprisingly, such viruses have not been definitively found in Kingdom Fungi, though genomic and metagenomic work suggests a putative association. Here we report both “viral fossils” and active infection by giant viruses in fungi, particularly in the zoosporic phyla Blastocladiomycota and Chytridiomycota. The recovered viral genomes span up to 350 kb, encode over 300 genes, and form a monophyletic family-level clade within the *Nucleocytoviricota* related to orders *Imitervirales* and *Algavirales*, which we name *Mycodnaviridae*. We observed variation in infection status among the isolates including apparent active infection and transcriptionally-suppressed states, suggesting that viral activation may be constrained to certain life stages of the host. Our experimental findings add to the limited natural virus-host systems available in culture for the study of giant viruses. These viruses may have shaped the early evolution of these fungal lineages by the shuttling of genes between major kingdoms and domains of life. Our findings expand the known host range of *Nucleocytoviricota* into a new kingdom that contains many model species. *Mycodnaviridae* have a global distribution, which invites inquiry into the implications of these infections for host traits, host genome evolution, and the metabolic impacts to ecosystems.

Main

In the entire Fungal Kingdom, only a handful of DNA viruses are known–all small circular single-stranded (CRESS) viruses (Li et al. 2020; Yu et al. 2010). This apparent rarity of DNA viruses in fungi likely results from taxonomic biases in searches, rather than a true biological phenomenon. The study of mycoviruses has historically focused on their application for biological control of fungal pathogens, particularly in agricultural contexts, and as a result most research has involved viruses infecting fungi in the Ascomycota—one of two phyla comprising the subkingdom Dikarya, which contains the majority of described fungal taxa. We previously demonstrated that by expanding the taxonomic scope of considered fungal hosts to include lineages outside of the Dikarya the known RNA mycoviral diversity was greatly increased (Myers et al. 2020). The current fungal taxonomic division includes eight phyla of zoosporic fungi that reproduce with motile spores and represent a particularly understudied paraphyletic assemblage that includes the last common ancestor of fungi (Hyde et al. 2024). The presence of DNA viruses in these fungi has been almost entirely unexplored (but see Clemons et al. 2023), and indeed, the finding of giant viruses, which we report here, was a serendipitous discovery resulting from a large genomic sequencing effort across these fungal phyla (Amses et al. 2022).

While only a few active DNA viruses in fungi have been documented, previous studies have suggested putative associations between *Nucleocytoviricota* and fungi through the identification of fungal homologs in metagenomic-assembled giant viruses (Schulz et al. 2018, Schulz et al. 2020, Bhattacharjee et al. 2023) and giant virus EVEs in fungal genomes (Gallot-Lavallée and Blanc 2017, Gong et al. 2020, Zhao et al. 2023, Nobre 2024). These links vary in their robustness but nonetheless the cumulative evidence suggests a rich evolutionary history between fungi and giant viruses. Despite these tantalizing findings, active giant viral infections in fungi have not previously been evidenced nor have novel clades that include active giant viruses in fungi been characterized.

Results

Zoosporic fungi contain giant virus genes and genomes with global distribution

We identified homologs of the giant virus major capsid protein (MCP; NCVOG0022) in the genomes of 17/141 fungi with HMM searches (Fig S1, Table S1). The distribution of MCP homologs is restricted to non-Dikaryotic lineages, and, particularly, the zoosporic fungi; no MCPs were found by hmmsearch in non-zoosporic lineages (n= 40). We further searched all fungal genomes in Mycocosm (Grigoriev et al. 2014; n = 2,543) for MCP homologs by blastp, which resulted in one hit to a non-zoosporic fungal species: *Dimargaris cristalligena* (phylum Zoopagomycota), a mycoparasite. Among the zoosporic lineages, MCPs were found in Chytridiomycota (n=12), Monoblephidomycota (n=2), and Blastocladiomycota (n=3). Blastocladiomycota are a particularly poorly sampled group, and thus our genomic searches

were limited (n=7). However, MCPs appear to be common in the most highly sampled genus within this phylum, *Allomyces.* Based on available sequences, we designed primers targeting the MCP gene of *Allomyces* and found 30/58 unique isolates were positive for capsid by PCR.

The contigs containing MCP genes vary widely among the fungal isolates in gene content, GC composition, intergenic distance, and other metrics, suggesting numerous independent endogenization events (Fig 1 A). In three isolates (*Podochytrium* sp. JEL797, *Gaertneriomyces semiglobifer* Barr43, and *Fimicolochytrium jonesii* JEL569), MCP was the only gene of viral provenance on contigs which otherwise contain primarily fungal genes and have GC content and intron density similar to other host contigs (Fig S2). In other isolates, contigs with MCPs appear as mosaics of virus, prokaryote, and fungal genes, which include multiple additional giant virus orthologous genes (GVOGs), and have markedly greater gene density compared to host contigs, consistent with reference giant virus genomes (Fig 1 A, B; Fig S2). These giant virus-like contigs range up to nearly 350 kb and are suggestive of either very recent endogenization or proviral state. Interestingly, in five isolates the MCP contig contains a gene encoding an adintovirus-like integrase.

Based on MCP data previously extracted from global metagenomics datasets, we assessed the global distribution of these novel fungal MCPs. Environmental MCP homologs with bidirectional best blast hits to fungal MCPs have a global distribution (Fig 1 C). The *Allomyces* isolates tested by PCR for MCP have worldwide provenance, thus also suggestive of a long history of viral infection.

The most contiguously assembled viral genomes were found from two strains of *Allomyces*. A. arbusculus Burma1F mycodnavirus 1 includes two contigs of total size 345,111 bp, encoding 314 genes with likely provenance, based on homology, assigned as follows: 10% fungi, 14% bacteria, 20% virus, 4% putatively *Mycodnaviridae*-specific, and 52% unknown/ORFan (Fig 1 B). The isolate *A. javanicus* California12 has multiple large contigs (ranging between 80-288kb) with giant virus signatures (Fig S3). Interestingly, when binned by tetramer frequency, the viral contigs in *A. javanicus* California12 form two bins suggesting the presence of two distinct viruses. The two bins are roughly similar in size (212,591 bp and 287,844 bp) and show some duplication of hallmark GVOGs: each bin contains copies of MCP, D5-like helicase-primase, DNA PolB, mRNA capping enzyme large subunit, and superfamily II helicase. The larger bin contains an additional five GVOGs (YqaJ recombinase, RNA ligase, disulfide (thiol) oxidoreductase, transcription initiation factor IIB, and A32-like packaging ATPase). *A. javanicus* strains are believed to be hybrids (Emerson 1941) and the two viruses could have originated from the two parents of this diploid sporophyte.

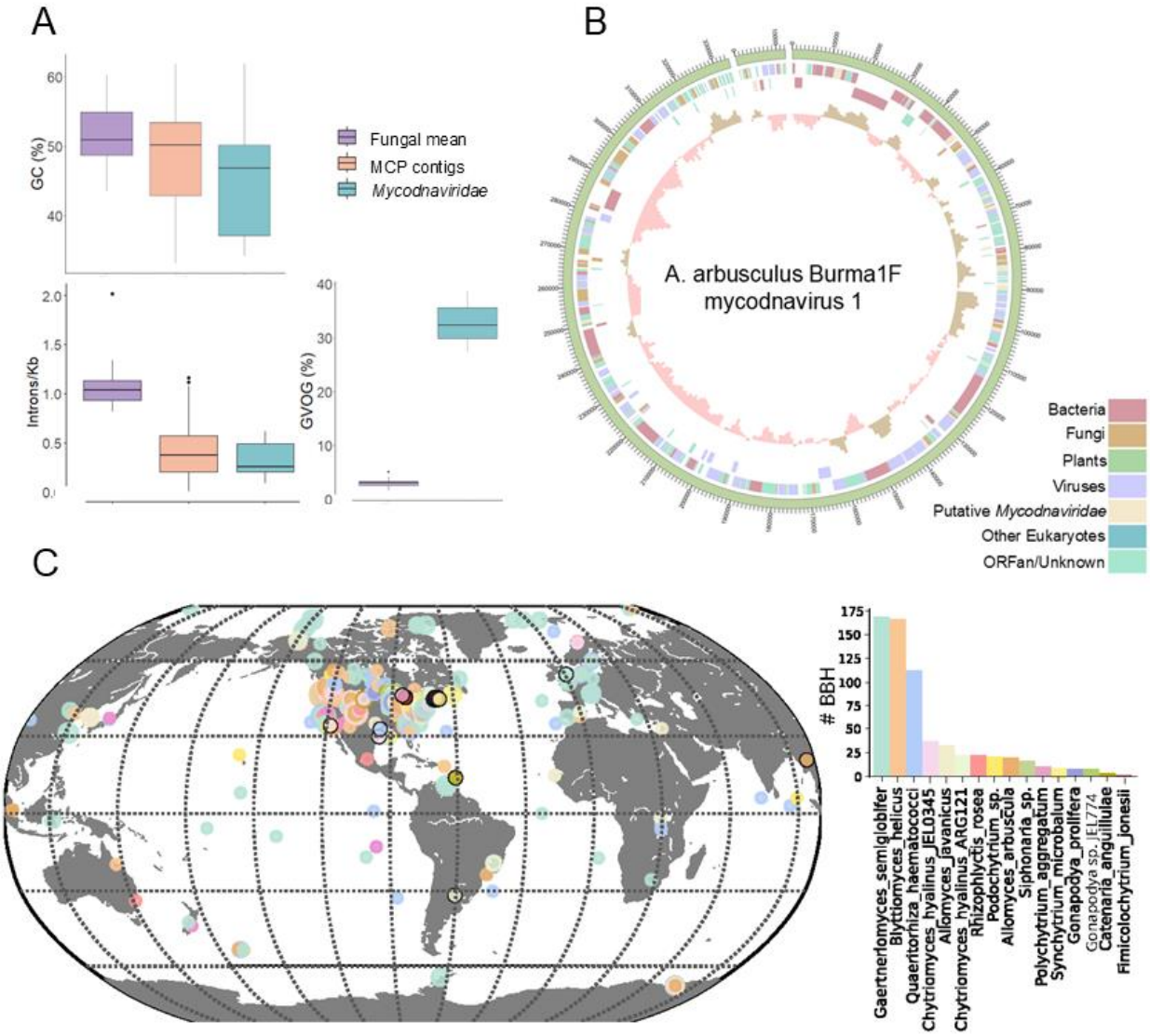

Fig 1. Genomic features of *Mycodnaviridae* (A) Boxplots of genome statistics for the fungal genomes found to contain MCP genes (n=17): mean values across all contigs ("Fungal mean", purple), contigs containing at least one major capsid protein (MCP) gene ("MCP contigs", pink), and MCP contigs that met the criteria set for orthology analysis (ViralRecall score >2, viral region length > 70%, intron density >25% different from host; "*Mycodnaviridae"*, teal). (B) The genome of Allomyces arbusculus Burma1F mycodnavirus 1 is a mosaic of bacteria; viral, and eukaryotic genes. Contigs are represented by the outermost ring in the circularized genome plot. From outside to inside: ORFs on the forward strand, ORFs on the reverse strand (both color coded by provenance), GC skew. (C) Global distribution of the *Mycodnaviridae*-like sequences based on the major capsid protein. Colored circles correspond to metagenomic MCP sequences with best blast hit (BBH) to *Mycodnaviridae* identified in this study and circles with a black outline indicate geographic location of *Mycodnaviridae* and hosts described in this study.

Mycodnaviridae is a monophyletic clade of fungal giant viruses

A phylogeny of representative giant viruses including the 7 fungal samples with at least 4/7 hallmark *Nucleocytoviricota* genes (GVOG7 marker set; Aylward et al. 2021) show that fungal giant viruses form a monophyletic clade together with three giant virus metagenome-assembled genomes (GVMAGs) of the previously proposed *Imitervirales* family IM_02 and in a sister position to *Imitervirales* IM_18 (Fig 2A). Our phylogenetic analysis showed that less complex phylogenetic models (WAG) and reconstruction algorithms (FastTree, IQ-tree2 -fast) place the IM_02-IM_18 cluster basal to other *Imitervirales*. However, with more complex phylogenetic models (IQtree2 with LG+I+G4, LG4X) the IM_02-IM_18 cluster was split off from *Imitervirales* and groups sister to *Pandoravirales* and *Algavirales*. The monophyletic grouping of the fungal giant viruses with IM_02 was well supported across supermatrix-based species trees and single protein trees for each of the markers in the GVOG7 set. Both IM_02 and IM_18 previously consisted exclusively of GVMAGs but since the new genomes added can be connected with fungal hosts, we dub IM_02 *Mycodnaviridae* (Fig 2 A). Our analysis also suggests that IM_02 together with IM_18 is not part of the order *Imitervirales* but may represent a novel order in the *Nucleocytoviricota*. Further detailed phylogenomic analysis after improved taxon sampling will be required to provide additional evidence for such a relationship. The hosts of IM_02 and IM_18 are unknown, but the clustering of these clades with giant viruses from fungi strongly suggest a fungal association.

We investigated whether a core suite of genes exist that functionally define the *Mycodnaviridae* by conducting orthology analysis on the most complete viral genomes–five have at least 9 of the 20 core GVOGs present in the majority of the *Nucleocytoviricota* (Yutin et al. 2009). This resulted in 27 gene clusters shared by all five (Fig 2 B) though this is likely an underestimate due to variation in genome completeness, which is suggested by variation in viral genome assembly size. In addition to GVOGs such as MCPs, VLTF3, and YqaJ-like recombinase, the core set is comprised of gene orthogroups involved in DNA biosynthesis such as DNA helicase, ribonucleotide reductase, and proliferating cell nuclear antigen, which are also GVOGs found in other large and giant viruses. Other shared gene clusters include a cysteine protease, protein with N-acetylglucosaminyltransferase activity (possibly involved in cell wall modification), six genes of unknown function, and five novel genes apparently unique to these viruses.

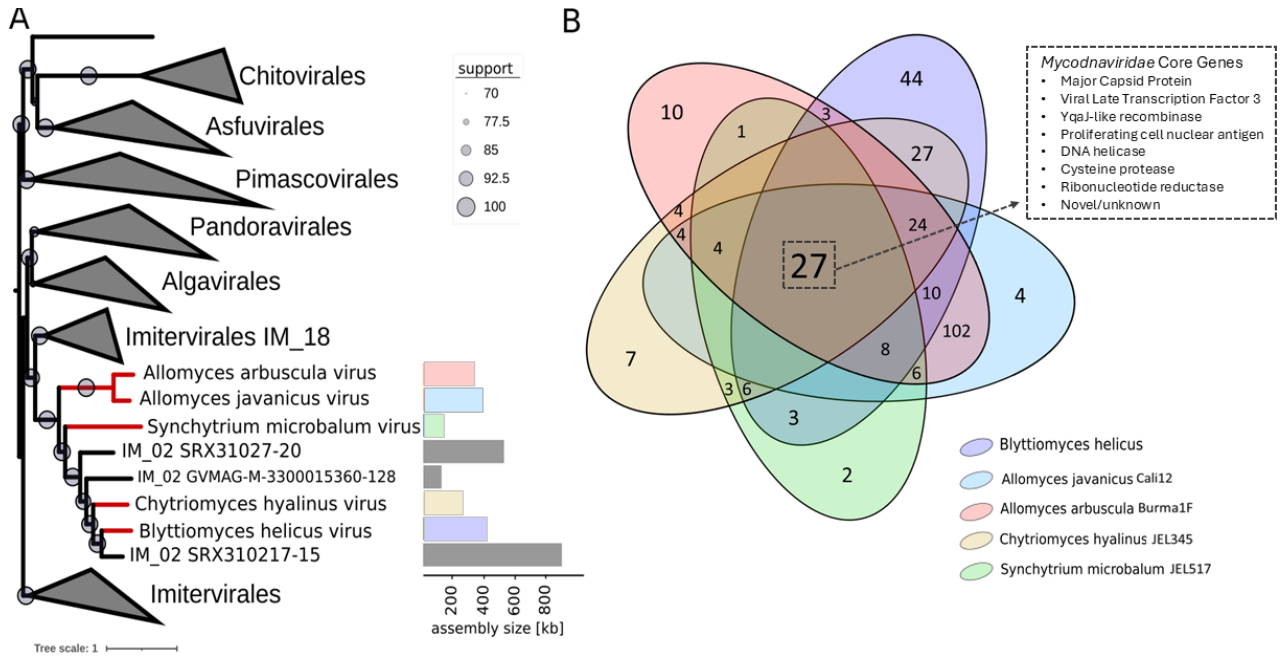

Fig 2. Phylogenetic position of the *Mycodnaviridae* and core genes. (A) Position of the *Mycodnaviridae* clade in the species tree of the *Nucleocytoviricota*. Red branches indicate *Mycodnaviridae* genomes. The tree is rooted at Pokkesviricetes. (B) Venn diagram indicating the number of orthologous gene clusters shared between the 5 most complete *Mycodnaviridae* genomes.

Allomyces virus is integrated in the nuclear genome, with the capacity for productive infection

One critical question was whether these giant virus contigs are merely relics of past infections that have been maintained as EVEs in fungal genomes, or whether they are capable of replicative infection. We have accumulated evidence that supports both scenarios. In *Allomyces arbusculus* Burma1F, long-read whole-genome sequencing by Oxford Nanopore revealed the viral sequence integrated into a chromosome that harbors telomere repeats on both ends (contig 03: 1397320-1743578; (Figure 3 A & B). Nanopore-based genome assembly also revealed 13 clusters of LTR-transposons, only one per contig, that potentially represent centromeres similar to those observed in the dikaryotic fungus *Cryptococcus neoformans* (Yadav 2018, Janbon 2014). Furthermore, 26 telomere repeat-containing ends were identified across all contigs, which combined with 13 putative centromeres, indicate Burma1F harbors 13 chromosomes. With interest in whether the viral genes are expressed and, if so, whether expression occurs throughout host tissue or is constrained to some structures or life stages, we sequenced RNA of *A. arbusculus* Burma1F at three different stages. We found almost no evidence of expression from the viral genes, with the exception of genes in the flanking region (Fig 3 B). Interestingly, CpG methylation was not detected in these viral regions per the Nanopore-generated data. These data suggest active silencing of the viral genes by the host, and the expression data help define the border of the integrated virus. The mechanism of silencing employed by the host is as yet unknown.

An additional distinguishing feature between viruses and EVEs is read coverage of the viral contigs because sequencing during viral replication is expected to result in extremely high coverage of viral reads. Overall, we found no clear pattern between coverage of fungal contigs containing MCPs and coverage of other genomic contigs, with one exception. We sequenced genomes of an additional 9 strains of *Allomyces;* one isolate, *Allomyces arbusculus* Cali8, included 8 contigs (total 309,231 bp) that averaged nearly 50-fold higher coverage than the rest of the genome (Fig 3 C). Of those, two contigs include MCPs and a combined 7 additional core GVOGs as well as a viral-like integrase. These results strongly indicate an active infection by a giant virus in this strain of *Allomyces.* Other sequenced *Allomyces* strains had contigs similarly rich with giant virus hallmark genes but lacking differences in coverage from the genome-wide median estimated by read depth. It is plausible that in these strains the conditions for triggering viral release were not met under laboratory culture conditions, and thus the virus remained in a proviral state. Given the high occurrence of MCPs we suspect that additional *Allomyces* strains contain provirus that is perhaps capable of activating and forming an infectious propagule, though the triggering mechanism for viral release is unknown.

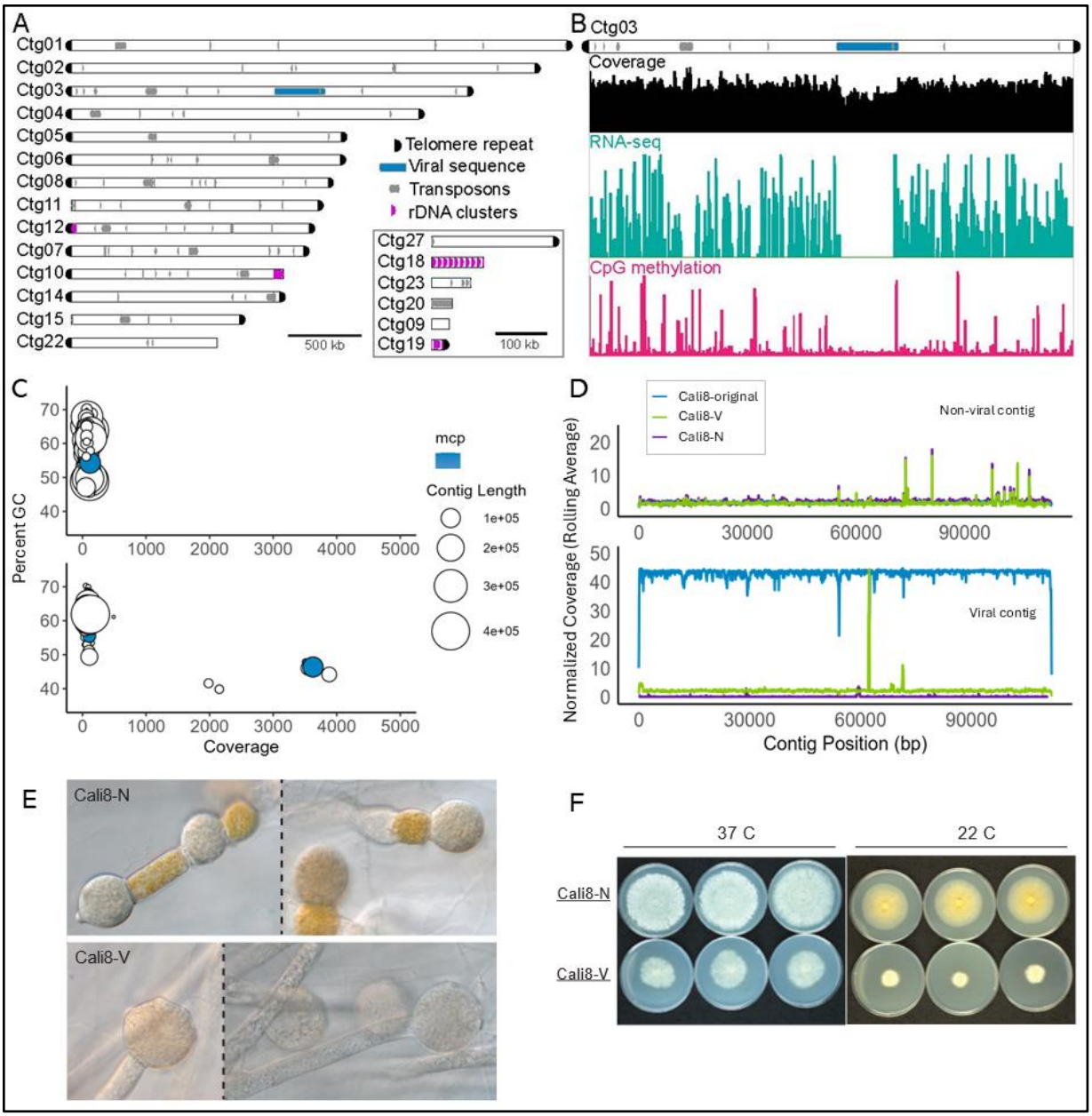

Figure 3. *Allomyces* viruses are integrated into the nuclear genome, with complex patterns of expression/suppression and phenotypic effects. (A) Assembly of *A. arbusculus* Burma1F shows the virus integrated into the host nuclear genome (Ctg 03) (B) Mapping of Nanopore reads (genome coverage), RNAseq reads, and CpG methylation extracted from Nanopore sequencing reads across the virus-harboring contig of *A. arbusculus* Burma1F, showing silencing of the integrated virus unrelated to methylation. (C) Plots of the GC content and genomic read coverage for assemblies of *A. anomalus* BEA2 (upper) and *A. arbusculus* Cali8 (lower). Contigs that include a major capsid protein (MCP) are shaded blue and size of data points reflect contig length. Cali8 includes two contigs with both MCPs and very high coverage. (D) Rolling average of normalized read coverage of a non-viral (upper) and viral (lower) genome segment in three isolates of Cali8. (E) Cali8-N gametophytes display normal phenotype with both male and female gametangia while Cali8-V nearly exclusively produces malformed and unproductive male gametangia (F) Cali8-N and Cali8-V grown at 37 C and 22C. At both temperatures Cali8-V had dramatically reduced growth rate.

In an initial effort to assess functional impacts of giant virus presence, strains of *Allomyces* with and without detectable MCP genes were grown on agar and liquid Emerson's YpSS media to test if the presence of MCP influenced growth rate of the fungal strain. No significant differences in growth rate ($t(10) = -1.10$, $p = 0.30$) and biomass accumulation ($t(10) = -0.06$, $p = 0.95$) were observed (Fig S4, A & B). This is likely due to the absence of expression of virus genes when present in the fungal chromosome (as in Fig 3 B). We also compared the thermal maximum of *Allomyces* strains with and without MCP grown on solid YpSS media. We found no significant difference ($t(10) = -0.25$, $p = 0.80$; Fig S4 C) depending on presence of MCP, however, one of the strains, Cali8, showed sectoring at 42 C. With archived samples of Cali8, we generated meiospores by germinating resting sporangia. These spores mostly showed abortive growth, however, two single spore isolates with divergent morphologies were obtained (Cali8-V and Cali8-N) (Fig 3 E). Cali8-V mycelium was consistent with a gametophyte stage, however, the strain primarily produced male gametangia that were enlarged, fewer in number than Cali8-N, and no gametes could be released. In comparison, the Cali8-N mycelium appeared normal, and growth rates of Cali8-N at both 22 C and 37 C were much higher than Cali8-V (Fig 3 E & F). These two strains were then subjected to whole genome sequencing in an effort to discern whether viral titer was responsible for the altered morphology. After normalization, average coverage of the viral region in the sector demonstrating abnormal hyphal morphology was 25 times greater than the sector with normal growth; this region in the originally sequenced *Allomyces arbusculus* Cali8 strain (polysporous isolate) had 490 times greater coverage (Fig 3 D). These results highlight that viral release is complicated and may be linked with environmental and host lifecycle triggers as well as the complex life history of this fungus. These results also indicate that the host possesses mechanisms for avoiding viral release and/or restricting the spread of viral particles.

Transmission electron microscopy reveals virus-like particles

Arguably the presence of active viruses is most directly evidenced by images of the viral particles themselves. We took two approaches to this end: we searched the literature on zoospore ultrastructure for unrecognized or mis-identified electron-dense bodies that could be of viral origin and also prepared zoospores of *Allomyces* for transmission electron microscopy (TEM). Both approaches resulted in electron micrographs of giant virus-like particles with similar morphologies (VLPs, Fig 4).

The literature on zoosporic fungi ultrastructure is rich with unidentified bodies with uncertain functions. Zoospores are relatively simple, single cells, and yet are home to unique organelles, vacuoles, lipid bodies, and inclusions that have proven difficult to assign a functionality. Indeed, some of these appear remarkably virus-like. Letcher and Powell (2018) describe a chytrid isolate *Polyphlyctis willoughbyi* PL AUS 26, writing "occurring sparsely in the peripheral cytoplasm were electron-opaque hexagonal crystals ~0.1 µm in the broadest dimension" (Fig 4 C-E). Simmons et al (2009) describe the chytrid *Clydaea vesicula*, which is so named for the "aggregation of vesicles with electron-opaque contents" within the zoospore cytoplasm (Fig 4 F & G). Our in-house transmission electron microscopy of *A. javanicus* California12 zoospores also shows cytoplasmic VLPs (Fig 4 A & B). The VLPs are round to hexagonal, 150-175 nm diameter with an approximately 100 nm granular core, and appear in low abundance in

zoospores. Taken together, it is striking that the VLPs of these three zoosporic fungi converge on a shared morphology, which may represent that of the *Mycodnaviridae*. We report these findings with caution, however, as VLPs have not yet been isolated and sequenced directly.

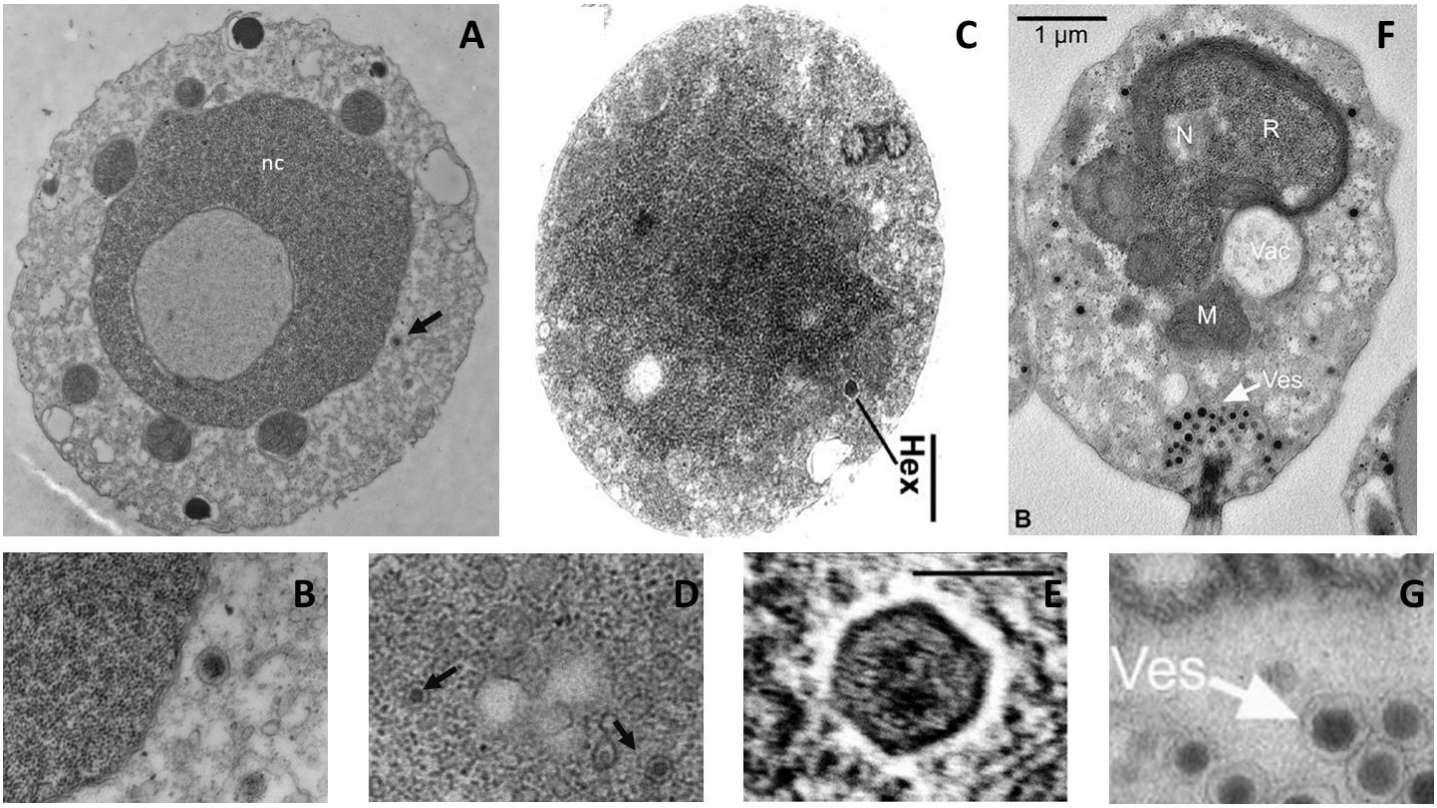

Fig 4. Cross-sections of zoospores including virus-like particles (VLPs) visualized by transmission electron microscopy. (A) *A. javanicus* California12 zoospore cross-section at 2500x containing a VLP (arrow) near the nuclear cap (nc), and (B) the VLP at 15000x magnification. (C) Cross-section of *Polyphlyctis willoughbyi* PL AUS 26 zoospore containing "hexagonal inclusions", magnified in (D & E). (F) *Clydaea vesicula* cross-section with electron-dense vesicles (Ves, arrow), magnified in (G). Images reused with permission (C & E, Elsevier license number 5995480828397; F & G, Elsevier license number 5995480495911).

Discussion

In this paper, we report the discovery of *Mycodnaviridae*, a new family of *Nucleocytoviricota* with close phylogenetic affinities to *Algavirales* and *Imitervirales*. The three phyla of fungi in which we have found evidence of giant viruses—Blastocladiomycota, Chytridiomycota, and Zoopagomycota—have life cycle stages similar to some algae and amoebae, which may facilitate infection by viruses with persistent infection strategies, such as the phaeoviruses in *Algavirales*. In both algae and zoosporic fungi there exist life-stages (spores and gametes) consisting of flagellated single-cells lacking a protective cell wall. The phaeoviruses have evolved stable coexistence with their hosts through a persistent viral strategy whereby these spores or gametes are infected by virus particle(s) that then integrate into the host genome

(Müller and Parodi 1993). Development proceeds with one copy of the virus integrated into the DNA of each cell (Meints et al. 2008). Thalli often appear symptom-free until some unknown mechanism triggers virus emergence from the genome. Reproductive organs may develop irregularly as they become factories for viral replication (Henry and Meints 1992; Stevens et al. 2014). Very recently, another integrated viral genome was identified within a green alga and was shown to actively produce viral particles (Erazo-Garcia 2025). We hypothesize that fungal giant viruses in zoosporic lineages share similar replication cycle characteristics.

Genome coverage data, transcriptional patterns, and the presence of an integrase gene in *Mycodnaviridae* are consistent with this hypothesized replication cycle. In most genomes viral contigs have similar coverage to the host average, as would be expected for a provirus integrated into a single-celled spore/gamete that propagates the organism. Genome coverage data in one isolate of *Allomyces* (Cali8), however, suggests active replication at the time of initial sequencing. Subcultures of the isolate are consistent with either viral endogenization into the nuclear genome or near-complete loss of the virus. In another isolate of *Allomyces* (Burma1F), transcriptional patterns suggest that the provirus may be silenced in the host genome, consistent with transcriptional patterns of phaeoviruses integrated in their algal hosts (Cock et al. 2010). The reason that we failed to observe any viral effect on fungal host phenotypes in this isolate may be related to this absence of viral gene expression.

Electron micrographs of putatively infected zoospores show virus-like particles (VLPs) with similar morphology to the phaeoviruses: round to hexagonal VLPs of 150-175 nm diameter with an approximately 100 nm granular core. VLPs typically appear in low abundance in zoospores, consistent with expectations that zoospores are recipients of infective viruses that migrate to the nucleus and, upon zoospore encystment, integrate into the host genome. The apparent near-restriction of *Mycodnaviridae* to zoosporic lineages is consistent with this hypothesis for the replicative cycle, though a mechanism triggering viral release remains unknown.

Like all cellular organisms, the evolution of the fungi has been impacted by interactions with viruses. Giant viruses are hypothesized to have originated with the diversification of eukaryotes, and metagenomic sequence data demonstrates their abundance in the environment. The lack of evidence of active infection by giant viruses in fungi before now is therefore surprising, though previous reports have shown their association (Zhao et al. 2023; Bhattacharjee et al. 2023; Gong 2020; Schulz et al. 2020; 2018). Most interestingly, the EVEs reported by others are of distinct and distant relation to the clade of giant fungal viruses we report here. Phylogenetic reconstruction of the DNA polymerase B (PolB) using data from four such studies as well as *Mycodnaviridae* shows at least five unique clades of giant virus PolBs associated with Fungi (Supplemental Figure S5). Taken together, these data suggest that fungi have historically and, perhaps, currently been hosts to multiple distinct lineages of giant viruses.

It is intriguing to consider the implications of a possible ancient association between fungi and giant viruses in relation to fungal evolution because viral gene acquisition can be an impetus for evolutionary innovation (Frank and Feschotte 2017). In the fungi, a transcription factor involved in cell cycle regulation that is, surprisingly, unique to fungi is hypothesized to have been acquired by the fungal ancestor through lateral gene transfer from a virus (Medina et al. 2016).

Phylogenetic evidence points to the source as either a giant virus or phage-infected bacterium. We searched MCP-containing contigs but found no homologs containing the diagnostic viral-like KilA-N domain of this cell-cycle protein, though this does not preclude giant viruses as the original source. Other evolutionary innovations resulting from giant virus association could involve novel metabolic functions. Especially in GVMAGs, a vast diversity of metabolic genes has been found (Schulz et al. 2020, Moniruzzaman et al. 2020) including many involved in carbon and nitrogen metabolism. Assessing the genetic and functional impact of giant virus infection on fungal metabolism should be a future research priority. To understand the impact of the viruses, we need to resolve when and under what conditions the viruses are expressed during the life cycle.

Notably, the most contiguous viral genomes were found in *Allomyces* spp., from cultures that were recovered from filter paper stocks after 30 years of storage. This fact demonstrates desirable aspects of this fungus that bolster motivation for its revival as a model system. *Allomyces* has historically, and currently though with much less frequency, been a model for studying biological phenomena such as anisogamy (Olsen 1984; Phadke 2018), sensorimotor systems including phototaxis and chemotaxis (Machlis 1969; Swafford and Oakley 2018), and even cellular differentiation (Cantino and Lovett 1964). The development of *Allomyces* as a model for studying giant viruses has enormous potential. Much of what is known about giant virus biology has been learned from protist-virus interactions or metagenomics where the host is unknown. The development of additional laboratory model systems of giant viruses and their natural hosts could advance our understanding of virus-host interactions, including immune response, viral “reprogramming” of host cells, metabolic impacts, and their ecosystem-level consequences.

The discovery of the first giant viruses in fungi that are restricted primarily to the zoosporic fungi inspire numerous questions that beg further investigation. How is viral expression and virion production related to the host life cycle, and vice-versa? What are the metabolic impacts of infection, and how important are they on the global scale? What defense systems do fungi employ against giant viruses, and do they explain why Dikarya fungi are not infected? To what extent have giant viruses shaped genome evolution in fungi? Giant virus model systems in zoosporic fungi may be the key to exposing these latent revelations.

Data Availability

Mycodnaviridae genome assemblies and alignment for the *Nucleocytoviricota* phylogeny were deposited in Deep Blue Data, the University of Michigan’s institutional data repository (DOI: https://doi.org/10.7302/p5ay-ng61). Fungal genome assemblies, and RNAseq data are available in NCBI BioProject PRJNA1257431.

Acknowledgements

Cultures and DNAs were obtained from the Collection of Zoosporic Eufungi at the University of Michigan. Next-generation sequencing was performed at the Advanced Genomics Core at the

University of Michigan. Transmission electron microscopy was conducted at the University of Michigan's Department of Molecular, Cell, and Developmental Biology Imaging Core; we heartily thank Gregg Sobocinski for his technical assistance with sectioning and training on the Jeol. We also thank Dr. Pete Letcher for contributing previously prepared grids and blocks of embedded zoospores for TEM. We thank Mia Sinks for technical assistance with *Allomyces* cultures. This project was funded in part by the National Science Foundation grants DEB-1929738 and DEB-2403677 to TYJ. KRA was supported by the following National Institutes of Health training grant: "Michigan Predoctoral Training in Genetics" [T32GM007544]. JES, TYJ, and JH are fellows and co-director (JH) of the CIFAR program Fungal Kingdom: Threats & Opportunities, and these studies were supported in part by a CIFAR Catalyst Award. The work conducted by the U.S. Department of Energy Joint Genome Institute (https://ror.org/04xm1d337), a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy operated under Contract No. DE-AC02-05CH11231. Efforts by SS, VY, and JH were supported in part by NIH/NIAID R01 grants AI39115-27 and AI050113-20.

Competing Interests

FS is the co-founder and CEO of SampleX, a decentralized marketplace for biological samples, and co-founder and CTO of BioKEA, a consulting company for AI in biology. These affiliations did not influence the design, execution, or interpretation of the research presented in this manuscript.

Author Contributions

JES, TYJ, and JMM conceived of and planned the study. Culturing and DNA preparation was conducted by MOQ and JMM. RNA preparation, PCR, transcriptome assembly, viral content identification, and orthology analysis was conducted by JMM. Giant virus phylogeny and distribution analyses were performed by FS. Genome assembly and annotation was conducted by JMM, KRA, and SR. Transcriptome analysis was conducted by SR. Nanopore sequencing DNA extraction, as well as genome assembly and analysis of strain Burma1F was conducted by VY, SS and JH. Summary statistics were conducted by JES and JMM. Growth experiments were performed by TYJ. Transmission electron microscopy was performed by JMM and DRS. Manuscript was written by JMM with contributions from TYJ and FS. All authors read and approved the final manuscript.

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Methods

Fungal strains, nucleic acid preparation, and sequencing:

We initially searched 135 genomes in this study including 131 fungi, 92 of which are zoosporic *(Table S1)*. For full details about DNA preparation and sequencing of these isolates see (Amses et al. 2022). An additional nine strains of *Allomyces* spp. were whole-genome sequenced from DNA archived in the CZEUM collection (Simmons et al. 2020). Library preparation and sequencing was performed by the Advanced Genomics Core at the University of Michigan by NovaSeq S4 300 cycle on 4% of a flowcell.

Allomyces javanicus California12 and *Allomyces arbusculus* Burma1F were grown in PmTG liquid media for 7-10 days at 22℃ with shaking at 180 rpm. Tissue was subsampled with sterile tweezers and immediately frozen and ground to a powder in liquid nitrogen. Approximately 50 mg was apportioned to 1.5mL tubes, 1 mL of TRIzol applied, and stored at -20℃ until further processing. For *A. arbusculus* Burma1F, the remaining tissue was decanted, rinsed once with sterile water, and replenished with sterile water. After overnight incubation at 22℃, tissue was decanted, rinsed once with sterile water, subsampled and processed as described above, then transferred to a sterile tea strainer and submerged in 50 mL sterile water for two hours on the benchtop. The tea strainer was removed, and the remaining liquid observed under a microscope for zoospore presence, followed by centrifugation at 4000x g and 4℃ for 12 minutes. The zoospore pellet was decanted, 1mL of TRIzol added, ground with a pestle, then stored at -20℃. The RNA extraction for all samples was completed per manufacturer protocol. Total RNAs were quality-checked on a Tapestation and had RIN values between 6.8-10. Samples were Poly-A enriched, and libraries were prepared and sequenced by the University of Michigan Advanced Genomics Core by NovaSeq S4 300 cycle on 1% of the flowcell.

Genome and transcriptome assembly, annotation, and statistics:

Transcriptome data were quality filtered by removing reads with Phred score <20 using Fastx-toolkit (Hannon 2010), then assembled *de novo* using Trinity 2.12 (Grabherr et al. 2011). Hisat2 v.2.2.1 (Kim et al. 2019) was used for the RNAseq analysis: the hisat2-build function was used to build an index from genome assemblies, then RNA sequencing reads were aligned to the indexed assemblies using default parameter settings. Alignments were visualized with SeqMonk v.1.48.1 (RRID:SCR_001913).

Paired-end genomic data of *Allomyces* strains were quality-filtered by trimmomatic (Bolger, Lohse, and Usadel 2014) with a sliding window of 4bp and threshold of Phred 20, then assembled by Spades 3.15.5 (Prjibelski et al. 2020) and annotated with both Prodigal v. 2.6.3 (Hyatt et al. 2010) and Augustus 3.2.1 (Stanke et al. 2006) trained with *Rhizopus oryzae*.

For the genome plots of *Allomyces arbusculus* Burma 1F *and Allomyces javanicus* California12, we used Prodigal v. 2.6.3 (Hyatt et al. 2010) for gene prediction of soft-masked viral contigs with inputs from Funannotate v. 1.8.16 (Palmer and Stajich 2023) which is a fungal gene annotation

tool and GeneMarkS (http://exon.gatech.edu/genemark/index.html) using the viral option for input sequence type. GC skew was calculated in a 5-kb sliding window with a 1-kb step size. Circos v. 0.54 (Krzywinski et al. 2009) was used to construct the circular genome visualization.

Identification of viral content:

We used the program ViralRecall (Aylward and Moniruzzaman 2021) to identify putative viral genes, viral contigs, and viral regions within contigs. We first ran ViralRecall 1.0 on all assemblies with both stringent (-s 15 -m 30 -v 10) and relaxed (-s 15 -m 2 -v 2) parameters–the latter for poorer quality genome assemblies–and identified isolates containing MCP homologs with e-value <$1e^{-10}$. ViralRecall searches gene predictions generated using Prodigal (Hyatt et al. 2010); we also performed hmmsearches (HMMER3 v3.1b2; S. Eddy 2009) with the ViralRecall 2.0 GVOG hmm on a subset of our Augustus-predicted genomes. We found no differences in the number of MCPs called, and so continued characterizations for the isolates that these initial ViralRecall results predicted at least one MCP homolog. For each MCP-containing contig, we calculated GC content, intron density per the Augustus annotation, and performed BLAST searches (Altschul et al. 1990) on both gene prediction datasets.

To aid in distinguishing viral genomes from within the fungal assemblies, we searched the 16 prodigal annotated genomes previously identified to contain MCPs with HMMER3 (Eddy 2009) as in Schulz et al. 2020 using hmms of a subset of the nucleocytoplasmic viral orthologous genes (NCVOGs)— the 20 NCVOGs most likely to have been vertically inherited (Yutin et al. 2009). We made individual gene trees including all hits with e-values less than 1e-10 by aligning gene sequences with MAFFT version 7 using the E-INS-i algorithm (Katoh and Standley 2013), trimming the resulting alignments with the -automated1 method in TrimAl (Capella-Gutiérrez 2009), and reconstructing trees with the approximately maximum-likelihood approach implemented in FastTree (Price, Dehal, and Arkin 2009) with 100 bootstrap replicates, rooting at the midpoint. We also ran the latest version of ViralRecall (2.0; Aylward and Moniruzzaman 2021) on these 16 assemblies and used these results in combination with genome statistics to discern putatively viral contigs, which we classified as belonging to a viral genome if they met the following criteria: ViralRecall score >2, viral region length (per ViralRecall) >70% of the total contig length, and intron density > 25% different from the host average. One isolate appeared to contain two distinct NCLDVs; we used MetaBAT2 (Kang et al. 2019) on the entire genome of *Allomyces javanicus* California12 under the default settings to separate the two NCLDV genomes.

We conducted BLASTP searches of the hybrid proteomes against nr to identify homologs (e-value <1e-10). Because many symbiont genes are likely misidentified as their hosts' in the databases, we labeled a gene as "viral" or "bacterial" if any of the top hits were to a virus or bacterium. Some genes have homologs only in the fungi which we have shown also encode MCP; we labeled these "putative FNCLDV".

To assess prevalence of fungal giant virus-like MCP throughout the fungal kingdom, we used blastp to search all unmasked fungal genomes in Mycocosm (Grigoriev et al. 2014) using MCP of *Allomyces arbusculus* Burma1F as query (total 2,543 genomes; conducted on Dec 30, 2023).

Giant virus phylogeny, orthology analysis, and KilA-N domain search:

To build the giant virus species tree the nsgtree pipeline was used (https://github.com/NeLLi-team/nsgtree) on genome representatives of the viral phylum Nucleocytoviricota (Aylward et al. 2021). In brief, 7 giant virus orthologous groups (GVOGs; Aylward et al. 2021) were identified using hmmsearch, extracted GVOGs were aligned with MAFFT (version 7.31; Katoh and Standley 2013), trimmed with trimal (-gt 0.1, v1.4; Capella-Gutiérrez, Silla-Martínez, and Gabaldón 2009) and concatenated. To limit the amount of missing data we removed genomes that had less than 4 out of 7 GVOGs. A species tree was built from the supermatrix alignment using IQ-Tree (version 2.03); (Minh et al. 2020; Kalyaanamoorthy et al. 2017) with LG+I+F+G4 and ultrafast bootstrap replications (Hoang et al. 2018) and visualized in iToL (Letunic and Bork 2021).

To analyze the global distribution of *Mycodnaviridae* we used the MCP as a query for a blastp using diamond (--query-cover 30 --subject-cover 30; Buchfink, Xie, and Huson 2015) against all MCP that we previously extracted (Schulz et al. 2020) from publicly available IMG metagenomes (Chen et al. 2023). All blastp hits were then extracted and used as query for another blastp against a database consisting of all MCP extracted from representative giant virus genomes (Aylward et al. 2021) and the initial *Mycodnaviridae* MCPs. We considered as bidirectional best blastp hits (BBH) the environmental MCP that had the best hit based on bitscore to the initial *Mycodnaviridae* MCPs. Geolocation of the BBHs was then used to plot the distribution of MCPs related to *Mycodnaviridae* on a world map projection.

To search for homologs of the viral KilA-N domain in *Mycodnaviridae* genomes, the source data from figure 6 of Medina et al. 2016 (219 protein sequences) was downloaded. Sequences were aligned using mafft (version 7.31; Katoh and Standley 2013), and an hmmprofile made with HMMER hmmbuild (Eddy 2009), which was then used to hmmsearch the fungal giant virus genomes.

Orthofinder 2.5.2 (Emms and Kelly 2019) was used to identify orthologous gene clusters from the prodigal predictions of the most complete viral genomes, determined as the viral genomes containing the greatest number (at least nine) of the NCVOGs20. Visualization of the shared orthologous gene clusters was by adaptation of https://commons.wikimedia.org/wiki/File:Symmetrical_5-set_Venn_diagram.svg. Sequences of orthologous gene clusters were fed into EggNOG-mapper 5.0 (Huerta-Cepas et al. 2019) with default parameters but enabling SMART annotations.

Allomyces capsid PCR and growth experiment:

Primers were designed from an alignment of the MCP sequences of *A. javanicus* California12 and *A. arbusculus* Burma1F (F 5’ TACACCAACTTTGCGATGGA3’ R 5’ GTAGCTCGTTGACCCGACAT3’). DNA was provided by the Collection of Zoosporic Eufungi at the University of Michigan. A standard PCR was performed using GoTaq (Promega) and the products visualized by agarose gel electrophoresis for absence/presence. Burma1F was used

as a positive control, and Burma3-35 served as a negative control based on absence of the gene in the sequenced genome.

We chose 6 strains that tested positive for MCP (BEA2, Burma1F, Cali8, Cali12, FijiF1, NorthCaro2), and 6 testing negative (ATCC10983, Burma3-35, CubaS7, DJ02, DJ07, Mex58) to determine phenotypic effects of the gene's presence. Strains were grown in triplicate on Emerson's YpSS media (Emerson 1958). The inoculum for each plate was made by growing strains for 48 hours on fresh YpSS and removing a small circular inoculum by punching discs with the back of a glass pipette. After 72 hrs of growth at 35 C, linear growth was measured for each plate with two perpendicular radii. We also estimated biomass accumulation of the same strains using liquid YpSS. Triplicate 250 ml flasks with 100 ml of media were inoculated with 4 discs cut using the back of glass pipettes. The strains were grown at 35 C for 48 hours with shaking at 120 rpm. The mycelium was harvested using miracloth filtration, transferred to pre-weighed filter papers, dried completely, and dry weight was measured. A final experiment measured thermal maxima of strains by inoculation of YpSS plates and placing them at multiple temperatures: 38, 40, 42, 44, and 46 C. Duplicate plates were prepared from 9 day old YpSS plates using discs excised using glass pipettes. Plates were scored for evidence of growth, where < 2 mm of growth after 1 week was considered negative.

Nanopore Sequencing and Genome Assembly:

To obtain High Molecular Weight DNA for long read sequencing, the strain was first grown on YpSS solid medium for 3 weeks. A block (3mm x 3mm) from the edge of the growing culture was then excised and immersed in DS buffer to induce zoospore release. The spore suspension was used to inoculate 100 ml fresh YpSS liquid medium, which was then incubated for 36 hours at 33 °C in a floor shaker. After incubation, the cells were collected with strainer, washed once with PBS, frozen at -80 °C, and lyophilized. The frozen-dry cells were broken into powder using 4 mm glass beads and vortex at the maximum speed. Genomic DNA was then extracted using a modified CTAB protocol, and DNA quality was inspected with CHEF gel electrophoresis as previously described (https://doi.org/10.1073/pnas.2416656121).

Purified DNA was processed for library preparation and sequencing on an Oxford Nanopore MinION Mk1C device using R10 flow cells (FLO-MIN114) with MinKNOW UI (v23.07.12) following the sequencing kit guidelines. The generated Pod5 data was subjected to base calling using Dorado (v0.6.0) followed by genome assembly using Canu (v2.1.1). The resulting genome assembly was analyzed for the presence of the capsid sequence using BLASTn analysis and transposons were identified using Repeatmasker (v4.0.7; http://www.repeatmasker.org), supplemented with Dfam (v3.3), RepBaseRepeatMaskerEdition-20181026 libraries, and RepBase EMBL database (v26.04; Bao 2015, Storer 2021). CpG methylation analysis was performed using the nanopore Modkit tool (v0.2.5-rc1).

Transmission Electron Microscopy:

Plate cultures were flooded with WC media for 2 hours. Recovered liquid containing discharged zoospores was fixed with equal volume 3% glutaraldehyde in 0.01M Sym-collidine, incubated

for two hours at room temperature, and then overnight at 4℃. Zoospores were pelleted and washed thrice with 0.05% Sym-collidine. The pellet was secondarily fixed with 1% osmium tetroxide at room temperature for 30 minutes, followed by two washes each with 0.05% sym-collidine and water. Samples were embedded in 1% noble agar, cut into small blocks (~1mm^3) washed twice with 10% acetone, and incubated in 2% uranyl acetate at 4℃ overnight. Blocks were serially dehydrated with increasing concentrations of 10%, 30%, 50%, 75%, 95% acetone, followed by two washes with 100% acetone. Samples were infiltrated with a 3:1 acetone:Epon-Araldite resin, and incubated for 1 hr, followed by incubation in 1:1 acetone:resin for 1 hr, and again in 1:3 acetone:resin. Samples were then set in 100% resin, vacuum centrifuged overnight, and polymerized at 60℃ for 48 hours. Embedded material was sectioned at 80nm with a diamond knife, sections collected on 200 mesh formvar/carbon nickel thin bar square mesh, stained with lead citrate, and visualized on a Jeol 1400plus transmission electron microscope at the University of Michigan's Department of Molecular, Cell, and Developmental Biology Imaging Core. *Polyphlyctis willoughbyi* PL AUS 21 was previously prepared (see Letcher and Powell 2018), stored, and imaged on the Jeol 1400plus transmission electron microscope at the University of Michigan's Department of Molecular, Cell, and Developmental Biology Imaging Core.

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