Giant *Starship* elements mobilize accessory genes in fungal genomes

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Abstract

Accessory genes are variably present among members of a species and are a reservoir of adaptive functions. In bacteria, differences in gene distributions among individuals largely result from mobile elements that acquire and disperse accessory genes as cargo. In contrast, the impact of cargo-carrying elements on eukaryotic evolution remains largely unknown. Here, we show that variation in genome content within multiple fungal species is facilitated by *Starships,* a novel group of massive mobile elements that are 110 kb long on average, share conserved components, and carry diverse arrays of accessory genes. We identified hundreds of *Starship*-like regions across every major class of filamentous Ascomycetes, including 28 distinct *Starships* that range from 27-393 kb and last shared a common ancestor ca. 400 mya. Using new long-read assemblies of the plant pathogen *Macrophomina phaseolina*, we characterize 4 additional *Starships* whose past and ongoing activities contribute to standing variation in genome structure and content. One of these elements, *Voyager*, inserts into 5S rDNA and contains a candidate virulence factor whose increasing copy number has contrasting associations with pathogenic and saprophytic growth, suggesting *Voyager*'s activity underlies an ecological trade-off. We propose that *Starships* are eukaryotic analogs of bacterial integrative and conjugative elements based on parallels between their conserved components and may therefore represent the first known agents of active gene transfer in eukaryotes. Our results suggest that *Starships* have shaped the content and structure of fungal genomes for millions of years and reveal a new concerted route for evolution throughout an entire eukaryotic phylum.

1 Introduction

A landmark realization of modern evolutionary biology is that genome content is highly variable among

individuals of the same species [1]. Intraspecific genome content variation comprises a species' pangenome,

which consists of core (i.e., consistently present) and accessory (i.e., variably present) orthologs. The

pangenome concept has adjusted prior expectations of the rate of adaptive trait emergence and dispersal

in populations, as differences in accessory sequence distributions across individuals have been found to

arise rapidly and fuel phenotypic divergence [2–4]. For example, bacterial animal pathogens quickly evolve

resistance to antibiotics through the gain of entire genomic regions encoding resistance phenotypes [5] and

the host range of many fungal plant pathogens often expands through the gain of entire chromosomes

encoding host-specific virulence factors [6]. Identifying the mechanisms generating selectable variation in

the distribution of accessory genes among individuals is thus crucial for understanding the genetic bases

of adaptation [7].

Genome content variation in prokaryotes is primarily driven by mobile elements that in addition to

encoding functions for their own movement, also encode accessory genes [1]. These "cargo-carrying"

mobile elements range in size, from smaller transposons to larger genomic islands to entire DNA

molecules such as plasmids, and interact with their host genomes in diverse ways [8–10]. While some mobile

elements resemble parasites because their proliferation is predicted to come at the expense of host fitness

[11], cargo-carrying elements resemble mutualists that cooperate with host genomes because they often

carry genes encoding adaptive host traits [1]. For example, several well known integrative and conjugative

elements carry accessory genes mediating advantageous symbiosis and antibiotic resistance phenotypes

[12,13]. Cargo-carrying mobile elements are frequently transmitted through both horizontal gene transfer

(HGT) and vertical inheritance, which together generate extensive genome content variation within and

across species [1].

In contrast with prokaryotes, the contribution of mobile elements to genome content variation in

eukaryotes remains unclear, although is of increasing interest [14–16]. Eukaryotic genomes are often large

and repeat-rich, which complicates the characterization of accessory genes using short-read sequencing

methods [1]. However, long-read sequencing technologies that resolve repetitive sequence content now

enable more contiguous genome assemblies in which the movements and associations of mobile elements

can be tracked [14]. Several small cargo-carrying eukaryotic mobile elements in specific species have

recently been shown to generate genome content variation by increasing the copy numbers of their

accessory genes [17,18]. Larger eukaryotic mobile elements have only been identified recently, and though

little is known about these elements, initial studies suggest they impact host fitness, that some are horizontally acquirable, and are pervasive across multiple species [16,19–22]. Yet despite compelling preliminary evidence, the diversity, phylogenetic relationships, and contributions towards genome and pangenome evolution of large mobile elements are not well understood. Fungi are ideal for investigating these aspects of large mobile element biology due to their relatively small genomes with resolvable repeat content, notable associations between several recently described large mobile elements and phenotypes [19–21], and a vast diversity of sequenced genomes that enable kingdom-wide analyses.

Here, we investigated the contributions of large cargo-carrying mobile elements to the evolution of Ascomycete fungi, one of the most speciose, ecologically diverse and economically important groups of microbial eukaryotes. We sequenced and searched 12 genomes of the plant pathogen *Macrophomina phaseolina* for evidence of large cargo-carrying mobile elements. We found several giant mobile elements that prompted us to search for similar elements in other Ascomycetes, and test the hypothesis that they all share a common origin. Using *M. phaseolina* as a case study, we then ask how these elements shape the evolution of genome structure, genome content, and ecologically-relevant phenotypes.

Results and Discussion

M. phaseolina genomes harbor insertions that resemble mobile elements

We sequenced 12 *M. phaseolina* isolates from soybean fields in Ohio, U.S.A. and Paraguay using a combination of Nanopore long-read and Illumina short-read technologies. We used multiple assemblers and gene annotators to obtain near-chromosome level quality draft assemblies and annotations (avg. N50 = 2.43mb; avg. assembly size = 52.31mb)(Figure S1, Tables S1, Table S2, Table S3, Methods). Isolates differed by at most 1% nucleotide identity across all alignable genomic sequence, as was expected given the predominance of clonal reproduction in this species [23]. In contrast, we found that isolates harbor extensive variation in accessory genes, with gene content diverging 20 times faster than nucleotide sequence per unit of evolutionary time and differing by up to 36% (Figure S2, Table S4). *M. phaseolina*'s pangenome is "open" (Heaps' alpha = 0.46), meaning that the size of its pangenome is predicted to increase with each newly examined genome. *M. phaseolina*'s pangenome is more open than most other fungal species examined, suggesting that individuals within this species generally harbor high proportions of accessory genes (Figure S2, Table S5, Table S6). We tested whether differences in genome content arise randomly across the genome, or whether they are enriched in specific regions, and found that highly variable accessory genes were significantly enriched in "hotspot" regions (Figure S2, Table S7, Table

S8). Accessory gene hotspots do not differ from the overall genome in proportion of SNPs or indels <=

1kb, but are enriched for indels >1kb (Methods), suggesting that gene variability is associated with large

structural mutations (Figure S2, Table S9).

We hypothesized that the variation in *M. phaseolina*'s accessory genome and the generation of large

structural mutations in hotspots is facilitated by large cargo-carrying mobile elements. We searched the

12 *M. phaseolina* genomes for large insertions that had three key features typified by the recently

described giant fungal transposons *Enterprise* and *HEPHAESTUS* [19,20]: (1) a predicted gene containing a

DUF3435 domain (Protein family accession: PF11917) located at the 5' end of the element in the 5'-3'

direction. DUF3435 domains are homologous to and share conserved active sites with tyrosine site-

specific recombinases that catalyze the excision and integration of bacterial integrative and conjugative

elements [19,24,25]; (2) flanking target site duplications (TSDs) that arise when DNA transposons integrate

into their target site through staggered double stranded breaks [26]; (3) terminal inverted repeats (TIRs),

which are typically required for the excision of DNA transposons by transposases, although are not

present in *Enterprise* [19,26]. We found four very large (76,706 - 195,340 bp) elements we call *Voyager,*

Argo, Phoenix, and *Defiant* that all have a DUF3435 gene in the required position and often have TSDs

and TIRs as well (Figure 1B, Table 1, Table S10). These four elements are discrete and distinct entities

that carry diverse arrays of accessory cargo and differ in their target sites and TIRs. However, despite this

variability, DUF3435 genes in these elements often co-localize with four additional genes that are present

in variable copy numbers and conserved across elements, consisting of DUF3723s, ferric reductases

(FREs), patatin-like phosphatases (PLPs) and NOD-like receptors (NLRs) with different functional

domains (Table 2, Table S11). We refer to these genes by their predicted function throughout the text.

Starships **are a novel group of giant mobile elements widespread across Ascomycetes**

We hypothesized that the conserved associations between DUF3435s, DUF3723s, FREs, PLPs, and

NLRs are indicative of a "winning" coalition of co-functioning genes maintained through selection [27],

much like the multi-component translocative machinery of bacterial integrative and conjugative elements

[10]. We therefore predicted that they would appear together in other Fungi. We used the genes in *M.*

phaseolina's elements to query a database of 1274 Ascomycete genomes, and found 134 regions

containing homologs to DUF3435 that also colocalized to varying degrees with DUF3723s, FREs, PLPs,

and NLRs (Figure 1D, Table S12, Table S13). Using whole genome alignments, we manually annotated

the insertion sites, TSDs, and TIRs for 28 of these elements sampled across all extant classes of

filamentous Ascomycetes, providing evidence of pervasive and recent mobile activity across one of the

most diverse and speciose eukaryotic phyla (Figure 1B, Table S14, Figure S4, Figure S5).

We used the phylogenetic history of DUF3435 genes at the 5' element ends, which we refer to as

"captains", to characterize the evolutionary relationships between the various elements (Methods). Not

only did we find that captains from different elements fall into distinct clades (Figure 1A, Figure S5,

Figure S6), but we also found that the tyrosine recombinase domains of all DUF3435 captains group into

a monophyletic clade that is as distantly related to bacterial tyrosine recombinases as it is to described

tyrosine recombinases found in either fungal *Crypton* DNA transposons [28] or eukaryotic retrotransposons,

such as DIRS elements [29](Figure S7). We conclude that DUF3435-containing genes represent a novel

group of eukaryotic tyrosine recombinases that mobilize large amounts of DNA. A study that was

simultaneously published with our own independently demonstrated that elements with DUF3435-

containing genes are a new monophyletic group of transposable elements (cite V. Kapitonov here). We

consider regions bounded by TSDs that contain a DUF3435 sequence at the 5' end to be a new class of

eukaryotic mobile element that we call *Starships* (Figure 1C). Following Vogan et al., 2021, We

distinguish between different types of *Starship* elements based on their insertion sites and the

phylogenetic relationships between their DUF3435 captains. Regions that contain DUF3435 sequences

that co-localize with DUF3723s, FREs, PLPs, and/or NLRs but lack TSDs are referred to as *Starship*-like,

and regions that contain at least 3 hits to any of these conserved genes but lack DUF3435 sequence are

considered to be degraded *Starships*.

The DUF3435 phylogeny revealed that the captains of different *Starships* likely last shared a common

ancestor prior to the divergence of the Pezizomycotina, ca. 400 million years ago [30] and thus have a single

origin (Figure 1A). This phylogeny unites previously disparate phenomena in fungi, including

Enterprise[19], *HEPHAESTUS*[20], a large mobile region containing the ToxhAT transposon and ToxA

effector found in numerous wheat pathogens [21], and multiple previously described large structural variants

whose origins were unclear (Galactica_Afla, Starship-1_Afum)[31,32], by demonstrating that they are all

Starships. The majority of DUF3435s in the Ascomycota are largely restricted to clades consisting of

sequences from the same taxonomic class and multiple class-specific clades exist, consistent with an

evolutionary scenario dominated by the vertical inheritance of ancient homologs (Figure S4). This same

trend is recapitulated within individual taxa, such as *Macrophomina, Aspergillus* and *Fusarium,* that

contain multiple highly diverged and distantly related DUF3435s within their genomes (Figure S5).

However, HGT has been implicated in *Starship* transfers among closely related taxa in at least two

independent examples [20,21], and rates of HGT are expected to increase with donor-recipient relatedness [33],

suggesting HGT may be more prevalent at finer grained taxonomic scales beyond the scope of this study.

Starship accessory cargo suggests a range of impacts on host fitness

The diversity of accessory cargo found across *Starships* suggests their interactions with host genomes
span the conflict-cooperation spectrum. For example, some predicted cargo suggests mobile element
fitness could come at the host's expense: numerous *Starships* contain homologs to spore killing (*Spok*)
genes that encode a toxin-antitoxin meiotic drive system capable of killing developing sexual progeny in
Enterprise [19,34] (Figure 1B, Table S13). In contrast, many cargo genes have putatively adaptive functions
that would hypothetically benefit the host in certain environments: several carry biosynthetic gene
clusters (BGCs) involved in the production of secondary metabolites (e.g., *Defiant* and *Galactica*); others
carry characterized virulence factors or homologs of known virulence factors (e.g., *ToxA* [21], Ago1 in *Argo*
[35], OCH1 in *Voyager and Derelict* [36–38]); *HEPHAESTUS* contains several genes characterized to
contribute to heavy metal resistance[20]; finally, FREs that are conserved across multiple *Starships* are part
of a large metalloreductase family with several characterized members, suggesting some confer benefit to
the host, for example, in the acquisition of iron in growth limiting environments or contributions to
pathogen virulence [39–41]. While the few *Starship*-like regions we identified in our Basidiomycete outgroup
lacked FRE homologs, they contain homologs to Fet3p and Ftr1p which form a high affinity iron
permease complex in yeast [42], suggesting a functional association convergently evolved between iron
metabolism and *Starships* (Figure 1B). Carrying host-beneficial cargo may be an especially important
trait for elements that are predominantly inherited through vertical means, as their own fitness is closely
aligned with their host's reproductive success [43].

Starship activity shapes the distribution of accessory genes in *M. phaseolina* populations

We carried out an in-depth characterization of *M. phaseolina*'s *Starships* to better understand how these
larger elements shape variation in accessory genome content. *Starship* elements in *M. phaseolina* differ in
copy number, genetic cargo, and generally have little alignable sequence in common such that they each
contribute in distinct ways to content divergence across *M. phaseolina* isolates (Figure 2A, Table 1, Table
S15). Extensive content variation even exists within certain *Starship* element types, such as *Phoenix* and
Defiant. For example, we found a putative cargo turn-over event in two *Defiant* elements that carry either
a carotenoid BGC or a non-ribosomal peptide synthetase (NRPS) BGC (Figure 2D). Within any given
genome, 85-100% of *Starships* overlap with accessory gene hotspots indicating that they are hotspots for
gene gain and loss (Methods, Figure 2C, Table S16). Specific *Starship* elements are further linked to the
expansion of accessory gene families they carry as cargo (Figure 2B). For example, over half of the

OCH1 homologs (59/109) and just over a third of the FRE homologs (112/316) in the *M. phaseolina*

population are located in *Starship* or *Starship*-like regions (Figure 2B, Figure S8, Figure S9). Conserved

cargo genes and functional domains carried on *Starships*, including OCH1, PLP, FRE, DUF3723 and the

HET and NACHT domains in NLRs, are typically restricted to clades of *Starship*-specific sequences,

indicative of cargo-*Starship* co-diversification (Figure 2B, Figure S8, Figure S9, Figure S10, Figure S11,

Figure S12, Figure S13). Content diversity and flexibility, coupled with a transposition mechanism,

underscores the potential for *Starships* to shape the distribution of accessory genes among individuals.

Not only do *Starship* elements actively generate new variation, but remnants of degraded *Starship*-like

regions found throughout the genomes of *M. phaseolina* suggest their impact extends beyond the life of

the element. For example, we discovered a group of regions, which we call *Derelict,* that have *Starship*-

specific cargo but lack DUF3435 homologs (Figure 1A, Table S11). Like active *Starships, Derelicts* show

large swaths of similarity among isolates and copies (avg. 41% alignable sequence), but otherwise are

distinct from other elements (Figure 2A). They have homologs of FRE, DUF3723, and OCH1 that form

large monophyletic clades within their phylogenies, and these sequences are in general more closely

related to their homologs in other *Starships* than they are to non-*Starship* associated sequences,

suggesting that *Derelicts* are old copies of a *Starship* whose captain degraded but whose cargo has

persisted (Figure 2B, Figure S9, Figure S8, Figure S13). We find many additional *Starship*-like regions

that similarly lack DUF3435 homologs but have conserved *Starship* content and occasionally aligned to

characterized *Starship* elements, suggesting they have recently degraded (Table S11). Across all

genomes, 72-100% of these degraded regions overlapped with an accessory gene hotspot, indicating that

they have remained a source of genome content variation among individuals (Table S16). Across the 1274

Ascomycete genome database, we similarly found 102 *Starship*-like regions that did not have DUF3435

genes, but had at least four hits to FRE, DUF3723, NLRs, and PLP sequences, suggesting these regions

may also be degraded *Starship* elements (Table S13). Evidence of cargo that persists after element

degradation implicates *Starship* birth-and-death cycles as a fundamental mechanism shaping variation in

genome content among individuals.

***Starships* contribute to standing variation in the structure of *M. phaseolina*'s genome**

Many fungal genomes are compartmentalized into accessory chromosomes that are variably present

among individuals, and core chromosomes with large tracts of conserved synteny across individuals [44]. In

M. phaseolina, we identified 10 large "core contigs" that are largely syntenic, often have telomeric

repeats at either end, and are present across all individuals (Figure 3, Figure S3, Table S17, Table S18,

Methods). We found that on average, 88.6% of each *M. phaseolina* genome is captured by these core contigs, while the rest is located in unplaced contigs that do not align to the core contigs (Table S19). However, core contigs in the *M. phaseolina* population are highly rearranged and contain large tracts of variably present sequence, such that none of the core contigs in the eight highest quality assemblies share the same structure or content across all isolates (Figure S3), consistent with previous observations in this species [45].

We therefore examined associations between *M. phaseolina*'s *Starships* and large-scale differences in genome structure, which may be facilitated by their ability to insert into otherwise conserved sequence tracts (Figure 3B, event 3). We identified 37 large translocations, inversions and chromosome fusions in the *M. phaseolina* population, and 30% of these had *Starships, Starship*-like regions, or degraded *Starships* overlapping or near their breakpoints (Table S20). For example, a degraded *Defiant* element is found at the junction of a putative fusion between core chromosomes 9 and 1 in *M. phaseolina* isolate mp247, and a reciprocal translocation between core chromosomes 9 and 3 in *M. phaseolina* isolate mp042 occurs precisely at a *Voyager* insertion (Figure 3B, events 1 and 2). Due to their sheer size, sequence similarity, and the fact that they are themselves colonized by multi-copy mobile elements including LTRs (Table S10), *Starship* insertions may provide opportunities for rearrangements to occur through ectopic recombination [46], although our data cannot confirm whether they are the causal mechanism. However, 95% of the rearrangements overlapped with some sort of mobile element, in general agreement with previous observations that mobile elements are associated with large scale rearrangements in fungal genomes [46,47].

Starship activity is also linked to the active growth of large genomic regions whose presence varies among individuals. Gene phylogenies of DUF3435s and other conserved cargo suggest *Starships* can sequentially nest within each other, generating large tracts of accessory sequence (Figure S6). For example, a degraded *Voyager* insertion in *M. phaseolina* isolate mp247 has at least three *Starship* or *Starship*-like elements that have sequentially inserted into each other to produce a repeat-rich region spanning 221,058 bp (Figure 2D). Mobile element nesting is a common mechanism of bacterial integrative and conjugative element growth [48] and has also been observed in *HEPHAESTUS* [20]. In addition to generating variable regions on otherwise core contigs, *Starships* and *Starship*-like regions may also contribute to the birth and/or growth of accessory chromosomes. Contig ac11-1, a putative accessory chromosome in *M. phaseolina* isolate mp053, is ~ 800 kb, with *Starship-like* regions composing roughly 16% of its length.

 ***Voyager*: a massive mobile element targeting 5S rDNA**

 The most active *Starship* in the *M. phaseolina* population is *Voyager,* ranging in copy number from 0-4

 across individuals (Figure 4A). We resolved 6 different *Voyager* insertion sites that have clear

 breakpoints and high Illumina read coverage across occupied and empty sites (Figure 4C, Table S21). In

 isolates with a given *Voyager* insertion, flanking regions are syntenic with the corresponding insertion site

 in isolates that do not have the insertion, ruling out the alternative explanation that *Voyager* has moved by

 chromosomal translocation. Based on these resolved insertion sites, we recovered 15 full-length copies of

 Voyager with identifiable TSDs and TIRs (Figure 4B, Table 1). We also found an additional 7 fragmented

 copies from the two lowest quality assemblies (mp124 and mp194) that had recoverable DUF3435

 sequences, but were excluded from the following summary statistic calculations. *Voyager* elements range

 from 74,275 bp to 124,223 bp in length (avg. 96,567 bp), contain between 10-47 predicted protein-coding

 sequences (avg. 37), and are alignable from start to end. Variation in size and in protein-coding sequences

 among *Voyager* copies is largely due to variation in transposable element content, which ranges from 15-

 50% (avg. 31.2%; Table S21).

 Although *Voyager* copies are found at multiple sites in any given genome, they are always inserted into a

 copy of the 5S rDNA gene, 20 bp from the gene's 5' start site and 110 bp from the 3' end. The insertion

 of *Voyager* into the 5S rDNA gene shifts the free energy of the 5S rRNA's predicted secondary structure

 from an estimated -42.22 kcal/mol to -38.49 kcal/mol such that we predict it loses function. Due to

 experiencing strong purifying selection[49], rDNA sequences have been proposed as stable mobile element

 target sites enabling long-term persistence[50]. Similarly, many bacterial integrative and conjugative

 elements insert in or near tRNA genes which would also provide stable target sites [9,24]. We estimate that

 M. phaseolina has upwards of 45 copies of 5S rDNA genes dispersed across its genome that are fixed in

 the population, similar to copy numbers observed in other Ascomycete fungi[49]. *M. phaseolina* has at least

 2 distinct variants of the 5S rDNA gene, and *Voyager* insertions and target sites are only present in one of

 them, raising the possibility that changes to the 5S sequence of this species are selected to avoid being

 disrupted by *Voyager*. Additionally, we identified a *Starship* in *Alternaria tenuissima* that has distinct

 cargo but whose captain is closely related to *Voyager*'s and whose target site is similarly located inside 5S

 rDNA sequence (Figure 1B). This suggests that targeting conserved host genes is a broadly successful

 Starship strategy even though it may open the door to potential conflict with host genomes.

 ***Voyager* has contrasting associations with pathogenic and saprophytic growth**

Insight into the genetic bases of virulence in *M. phaseolina* is critically needed because this pathogen

represents an emerging threat to food security: it can infect more than 500 plant species, including many

agricultural crops, and is increasingly expanding into new geographic regions. Although mobile element

insertions in rDNA tend to negatively impact growth [51,52], the majority of *Voyager* elements also carry

homologs of the virulence factor OCH1, a conserved α-1,6-mannosyltransferase that contributes to cell

wall integrity and pathogenicity in diverse plant and animal pathogens [36–38]. We therefore hypothesized

that *M. phaseolina*'s growth on its host would increase with the number of OCH1-carrying *Voyager*

elements present in the genome. We inoculated soybean seedlings in a greenhouse, and measured the area

under the disease progress curve (AUDPC) as a proxy for pathogenic growth (n = 12 isolates, 12

replicates/isolate; Table S22). We similarly inoculated potato dextrose agar (PDA) plates placed at six

different temperatures in an incubator, and measured the area under the growth progress curves (AUGPC)

as proxies for saprophytic growth (n = 12 isolates, 3 replicates/isolate/temperature; Table S23). Using

linear models corrected for phylogenetic relatedness among isolates (Table S24), we found a significant

positive linear and negative quadratic relationship between *Voyager* OCH1 copy number and pathogenic

growth ($y = 138.3 + 93.6x - 22.5x^2$; $P_{linear}=0.05$, $P_{quadratic}=0.03$) that suggests the predicted contributions of

Voyager OCH1 to pathogenicity are greatest at intermediate copy number but then decline. Conversely,

we found a marginally significant negative linear and positive quadratic relationship for saprophytic

growth at 20ºC ($y = 201.1 -31.6x + 7.3x^2$; $P_{linear}=0.10$, $P_{quadratic}=0.07$) and a significant negative linear and

positive quadratic relationship for saprophytic growth at 30 ºC ($y = 306.7 - 35.9x + 7x^2$; $P_{linear}<0.01$,

$P_{quadratic}<0.01$) that together suggest the contributions of *Voyager* OCH1 to saprotrophy are lowest at

intermediate copy number. These results are consistent with a scenario where *Voyager* incurs an

ecological trade-off between *M. phaseolina*'s ability to grow on or off of its host, and suggests alternating

host and non-host environments help maintain variation in virulence phenotypes in natural populations.

However, our small sample size (n=12) demands that observed trends be confirmed once more

individuals are genotyped for *Voyager* insertions. The complex associations of *Voygager* with fungal-

plant interactions may predate the origin of the *Macrophomina* genus because we identified a putatively

vertically inherited copy of *Voyager* in the closely related plant pathogen *Botryosphaeria dothidea* that

has the same target site and also carries OCH1, but may not be presently active due to an LTR insertion

into the reading frame of the captain (Table S14, Figure S8). Vertical inheritance of the same element

strengthens the hypothesis that conserved host genes provide stable target sites, and underscores how this

strategy may enable persistence through speciation events.

Are *Starships* agents of active gene transfer in eukaryotes?

Starships are distinct from other eukaryotic mobile elements not only in their consistently large size, but

in that many of them share a conserved set of accessory genes (Table 2). Although the functions of these

genes are not known, they present parallels to translocative and dispersive modules in bacterial integrative

and conjugative elements, especially those found in Actinomycetes who produce coenocytic hyphal

networks resembling filamentous Ascomycete fungi. Like many of these bacterial elements, *Starships* are

likely mobilized by site-specific tyrosine recombinases located at the 5' end of the element [10,53].

DUF3723s are homologous to DNA-binding bacterial chromosome segregation genes (TIGRFAM

accession: TIGR02168), and present a functional analogy to DNA translocases that catalyze bacterial

element transfer [10]. NLRs with HET, NACHT and PLP domains govern hyphal fusion events in fungi [54,55]

and present parallels with Nudix hydrolase genes that govern Actinomycete hyphal fusion prior to

integrative and conjugative element transfer [10]. Genes with NUDIX domains are also found in several

Starships (*Galactica, Starship-2_Afla*) (Table S13). Together, these shared features suggest *Starships*

encode mechanisms not only for their own excision and integration but for their transfer between

individual genomes, and provide testable hypotheses for elucidating a mechanism of fungal HGT, which

has long been searched for.

Conclusion

Starships: new frontiers for eukaryotic genome evolution

Starships represent a dramatic shift in our understanding of how mobile elements shape eukaryotic

genome and pangenome evolution [14]. They add to the growing realization that eukaryotic genomes,

ranging from fungi to algae to animals, are frequently colonized by large mobile elements, and their

activity casts the impact of these other elements in a new light [14–16]. Using the plant pathogen *M.*

phaseolina as a model, we demonstrate that *Starships* link the processes of mobile element activity,

structural variant generation and genome content evolution such that *Starship* birth-and-death lifecycles

likely hold multi-faceted consequences for their hosts. Our work with *M. phaseolina* further suggests that

Starship characterization will be important for understanding the emergence of virulence in pathogen

populations. *Starships* carrying diverse accessory genes are found in fungi ranging from saprotrophs to

lichens to human and plant pathogens and are further known to encode adaptive traits [20,21], which coupled

with their potential role in mediating life history trade-offs, underscores their vast potential for shaping

ecological outcomes across an entire kingdom. The *Starship* activity that we observe in present-day

fungal populations likely represents only a fraction of their historical impact, as their activity has been

ongoing for millions of years. Together, our results establish a novel framework for investigating how

mobile element cargo carriers shape the (pan)genomic bases of fungal adaptation, pushing our

understanding of eukaryotic evolution to bold, new frontiers.

Starship element	Avg. length in bp (s.d.)	Avg. number of genes (s.d.)	Avg. % repeat content (s.d.)	Copies per isolate, min-max	Target site
Voyager	96,567 (13,781)	37 (9)	30.4 (10.4)	0-4	AGAATACAGGGC
Argo	75,148 (14,824)	26 (4)	9.5 (2.1)	0-1	n.d.
Phoenix	74,447 (94,611)	21 (25)	44.6 (25.7)	0-3	TTTACA
Defiant	108,860 (57,441)	28 (15)	37.7 (15.5)	0-5	ACTA

340   Tables

341   Table 1: Summary statistics of *Starship* elements in *Macrophomina phaseolina*
342
343
344   Table 2: Conserved *Starship* cargo genes

Gene name	*Voyager* reference gene(s)	Protein family domains	Predicted function
DUF3435	mp102_11332	PF11917 (DUF3435)	Tyrosine recombinase
DUF3723	mp102_11356	PF12520 (DUF3723)	Unknown
FRE	mp102_11352	PF01794 (Ferric_reduc)	Ferric reductase
		PF08022 (FAD_binding_8)	
		PF08030 (NAD_binding_6)	
PLP	mp102_11357	PF01734 (Patatin)	Patatin-like phosphatase
NLR	mp102_11337	PF17107 (SesA)	NOD-like receptor
		PF05729 (NACHT)	
		PF12796 (Ank_2)	
		PF00023 (Ank)	
	mp102_ofg737	PF06985 (HET)	NOD-like receptor
		PF05729 (NACHT)	

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Figure 1: Giant *Starship* elements found across the Ascomycota share conserved components and mobilzie diverse arrays of accessory genes. A) To the bottom-left is a circular maximum likelihood tree of 1890 DUF3435 homologs retrieved from a database containing 12 *Macrophomina phaseolina* genomes generated for this study and 1274 published Ascomycete genomes, where branches are color-coded according to taxonomic class. Black dots along the perimeter indicate DUF3435 sequences found in 194 *Starships* and *Starship*-like regions (60 of which are from *M. phaseolina*), and to the right is a maximum likelihood tree of these sequences and several others of interest, where clades are depicted as triangles for clarity and all branches with <80% SH-aLRT and <95% UFboot support have been collapsed. A clade of degraded Starships that lack DUF3435 sequences is juxtaposed below the tree for visualization purposes only. B) Fungal species, taxonomic class, Starship name and simplified *Starship* schematics, with approximate locations of genes of interest depicted as colored boxes or numbered. Genes are not drawn to scale. Target site duplications (TSDs), when indicated, were confirmed from alignments of insertion polymorphisms. Names of elements from *M. phaseolina* are in bold, and previously published elements are indicated with an asterisk. C) A schematic showing the general organization of *Starship* mobile elements. D) Proportion of 134 *Starships* and *Starship*-like regions containing homologs to five genes of interest. Gene name abbreviations: FRE (ferric reductase); PLP (patatin-like phospholipase); NLR (Nucleotide Oligomerization Domain-like receptor); Spok (spore killer); OCH1 (alpha-1,6-mannosyltransferase); NRPS (non-ribosomal peptide synthetase); Ago1 (Argonaute). Other abbreviations: BGC (biosynthetic gene cluster). All original trees can be found in the supporting data files.

Figure 2: Distinct Starship elements in *Macrophomina phaseolina* vary in copy number across individuals and generate intraspecific variation in genome content. A) A heatmap summarizing the percentage of pairwise alignable sequence between all *Starships* and *Starship*-like regions, as well as the degraded *Starship* region *Derelict*, found across 12 *M. phaseolina* isolates, where rows and columns represent individual regions arranged by hierarchical clustering according to similarity in their alignment scores. B) Maximum likelihood phylogenies of genes homologous to OCH1 and FREs, as well as genes containing and HET and NACHT domains found in fungal NLRs, retrieved from the *M. phaseolina* genomes, where all branches with <80% SH-aLRT and <95% UFboot support have been collapsed. Genes found in regions of interest are highlighted by different colors. C) A circos plot depicting all contigs >100kb in the *M. phaseolina* mp102 genome. Core contigs are partially alignable across all isolates, while unplaced contigs are not. Regions of interest are labeled and visualized on the inner track. Sites with resolved *Starship* insertions in other genomes, but absent in mp102, are labeled on the outside track. Accessory gene hotspots are labeled in orange on the outer track. D) A schematic depicting a sequential *Starship* insertion event where an unknown DUF3435 gene is found nested in *Phoenix*, which itself is nested in a degraded *Voyager* element in isolate mp247 on contig cc6-2. E) A schematic depicting a cargo turn-over event between two *Defiants* carrying either a carotenoid or non-ribosomal peptide synthetase (NRPS) biosynthetic gene cluster (BGC). Gene name abbreviations: OCH1 (alpha- 1,6- mannosyltransferase); FRE (ferric reductase); NLR (Nucleotide Oligomerization Domain-like receptor);

Figure 3: Several *Starship* elements are associated with large structural variants across *Macrophomina phaseolina* genomes. A) Alignment-based chromosome maps of four *M. phaseolina* isolates and a hypothetical ancestral genome, where contigs that share homology are colored and ordered according to their putative chromosome of origin. Putative telomeres are labeled at contigs ends, and consist of at least 5 sequential telomeric repeats, unless indicated otherwise by bracketed numbers. Unplaced contigs are not illustrated. B) From left to right, circos-based nucmer alignments of (1) a putative chromosomal fusion, (2) a reciprocal translocation, and (3) a *Voyager* insertion polymorphism. Contigs are labeled according to chromosome of origin, or otherwise colored gray. *Starships* and degraded *Starships* are drawn in dark gray, labeled and outlined in black. Labeled numbers correspond to events depicted in both the chromosome maps and the circos alignments.

Figure 4: Increasing *Voyager* OCH1 copy number has contrasting effects on *Macrophomina phaseolina*'s pathogenic and saprotrophic growth. A) A schematic of the reference *Voyager* element in *M. phaseolina* mp102 (ID: mp102lvoyagerIh11) depicting all predicted genes and annotated repetitive elements B) A multiple sequence alignment of the *Voyager* insertion cc10.1, with annotated target site duplications (TSDs) and terminal inverted repeats (TIRs) C) A circos plot of six fully-resolved *Voyager* insertions with 10kb flanking regions, labeled by insertion ID and length. Tracks 1-2 depict genes in the + and - orientation, respectively. Tracks 3-14 depict the percent nucleotide identity of sequence alignments at each locus across 12 *M. phaseolina* genomes, arranged by increasing isolate number. The density plot in Track 15 depicts median Illumina coverage across intact 5S rDNA insertion sites in isolates with empty insertion sites, and ranges from 0-77x. The density plot in Track 16 depicts median Illumina coverage across *Voyager* insertions and their flanking regions, and ranges from 0-100x. D) Scatterplots (jitter = 0.05) depicting mean area under the disease progress curve (AUDPC; top) and mean area under the growth progress curve (AUGPC; bottom) phenotypes as a function of the number of OCH1 homologs found in *Voyager Starships*, for each of the 12 isolates. AUGPC was measured at 6 different temperatures. Error bars indicate +/- standard error, and are visible if they exceed the area of the point. Predicted quadratic equations with shaded 95% confidence intervals are superimposed on the plots. Pvalues for significant (P <0.05) and marginally significant (P<0.10) linear and quadratic coefficients are shown, and others in Table S24. Abbreviations: OCH1 (alpha-1,6- mannosyltransferase); PDA (potato dextrose agar).

 Supplemental information

Supplemental Tables:

Table S1: Macrophomina phaseolina isolate metadata

Table S2: M. phaseolina genome assembly quality assessment

Table S3: M. phaseolina contigIDs and corresponding accessions

Table S4: All pairwise M. phaseolina genome comparison statistics (used for Figure S2A)

Table S5: Metadata of fungal genomes used for pangenome analyses (used for Figure S2B)

Table S6: Pangenome rarefactions (used for Figure S2B)

Table S7: M. phaseolina accessory gene hotspot coordinates

Table S8: Accessory gene hotspot summary statistics

Table S9: Mutation densities in accessory gene hotspots and non-hotspot regions

Table S10: Starship and Starship-like region summary statistics (summarized from Table S11)

Table S11: Coordinates and annotations of all genes in all Starship regions in M. phaseolina

Table S12: All metadata of additional genomes that were searched for Starships

Table S13: Coordinates and annotations of all genes in all identified Starship and Starship-like regions

Table S14: Metadata of all annotated Starships identified in this study

Figure S15: All pairwise nucmer alignments between Starships and Starship-like regions in M. phaseolina

Table S16: Per M. phaseolina genome Starships and Starship-like regions summary statistics

(summarized from Table S11)

Table S17: All contigs that align to core M. phaseolina contigs (putative chromosomes)

Table S18: Telomeric repeats in Nanopore reads mapping to contig ends

Table S19: Summary statistics of core and unplaced contigs across all M. phaseolina isolates

Table S20: All coordinates and read coverage of large structural variants

Table S21: All coordinates of identified Voyager insertion sites plus 10kb flanking regions

Table S22: Area under the disease progress curve (AUDPC) measurements on soybean seedlings

Table S23: Area under the growth progress curve (AUGPC) measurements on PDA media

Table S24: Phylogenetic generalized least squares models and test statistics

Table S25: Sequence information gained by assembly merging

Table S26: RIPper output

Table S27: Mating-type loci in M. phaseolina

Supplemental Figures:

Figure S1: genome overview

Figure S2: pangenome overview and hotspots

Data availability: This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank

under the Biosample accessions SAMN17156263-SAMN17156274. All additional supporting data,

including predicted protein sequences, GFFs, repeat libraries, characterized *Starship* elements, sequence

alignments and newick tree files are available from the following Figshare repository (DOI:

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editing the manuscript.

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  Methods

Macrophomina phaseolina **isolation**

Composite soil samples were collected from six soybean fields in Paraguay (2014-2015) and six in Ohio,

USA (2013-2014)(Figure S1). A 10-hectare area was defined in each field and with a 2.5-cm-diameter

cylindrical probe, 20 to 25 soil cores were collected at a 25 cm depth and thoroughly mixed. From each

composite soil sample, *M. phaseolina* was isolated from a 1-gram subsample of air-dried soil as

previously described[56]. Colony forming units (CFU) morphologically identified as *M. phaseolina* were

transferred to a new PDA plate to obtain pure isolates.

Phenotyping on soybean seedlings

A modified soybean cut-stem inoculation technique was used to assess the pathogenicity of the *M.*

phaseolina isolates[57,58]. In the greenhouse, soybean plants were grown to the second stage of vegetative

growth[59]. The stem was cut 25 mm above the second unifoliate node with a surface sterilized scalpel. A

mycelial plug from each *M. phaseolina* isolate was placed on top of the cut stem. Control plants were

prepared by placing an uninoculated PDA plug on the cut stem. A 200 µl pipette tip was used to cover the

stem and mycelial plug to ensure contact of the fungus and the plant and to prevent the plug from drying

out. Plants were arranged in a randomized complete block design with 6 blocks and two plants per

experimental unit. Soybean seeds were sown in 48-cell 4×12 plastic trays filled with potting mix (ProMix

BX, Premier Horticulture Ltd.) and thinned to one plant per cell post emergence. Two adjacent plants

were considered an experimental unit, and one block was composed of a flat containing 26 plants (used

for 12 *M. phaseolina* isolates and 1 control). A total of six blocks were placed on a greenhouse bench and

covered with a plastic tent, to maintain temperature and humidity. Plants were watered as needed,

temperature maintained at 27°C ± 3°C, and a 16-h photoperiod. Three days post inoculation (dpi), pipette

tips were removed, and the length of the necrotic lesion was measured at 3, 6, 9, and 12 dpi. The area

under the disease progress curve (AUDPC) was calculated using the agricolae package in R (version

4.1.0) for each isolate in each block[60,61]. We replicated the above experiment in 2018 and in 2021.

Analysis of variance revealed that the two experiments were not statistically different ($P = 0.532$),

therefore, data from both experiments was pooled. An average AUDPC for each isolate was then

determined by averaging all values across the 12 blocks from the two experiments.

Phenotyping on PDA media

From each *M. phaseolina* isolate, a 6-mm-diameter mycelial plug was placed in the center of a 100-mm-

diameter Petri dish containing PDA. Each Petri dish was considered an experimental unit and arranged in

a randomized complete block design with 3 blocks, each corresponding to a different shelf in an

incubator. Experimental units were incubated in the dark in the incubator for each of the above

temperatures. Colony diameter was measured every 24 hours for five days and these measurements were

used to calculate the area under the growing progress curve (AUGPC) for each isolate in each block using

the agricolae package in R (version 4.1.0) as previously described[60,62]. An average AUGPC for each

isolate was determined by averaging values across the 3 blocks. This experiment was repeated using

incubators set at 15, 20, 25, 30, 35, and 40°C

DNA extraction, library prep and genome sequencing

Isolates were grown in potato dextrose broth (PDB) by shaking cultures in the dark for 3-5 days at 27°C.

Mycelia were sieved through autoclaved Miracloth (EMD Millipore, Darmstadt, Germany) and rinsed

with sterile distilled water. Mycelia were flash-frozen in liquid nitrogen and stored at -20°C until genomic

DNA extraction. Frozen fungal mycelia were ground with a mortar and pestle and genomic DNA was

extracted using the DNeasy Plant Mini Kit (Qiagen, GmbH, Germany) following manufacturer's

protocol. DNA libraries were prepared for short-read Illumina Paired End 300 sequencing using the

Indexed Truseq DNA Library Preparation Kit (cat.#: FC-121-2001). Four samples were sequenced per

Mi-Seq Illumina flow cell with 600 cycles (Illumina, San Diego, CA, USA). DNA libraries for isolates

mp007, mp021, mp124, and mp194 were prepared for Oxford Nanopore long read sequencing using the

indexed Nanopore Ligation Sequencing Kit SQK-LS108, while all others were prepared using SQK-

LS109. Two DNA samples were sequenced per MinION FLO-MIN-106 flow cell with R9.4.1 1D

chemistry (Oxford Nanopore Technologies, New York, NY, USA). All library preparation and

sequencing was carried out by the Molecular and Cellular and Imaging Center (Wooster, OH, USA).

Read processing, genome assembly, and chromosome annotations

Illumina reads were trimmed and quality filtered using Trimmomatic v0.36 (ILLUMINACLIP:TruSeq3-

PE.fa:2:30:10:11 HEADCROP:10 CROP:285 SLIDINGWINDOW:50:25 MINLEN:100). Nanopore

reads were basecalled and de-multiplexed with Albacore v2.2.4 (default settings; Oxford Nanopore

Technologies), and filtered with Porechop v0.2.3 to remove adaptors, to only retain reads with at least 1

barcode, and to split chimeric reads (default settings; https://github.com/rrwick/Porechop). Given that

different genome assemblers often include different amounts of sequence in their final output, we merged

the non-redundant output of multiple assemblers to obtain the most complete assemblies possible. First,

filtered Illumina and Nanopore reads were used to generate hybrid assemblies with SPAdes v3.12 (--

careful, -k 55,77,99,127 --cov-cutoff auto)[63]. Then, unfiltered Illumina and filtered Nanopore reads were

used to generate hybrid assemblies with MaSuRCA v3.2.8 (default settings)[64] that underwent 10 rounds

of polishing by Pilon v1.22 using filtered Illumina reads to correct bases, fix mis-assemblies, and fill gaps

(default settings)[65]. All contigs from the SPAdes assembly were then aligned to the MaSuRCA assembly

using nucmer v4.0.0beta (delta-filter options: -q)[66], and contigs fully unique to the SPAdes assembly were

added to the MaSuRCA assembly to obtain the final assemblies, which were modestly larger than the

MaSuRCA assemblies alone (Table S25). We calculated pairwise percent nucleotide identity of alignable

sequence between all assemblies as "Length of alignable and identical sequence / Total length of

alignable sequence" using nucmer v4.0.0beta (--maxmatch).

We classified contigs into provisional core chromosomes using multiple criteria. First, to leverage

population-level data that could help in the annotation of telomeres from specific isolates, we mapped all

long-read sequences to the ends of each contig >10kb in each isolate. We kept all reads that aligned to at

least 1000bp at the contig ends, and considered at least 5 sequential fungal telomeric repeats $(TTAGGG)_n$

to indicate the presence of a putative telomere. We then grouped contigs into homologous sets using a

protocol inspired by graph-based methods to derive gene ortholog groups[67]. We conducted all possible

pairwise genome alignments using the minimap2 aligner in RagTag[68], which outputs a grouping

confidence score for all contig pairs calculated as the number of base pairs a query contig covers in its

assigned reference contigs divided by the total number of covered base pairs in the entire reference

genome. We took the best confidence score >0.5 for each contig pair, and input them into the MCL

clustering algorithm(inflation = 1.7)[69] such that sets of contigs with similar scores were designated as

homologous. However, this alignment-based method failed to account for large-scale rearrangements and

translocations, such that contigs that were clearly rearranged chromosomes were being grouped together.

We therefore took a conservative approach for our final classification of contigs into core chromosomes

by aligning the two highest-quality genomes that were most distantly related to each other (mp102 and

mp053; Figure S1), and designating all of their shared chromosome-scale contigs as 'core' (Figure S3).

We then re-aligned contigs >1kb in all genomes to mp102's core chromosomes using the nucmer aligner

in RagTag (-i 0.2 -a 0.2 -f 1000 --remove-small --nucmer-params '--mum -l 200 -c 500'), and designated

all contigs aligning to these core chromosomes as 'core', and all others as 'unplaced'. To generate maps

of putative chromosome structure among eight well assembled isolates, contigs were manually grouped

based on nucmer alignments to mp102, or to mp053 for the mp102 strain. Only contigs >500 kb were

evaluated. Of note, all strains except mp053 have a break in chromosome 1 at a large track ($>$ 50 kb) of

satellite sequence (satellite-7 in the repeat library) and are inferred to represent a single chromosome

based on shared presence of satellite-7 at contig ends and alignments to this region in mp053.

The features of these accessory contigs additionally provided new insight into the biology of *M.*

phaseolina. Many accessory contigs showed signatures of repeat-induced point mutations (RIP), a fungal

genome defense mechanism that introduces cytosine to thymine transitions into repetitive DNA, which

was not previously thought to be active in *M. phaseolina*[23]. Counter to the prevailing reports that *M.*

phaseolina reproduces strictly through asexual propagation, the contrasting patterns of RIP across the

twelve isolates suggest sexual reproduction has occurred at least once since their last common ancestor, as

RIP only occurs immediately prior to sexual reproduction and meiosis (Table S26). In support of this

hypothesis, we also observed two different mating type loci in isolates from both geographic regions

suggestive of an intact bipolar heterothallic reproductive system (Table S27).

***De novo* genome annotations**

All final assemblies were annotated using a pipeline that integrated the output of multiple *de novo* gene

prediction tools (Figure S14). Briefly, RepeatModeler v2.0.1 [70] using the Repbase database [71] was used to

generate a custom repeat library that was combined with previously published transcriptome and

annotated proteome data from *M. phaseolina*[23] to generate initial gene predictions using MAKER2[72].

High quality predictions were used to train SNAP[73], whose output was then combined with a *M.*

phaseolina-specific Augustus parameter file[74] and the output of self-trained GeneMark[75] in a second

round of MAKER2. High quality predictions were used to retrain parameter files for SNAP and

Augustus, combined with the output of GeneMark, and used in a final round of MAKER2 to generate

high-quality *de novo* gene annotations. To standardize annotations across all isolates, a crucial step for

downstream comparative analyses, OrthoFiller was used for each genome to annotate any missing genes

that were present in at least two other isolates[76]. All predicted proteins were annotated with Protein family

(Pfam) domains and Gene Ontology (GO) terms using interproscan v5.48-83[77]. All assemblies were

annotated for signatures of RIP using RIPper (default settings)[78]. To generate a custom repeat database for

M. phaseolina, the output of default runs from each *M. phaseolina* genome were inspected manually to

produce a fully classified and annotated library. The manual steps included removing redundancies and

putative cellular genes, and ensuring repeats were properly annotated from beginning to end. Due to high

divergence among individual repeat families (due to RIP), individual representative sequences were used

in lieu of consensus sequences. Classification of most elements was determined using the standard

schema provided by Wicker et al. [26]. Non-canonical helitrons were discovered, and classified and

annotated according to Chellapan et al. [79]. Initially, repeats containing DUF3435 domains were annotated

as *Cryptons*, and entered into the library. Many of these were later reclassified as *Starships* and given new

names.

Whole genome alignments and SNP tree

Whole genome alignments of the 12 new *M. phaseolina* genomes, a previously published *M. phaseolina*

reference[23] and an outgroup species *Botryosphaeria dothidea*[80] were generated with Cactus [81] and

projected against isolate mp102. MafFilter[82] with recommended parameters[83] was used to filter

alignments and extract 3,227,097 SNP sites that were then concatenated and used to generate a maximum

likelihood phylogeny with IQ-TREE v1.6.9 (-alrt 1000 -bb 1000 -bnni -m MFP+ASC)[84]. Total branch

length distance between all pairs of isolates was calculated on the resulting phylogeny as a measurement

of phylogenetic distance.

Pangenome annotations and comparisons

All pairwise sequence comparisons between predicted proteins in the 12 *M. phaseolina* genomes were

determined by diamond blastp (--more-sensitive -e 0.001 --max-hsps 1)[85], and then used as input to the

Pangloss pipeline(default settings)[86] to determine one-to-one ortholog relationships between predicted

genes using a combination of synteny- and sequence-based similarity metrics. In this way, each predicted

gene from each isolate was assigned to an ortholog group, and each ortholog group had at most 1

representative sequence per isolate. We calculated pairwise percent identity in genome content between

all isolates as the "Number of shared ortholog groups / Total number of non-redundant ortholog groups

present across both genomes". This process was then repeated for previously annotated genome datasets

from six other fungal species in order to provide us with comparison points for evaluating *M.*

phaseolina's pangenome diversity (Table S5, Table S6). Datasets from the other species were chosen,

when possible, to be composed of isolates from two distinct geographic locations, similar to the *M.*

phaseolina dataset.

Accessory gene hotspot analysis

We used BayesTraits v3.0.1 Multistate MCMC analysis[87] in conjunction with the whole-genome SNP

phylogeny to estimate the number of gains and losses experienced by each accessory ortholog group since

the last common ancestor of the 12 *M. phaseolina* isolates. Each analysis was run for 10 million

generations with a 2 million generation burn-in, sampling every 4,000 generations until 2,000 samples

were collected. Because we lacked *a priori* expectations of parameter distributions, we seeded the means

of all exponential priors from a uniform distribution ranging from 0-2 by using a hyper-prior, as

recommended by the software authors. For each ortholog group, we derived the median probability of it

being present or absent at each node in the phylogeny from the 2,000 samples of the posterior probability

distribution. We then calculated the probability of gains and losses across the phylogeny by examining

transitions between presence and absence states at each pair of parent and child nodes. To calculate the

probability of gain of an ortholog group at a given child node, we multiplied the probability of its absence

at the parent node by the probability of its presence at the child node. Similarly, to calculate the

probability of loss of an ortholog group at a given child node, we multiplied the probability of its presence

at the parent node by the probability of its absence at the child node. For example, if an ortholog group

had a p(absent) = 0.75 at a parent node, and a p(present) = 0.75 at one of that parent node's children, we

calculated the p(gain) = 0.5625, and p(loss) = 0.0625 at the child node. We summed across all losses, and

all gains in the phylogeny, to derive a phylogenetically- and probabilistically- weighted estimate of the

number of gains and losses experienced by each ortholog group. Since the frequent gain and loss of TEs

could obscure the gain and loss dynamics of host genes, we removed all ortholog groups where at least 1

member had either a predicted TE-associated protein domain or overlapped at least 50% with an

annotated TE feature.

To identify regions of *M. phaseolina*'s genome where accessory ortholog group gains and losses were

greater than expected by chance (i.e., hotspots), we derived a distribution based on the null expectation

that ortholog groups are randomly distributed across the genome by sampling with replacement 5 million

sets of 15 ortholog groups from randomly selected genomes. We calculated the total number of gains and

losses per set, and found that <=1% of sets had a total number of gains and losses >=24.22. For each

genome, we then calculated the total number of gains and losses in each 15 gene window on a 1-gene

sliding window basis, and designated all windows >= 24.22 as hotspots, so that any hotspot had a <= 1%

empirically-derived probability of being observed by chance. We then merged all overlapping hotspots

into hotspot regions.

Variant density

Genotyping structural mutations across a sample of individuals is challenging, especially on a genome-

wide basis. We therefore developed a statistic analogous to Nei and Li's average pairwise nucleotide

diversity[88], where we calculated the average length of different indel size classes in a given "focal"

genome across all pairwise comparisons with other "query" genomes. We conducted all pairwise

alignments of genome assemblies using minimap2 (-x asm10 -c --cs) and called SNP and indel variants

using paftools(-l 1000 -L 5000)[89]. Using these alignments and variant calls, we classified all sequences in

a focal genome into three types: sequence with no alignments (i.e., indels), sequence with 1:1 alignments

(which included SNPs), and sequence with multiple alignments, for each pairwise comparison. We then

overlaid this information with the coordinates of all hotspot regions for the focal genome, and calculated

the average length of sequence impacted by SNPs and indels of various size classes (0.1-1kb, 1-10kb,

>10kb) per 10kb of hotspot region. We then repeated this procedure for all background genomic regions.

Since we were interested in aligning homologous regions, we only considered alignments to focal regions

where <=5% sequence aligned to multiple locations in the query. To generate figures and calculate test

statistics, we only considered focal regions that had valid alignments to 11 query genomes. Significant

differences between the distributions of the average amount of sequence impacted by SNP and indels in

hotspot and background regions were determined using the unpaired two sample Mann-Whitney U test

implemented in the wilcox.test function in R.

Starship **search and classification**

We initially discovered the *Voyager* element in *M. phaseolina* through progressively aligning the flanking

sequences of large insertions to other genomes using MAFFT v7.407[90] in order to identify "empty"

insertion sites. We then used all predicted protein sequences within the *Voyager* reference element in *M.*

phaseolina isolate mp102 (region ID: mp102|voyager|h11) as well as the SPOK3 sequence from

Enterprise [19](GenBank accession: A0A516F180) as queries in a BLASTp search of the 12 *M. phaseolina*

proteomes and 1274 published Ascomycete proteomes curated from public databases (BLAST filtering

options: min. bit score = 50, max. Evalue = 1e[-4]; Table S12; gitlab.com/xonq/mycotools). Sequence

similarity searches present a more comprehensive approach for mobile element annotation because they

do not rely on the presence of polymorphic insertion sites. In *M. phaseolina*, all filtered hits to *Voyager*

queries located within 100,000 bp of each other were merged into regions, and all regions with at least 4

hits were designated as *Starship*-like regions. Full length *Starship* regions were then characterized by

progressively aligning flanking regions, as above. Regions whose insertion sites could not be resolved

were bounded by genes at the 5' and 3' terminal ends. We aligned all regions to each other using nucmer

and manually adjusted boundaries as necessary to include only sequence homologous to other regions.

We repeated the above procedure for the larger genomic database, except we implemented a more

conservative neighboring hit distance cutoff of 25,000 bp because in the vast majority of cases, we could

not manually verify the region boundaries through pairwise alignments due to insufficient data. Genes

within all regions were annotated with protein family (Pfam) domains using Interproscan v5.48-83[77], and

were additionally annotated with predicted functions and assigned to COG categories using eggNOG-

mapper[91].

Phylogenetic classification of *M. phaseolina Starships*

All *Starships* and *Starship*-like regions in *M. phaseolina* were classified into distinct element types by

comparing the phylogenetic relatedness of their DUF3435 captain sequences, following established

approaches for classifying bacterial integrative and conjugative elements [92]. Relationships were

additionally supported using phylogenies of DUF3723, FRE, OCH1, and HET and NACHT domains

from NLRs, and were used to identify the origin of degraded *Starship* regions. We first retrieved all hits

to the DUF3435, DUF3723, FRE, and OCH1 sequences from the *Voyager* reference using a BLASTp

search of the 12 *M. phaseolina* proteomes, and aligned all hits using MAFFT v7.407 (--auto)[90]. Due to the

functional domain complexity of NLRs, we built individual phylogenetic trees for the HET and NACHT

domains found most often in NLR genes (Table 2). We used hmmsearch to search the 12 *M. phaseolina*

proteomes using profile HMMs (--incE 0.001), and aligned all hits to the query profile HMM using

hmmalign [93]. Alignments were trimmed using ClipKIT (default settings)[94], and used as input to generate

maximum likelihood phylogenies with SH-like approximate likelihood ratio test (SH-aLRT) and ultra fast

bootstrap approximation (UFboot) support using IQ-TREE v2.0.5, where the best-fit substitution model

was selected using ModelFinder (-m MFP -alrt 1000 -B 1000)[84,95].

Tyrosine recombinase classification

An initial alignment of all DUF3435 sequences from the fully annotated starships (Table S14) was

generated using MAFFT (defaults). This alignment was used as input to HHPRED [96] which identified the

tyrosine recombinase domain of CRE recombinase (Protein Data Bank Accession: 1XO0) as the best hit

to a conserved region of the alignment. A database of repeat elements with known tyrosine recombinase

domains was collected from RepBase and HHPRED was used again to define the bounds of the tyrosine

recombinase domains for each repeat family. This approach was then used for prokaryotic and plasmid

elements from the ICEberg 2.0 database [97]. For each sequence, all amino acids within the bounds of the

tyrosine recombinase homology region were extracted, aligned using MAFFT v7.407 (--auto) [90], and

trimmed as submitted to IQ-TREE as above. The tyrosine recombinase tree strongly supports the

monophyly of the DUF3435 sequences in *Starship* elements in relation to other known tyrosine

recombinase domains.

Regression models

We built all phylogenetic generalized least squares regression models and assessed the significance of

their parameter estimates using the "gls" function in ape v5.0 in conjunction with the SNP-based isolate

tree (correlation = corBrownian, method = "ML")[98]. To test for the effect of *Voyager* on *M. phaseolina*

growth, we used *Voyager* OCH1 copy number as the linear and quadratic predictor variables and either

mean isolate AUDPC or AUGPC as the response variable for the pathogenicity and PDA experiments,

respectively (Table S24).

Statistical analyses and data visualization

All statistical analyses were conducted in R v4.1.1[99]. All Circos plots were generated using Circos[100] and

all other graphs were visualized using ggplot2[101]. All *Starship* schematics were either drawn manually or

visualized using genoplotR[102]. All phylogenetic trees were visualized using ete3[103].

Figure 1: Giant *Starship* elements found across the Ascomycota share conserved components and

mobilzie diverse arrays of accessory genes. A) To the bottom-left is a circular maximum likelihood tree

of 1890 DUF3435 homologs retrieved from a database containing 12 *Macrophomina phaseolina* genomes

generated for this study and 1274 published Ascomycete genomes, where branches are color-coded

according to taxonomic class. Black dots along the perimeter indicate DUF3435 sequences found in 194

Starships and *Starship*-like regions (60 of which are from *M. phaseolina*), and to the right is a maximum

likelihood tree of these sequences and several others of interest, where clades are depicted as triangles for

clarity and all branches with <80% SH-aLRT and <95% UFboot support have been collapsed. A clade of

degraded *Starships* that lack DUF3435 sequences is juxtaposed below the tree for visualization purposes

only. B) Fungal species, taxonomic class, *Starship* name and simplified *Starship* schematics, with

approximate locations of genes of interest depicted as colored boxes or numbered. Genes are not drawn to

scale. Target site duplications (TSDs), when indicated, were confirmed from alignments of insertion

polymorphisms. Names of elements from *M. phaseolina* are in bold, and previously published mobile

elements are indicated with an asterisk. C) A schematic showing the general organization of *Starship*

mobile elements. D) Proportion of 134 *Starships* and *Starship*-like regions containing homologs to five

genes of interest. Gene name abbreviations: FRE (ferric reductase); PLP (patatin-like phospholipase);

NLR (Nucleotide Oligomerization Domain-like receptor); *Spok* (spore killer); OCH1 (alpha-1,6-

mannosyltransferase); NRPS (non-ribosomal peptide synthetase); *Ago1* (Argonaute). Other abbreviations:

BGC (biosynthetic gene cluster). All original trees can be found in the supplementary data files.

Figure 2: Distinct *Starship* elements in *Macrophomina phaseolina* vary in copy number across

individuals and generate intraspecific variation in genome content. A) A heatmap summarizing the

percentage of pairwise alignable sequence between all *Starships* and *Starship*-like regions, as well as the

degraded *Starship* region *Derelict*, found across 12 *M. phaseolina* isolates, where rows and columns

represent individual regions arranged by hierarchical clustering according to similarity in their alignment

scores. B) Maximum likelihood phylogenies of genes homologous to OCH1 and FREs, as well as genes

containing and HET and NACHT domains found in fungal NLRs, retrieved from the *M. phaseolina*

genomes, where all branches with <80% SH-aLRT and <95% UFboot support have been collapsed.

Genes found in regions of interest are highlighted by different colors. C) A circos plot depicting all

contigs >100kb in the *M. phaseolina* mp102 genome. Core contigs are partially alignable across all

isolates, while unplaced contigs are not. Regions of interest are labeled and visualized on the inner track.

Sites with resolved *Starship* insertions in other genomes, but absent in mp102, are labeled on the outside

track. Accessory gene hotspots are labeled in orange on the outer track. D) A schematic depicting a

sequential *Starship* insertion event where an unknown DUF3435 gene is found nested in *Phoenix*, which

itself is nested in a degraded *Voyager* element in isolate mp247 on contig cc6-2. E) A schematic depicting

a cargo turn-over event between two *Defiants* carrying either a carotenoid or non-ribosomal peptide

synthetase (NRPS) biosynthetic gene cluster (BGC). Gene name abbreviations: OCH1 (alpha- 1,6-

mannosyltransferase); FRE (ferric reductase); NLR (Nucleotide Oligomerization Domain-like receptor);

Figure 3: Several *Starship* elements are associated with large structural variants across

***Macrophomina phaseolina* genomes.** A) Alignment-based chromosome maps of four *M. phaseolina*

isolates and a hypothetical ancestral genome, where contigs that share homology are colored and ordered

according to their putative chromosome of origin. Putative telomeres are labeled at contigs ends, and

consist of at least 5 sequential telomeric repeats, unless indicated otherwise by bracketed numbers.

Unplaced contigs are not illustrated. B) From left to right, circos-based nucmer alignments of (1) a

putative chromosomal fusion, (2) a reciprocal translocation, and (3) a *Voyager* insertion polymorphism.

Contigs are labeled according to chromosome of origin, or otherwise colored gray. *Starships* and

degraded *Starships* are drawn in dark gray, labeled and outlined in black. Labeled numbers correspond to

events depicted in both the chromosome maps and the circos alignments.

Figure 4: Increasing *Voyager* OCH1 copy number has contrasting effects on *Macrophomina*

***phaseolina*'s pathogenic and saprotrophic growth.** A) A schematic of the reference *Voyager* element in

M. phaseolina mp102 (ID: mp102|voyager|h11) depicting all predicted genes and annotated repetitive

elements B) A multiple sequence alignment of the *Voyager* insertion cc10.1, with annotated target site

duplications (TSDs) and terminal inverted repeats (TIRs) C) A circos plot of six fully-resolved *Voyager*

insertions with 10kb flanking regions, labeled by insertion ID and length. Tracks 1-2 depict genes in the +

and - orientation, respectively. Tracks 3-14 depict the percent nucleotide identity of sequence alignments

at each locus across 12 *M. phaseolina* genomes, arranged by increasing isolate number. The density plot

in Track 15 depicts median Illumina coverage across intact 5S rDNA insertion sites in isolates with empty

insertion sites, and ranges from 0-77x. The density plot in Track 16 depicts median Illumina coverage

across *Voyager* insertions and their flanking regions, and ranges from 0-100x. D) Scatterplots (jitter =

0.05) depicting mean area under the disease progress curve (AUDPC; top) and mean area under the

growth progress curve (AUGPC; bottom) phenotypes as a function of the number of OCH1 homologs

found in *Voyager Starships,* for each of the 12 isolates. AUGPC was measured at 6 different

temperatures. Error bars indicate +/- standard error, and are visible if they exceed the area of the point.

Predicted quadratic equations with shaded 95% confidence intervals are superimposed on the plots.

*P*values for significant ($P < 0.05$) and marginally significant ($P < 0.10$) linear and quadratic coefficients are

shown, and others in Table S24. Abbreviations: OCH1 (alpha- 1,6- mannosyltransferase); PDA (potato

dextrose agar).

787   Figure S1: Geographic origins and genomic characteristics of 12 newly sequenced *Macrophomina*

788   *phaseolina* strains from North and South America. A) The locations of soybean (*Glycine max*) fields

in Paraguay and Ohio, U.S.A. from which individual strains were isolated from soil samples (Table

macphIsolationData) B) *M. phaseolina* microsclerotia accumulation in a dissected soybean root,

characteristic of charcoal rot disease. C) To the left, a maximum likelihood tree based on a 3,227,097

single nucleotide polymorphism alignment depicting relationships between all sequenced strains and the

Botryosphaeria dothidea outgroup, with strain names highlighted according to their geographic origin. To

the right of each branch tip is a bar chart summarizing the lengths in megabases of various genomic

features in each draft assembly, followed by the assembly N50.

797   Figure S2:. **Chromosome maps depicting eight well assembled genomes of *M. phaseolina* isolates**

798   overlayed on the SNP-based phylogeny. A) The consensus genome is inferred based on the

chromosome structures of mp102 and mp053, the best assembled and most distantly related isolates.

Chromosomes are colored based on inferred synteny as calculated from pairwise nuncmer alignments.

Contigs are manually joined based on synteny to mp102, or to mp053 for the mp102 isolate. Contig

breaks are depicted with a black vertical line (|), putative telomeres are shown as a gold diamond,

Starships and *Starship*-like regions are colored dark gray. Thin, horizontal gray lines illustrate non-

syntenic regions. Only contigs > 500 kb are illustrated, unplaced contigs are not illustrated. The

phylogeny was edited from the maximum likelihood tree presented in Figure S1. B) Circos plot depicting

pairwise alignments between mp102 and mp053. Chromosomes are colored as in A). Nucmer output was

filtered to exclude alignments <14 kb, to avoid depicting conventional transposable element movement.

Contigs and contig regions with no links are presumed to represent accessory genomic regions.

810   Figure S3: Differences in *M. phaseolina*'s genome content accumulate rapidly and are concentrated

811   in hotspot regions enriched for large structural polymorphisms. A) A scatterplot depicting pairwise

nucleotide- and gene-based identity as a function of phylogenetic distance between all non-redundant

combinations of 12 *Macrophomina phaseolina* genomes. Predicted linear equations with shaded 95%

confidence intervals derived from linear models are superimposed for each comparison type, along with

p-value probabilities that the slopes do not differ significantly from 0. B) Rarefaction curves depicting the

number of unique ortholog groups (OGs) recovered in subsamples of different genomic datasets, color-

coded by fungal species consisting of *M. phaseolina* and several others included for comparison. Each

point represents the mean of 1000 permutations, with error bars representing +/- standard deviation. To

the right are estimates of Heaps Alpha, a measurement of pangenome openness, for each species. C) A

Manhattan plot indicating the total number of gains and losses associated with all OGs in a given sliding

window across the *M. phaseolina* mp102 genome. A dotted red line is drawn at the critical value used to

identify hotspot regions, determined empirically using a randomly generated null distribution of expected

gene gains and losses. D) Box-and-whisker plots summarizing the average amount of sequence per 10kb

of a given focal region impacted by SNPs and indels of various sizes in hotspot and background regions,

across all genomes. Significant differences between the distributions of sequence length in either region

were determined by a one sided Mann Whitney U test.

Figure S4: A maximum likelihood phylogeny of all 1890 DUF3435 homologs retrieved by BLASTp

from the 12 *Macrophomina phaseolina* isolates and a database of 1274 published Ascomycete

genomes using *Voyager*'s DUF3435 as a query. Branches are color coded according to taxonomic class

and sequences labeled by species. The phylogeny is rooted at a DUF3435 sequence retrieved from

Lentinula edodes (Basidiomycetes). Sequences found in *Starships* or *Starship*-like regions are labeled by

the region in which they are found, and captain sequences are additionally highlighted in red (Table S11,

Table S13). Captain sequences are defined as the DUF3435 sequence located nearest to the 5' terminal

end of a *Starship* element and oriented in the 5'-3' direction. Branch support labels indicate SH-aLRT and

UFboot support, respectively. All branches with <80% SH-aLRT or <95% UFboot have been collapsed.

A simplified, circularized version of this phylogeny is visualized in Figure 1A.

Figure S5: A maximum likelihood phylogeny of all DUF3435 homologs found across 195 *Starships*

and *Starship*-like regions from the 12 *Macrophomina phaseolina* isolates and a database of 1274

published Ascomycete genomes. Branches are color coded according to taxonomic class and sequences

labeled by species. The phylogeny is rooted at three DUF3435 sequences retrieved from Basidiomycetes.

Branch support labels indicate SH-aLRT and UFboot support, respectively. All branches with <80% SH-

aLRT or <95% UFboot have been collapsed. A simplified version of this phylogeny is visualized in

Figure 1A. To the right of the tree is a heatmap matrix summarizing the number of predicted genes within

each *Starship* and *Starship*-like region that are homologous to DUF3723, FRE, PLP and NLRs (Table

S11, Table S13).

Figure S6: A maximum likelihood phylogeny of all 165 DUF3435 homologs retrieved by BLASTp

from the 12 *Macrophomina phaseolina* isolates using *Voyager*'s DUF3435 as a query, rooted at the

last common ancestor of *Voyager*, *Argo*, *Defiant*, and *Phoenix* captain sequences. Captain sequences

are defined as the DUF3435 sequence located nearest to the 5' terminal end of a *Starship* element and

oriented in the 5'-3' direction. Sequences found in *Starships* or *Starship*-like regions are labeled by the

region in which they are found, and captain sequences are additionally highlighted in red (Table S11).

Branch support labels indicate SH-aLRT and UFboot support, respectively. All branches with <80% SH-

aLRT or <95% UFboot have been collapsed.

Figure S7: A maximum likelihood phylogeny of the tyrosine recombinase domain from the

DUF3435 captains of all fully annotated *Starships* presented in this study and reference sequences.

Sequences from characterized *Starships* are highlihgted in blue (Table S14). Tyrosine recombinase

domains from known eukaryotic TEs, prokaryotic integrative elements, phages, and plasmids are included

for reference. *Cryptons* are highlighted in green as they are mechanistically the most similar to the

DUF34335 domain found in *Starships*, but are clearly distantly related and polyphyletic. The phylogeny

is mid-point rooted. Branch support labels indicate SH-aLRT and UFboot support, respectively. All

branches with <80% SH-aLRT and <95% UFboot have been collapsed.

Figure S8: A maximum likelihood phylogeny of all 109 OCH1 homologs retrieved by BLASTp from

the 12 *Macrophomina phaseolina* isolates using *Voyager*'s OCH1 as a query, all OCH1 sequences

from *Botryosphaeria dothidea* strain LW-Hubei, and 5 reference sequences from functionally

characterized genes in other fungi. The phylogeny is midpoint rooted. Sequences found in *Starships* or

Starship-like regions are labeled by the region in which they are found (Table S11). Branch support labels

indicate SH-aLRT and UFboot support, respectively. All branches with <80% SH-aLRT and <95%

UFboot have been collapsed.

Figure S9: A maximum likelihood phylogeny of all 316 FRE homologs retrieved by BLASTp from

the 12 *Macrophomina phaseolina* isolates using *Voyager*'s FRE as a query, and 16 reference

sequences from functionally characterized genes in other fungi. The phylogeny is mid-point rooted.

Sequences found in *Starships* or *Starship*-like regions are labeled by the region in which they are found

(Table S11). Branch support labels indicate SH-aLRT and UFboot support, respectively. All branches

with <80% SH-aLRT and <95% UFboot have been collapsed.

Figure S10: A maximum likelihood phylogeny of all 1007 predicted amino acid sequences

containing HET domains (Protein family accession: PF06985) retrieved by HMMsearch from the

12 *Macrophomina phaseolina* isolates. The phylogeny is mid-point rooted. Sequences found in *Starships*

or *Starship*-like regions are labeled by the region in which they are found (Table S11). Branch support

labels indicate SH-aLRT and UFboot support, respectively. All branches with <80% SH-aLRT and <95%

UFboot have been collapsed.

Figure S11: A maximum likelihood phylogeny of all 1441 predicted amino acid sequences

containing NACHT domains (Protein family accession: PF05729) retrieved by HMMsearch from

the 12 *Macrophomina phaseolina* isolates. The phylogeny is mid-point rooted. Sequences found in

Starships or *Starship*-like regions are labeled by the region in which they are found (Table S11). Branch

support labels indicate SH-aLRT and UFboot support, respectively. All branches with <80% SH-aLRT

and <95% UFboot have been collapsed.

Figure S12: A maximum likelihood phylogeny of all 154 PLP homologs retrieved by BLASTp from

the 12 *Macrophomina phaseolina* isolates using *Voyager*'s PLP as a query. The phylogeny is mid-

point rooted. Sequences found in *Starships* or *Starship*-like regions are labeled by the region in which

they are found (Table S11). Branch support labels indicate SH-aLRT and UFboot support, respectively.

All branches with <80% SH-aLRT and <95% UFboot have been collapsed.

Figure S13: A maximum likelihood phylogeny of all 189 DUF3723 sequences retrieved by BLASTp

from the 12 *Macrophomina phaseolina* isolates using *Voyager*'s DUF3723 as a query. The phylogeny

is mid-point rooted. Sequences found in *Starships* or *Starship*-like regions are labeled by the region in

which they are found (Table S11). Branch support labels indicate SH-aLRT and UFboot support,

respectively. All branches with <80% SH-aLRT and <95% UFboot have been collapsed.

Figure S14: A flow chart describing the *de novo* annotation procedure of the 12 *M. phaseolina*

genomes. All steps indicating the use of particular software are visualized as rectangles with the software

name indicated in bold; all output materials are visualized as parallelograms; all binary decision points are

visualized as diamonds.

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