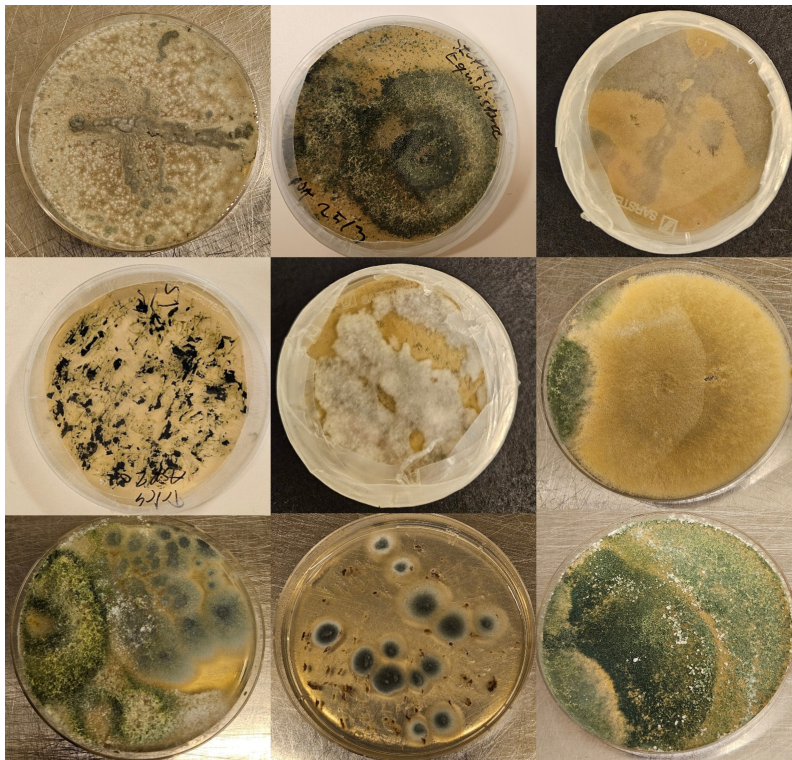




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Experimental Insights into Horizontal Transfer of Fungal *Starship* Megatransposons



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Abstract

Starships are a recently discovered class of cargo-carrying fungal megatransposons, whose phylogenetic distribution suggests that they are capable of horizontal transfer between species. In this project, I attempted to experimentally verify horizontal *Starship* transfer in a laboratory setting. I designed plasmid vectors to induce auxotrophy and insert a hygromycin resistance tag in the *Starship* of an *Aspergillus fumigatus* donor strain. I then confronted it with potential acceptor strains under different culturing conditions, intending to trigger horizontal transfer of the *Starship* element. I also used mycoparasitic *Trichoderma* as an acceptor strain, to investigate whether the close hyphal contact of mycoparasitic interaction would facilitate horizontal transfer. None of these experiments led to a detectable transfer event, emphasizing that the complexities of *Starship* transfer are not yet fully understood, and more work is needed to develop a working system for inducing it in laboratory conditions. Additionally, I bioinformatically identified three new putative *Starships* in *Trichoderma virens*, forming the first instance of *Starships* discovery in this genus.

Keywords

Horizontal gene transfer, fungal genetics, transposable elements, fungal evolution.

Introduction

Background

Fungal diseases have long been neglected as a field of research¹⁻³. Advances in modern medicine have led to an increased survival of immunocompromised people; this also means that there is an increasing pool of the population that is immunocompromised, and thus susceptible to fungal infection^{4,5}. This is also a problem that could become further exacerbated as a result of global warming⁶. Fungal infections are also a significant source of crop loss, which has massive economic implications¹, especially in developing countries⁷. Adding further complication is the fact that most classes of clinically used antifungal drugs are also used in agriculture; as most human fungal pathogens are also found in the environment, this leads to clinically relevant development of antifungal resistance⁸. Here, significant similarities can be drawn to the emerging threat of bacterial antibiotic resistance^{9,10}. More research into fungal biology, pathogenic trait acquisition and evolution is needed before the consequences for human health and food security become too severe^{1,3,11}.

Horizontal gene transfer, or HGT, is a term for when an organism acquires genetic material from a source other than vertical inheritance from its parent cells. In bacteria, common examples of horizontal gene transfer are through the conjugation of a plasmid, viral transduction, or transformation of environmental DNA¹². Genes from another organism are transferred to and might remain and be

expressed in the cell; they can be maintained in independent plasmids, or they might integrate into the chromosome, particularly in “hotspot” regions for HGT genes¹³. While transformation can involve the incorporation of “free” environmental DNA¹⁴, most other mechanisms of HGT involve mobile genetic elements such as viruses, plasmids, and transposable elements. Some mobile elements include accessory genes that can improve the fitness of the host, such as antibiotic resistance genes, which are thus spread via HGT^{15,16}. However, mobile genetic elements are often viewed as “selfish” elements under their own evolutionary pressure for proliferation. They have complicated interactions with both their host genome and with other mobile elements^{17,18}. Some of the aspects that can be deleterious to the host are the extra transcriptional burden, potential disruption of existing genes, and potential side effects of new proteins and their interactions¹⁹, and not all mobile elements carry any benefits at all¹⁶. Thus, most organisms have mechanisms to protect themselves against foreign horizontally transferred DNA, and the trade-offs for how and when to allow HGT in the cell are complex¹⁷.

HGT in eukaryotes is thought to play a much smaller role than it does in prokaryotes. In part, this is likely due to many eukaryotes having other mechanisms as their main evolutionary driving forces, such as sexual recombination, and gene duplication and divergence^{16,20}. Gene duplication is a powerful factor of evolution and comes with a complicated balance of deletion, neofunctionalization, pseudogenization and divergence, and though present in prokaryotes as well, it is thought to be a key factor in the evolution of eukaryotic complexity²¹⁻²³. Nevertheless, studies into the role of HGT in eukaryotes have become more feasible partly due to the new availability of long-read sequencing data²⁰, and horizontal gene transfer between eukaryotes has been shown on multiple separate occasions. Examples include the transfer of an antifreeze protein between fish species^{24,25}, extremophilic algae acquiring genes from extremophilic prokaryotes²⁶, and multiple events in the evolution of land plants²⁷. Horizontal gene transfer between different eukaryotic kingdoms has also been implied, with several oomycete genes used in plant pathogenicity being derived from plant-pathogenic fungi²⁸. There are multiple other examples of HGT in fungi: *Saccharomyces cerevisiae* strains used in winemaking contain genetic regions gained from a common wine contaminant fungus²⁹, and the wheat pathogenicity factor *ToxA*, causing significant economic damage in crop loss, has been horizontally transferred between three separate fungal pathogens^{30,31}.

In eukaryotes, transposable elements (TEs) have been especially highlighted for horizontal transfer^{32,33}. While many TEs are purely “selfish” elements, some carry auxiliary “cargo” genes within their borders, thus mobilizing these genes^{34,35}. In fungi, the recently described megatransposon *Hephaestus* carries multiple cargo genes conferring metal resistance. It also shows clear hallmarks of HGT: the phylogeny of different *Hephaestus* elements is inconsistent with, and much closer than, the phylogeny of the host species they reside in³⁶. The same kind of phylogenetic dispersion was noted for *Enterprise*, another fungal TE carrying spore-killing genes³⁷. A massive genetic island conferring

competitive advantages, transferred multiple times between cheese-related fungi³⁸, is also in fact a cargo-carrying megatransposon³⁹. It turns out that these are not isolated events: my research group recently discovered that these examples are all part of a whole superfamily of fungal megatransposons dubbed *Starships*^{39,40}.

What are Starships?

Starships are large transposable elements with an average size of 110 kb, but can span up to 700 kb⁴¹. They are widespread amongst the Ascomycete group *Peizizomycotina*, and thought to originate from the last common *Peizizomycotina* ancestor⁴². Traces of potential *Starships* have been found in other subphyla as well, including Basidiomycetes, but as these are rarely intact, they are theorized to be fragments of past HGT transfers⁴². All active *Starships* contain a tyrosine site-specific recombinase with a conserved DUF3435 domain, dubbed the “Captain”, which has been shown to be responsible for the transposition of the *Starship*⁴³. Certain auxiliary genes are often present as well, such as ferric reductases (FREs), patatin-like phosphatases (PLPs), DUF3723 domains, and NOD-like receptors (NLRs), which are suspected to be involved in the transfer of the elements³⁹. Unlike most transposable elements, *Starships* are usually only found as single copies in the fungal genome, with a presence/absence variation across populations³⁹. The single copy number likely protects them against being destroyed by repeat induced point mutation (RIP), a fungal genomic defense mechanism against TEs that causes extensive point mutations in repeated DNA sequences⁴⁴. Staying as a single copy is thought to facilitate the stable residence of *Starships* in the host genome, but it also means that a failed transposition event could lead to gene loss⁴³. Furthermore, *Starships* have been found in the breakpoints for chromosomal rearrangements³⁹, and has been found to actively transpose between chromosomes in a common fungal lab strain, something that could affect experimental reproducibility of experimental fungal research⁴⁵.

A key aspect of interest when it comes to *Starships* is the cargo genes they carry. In addition to its captain and common auxiliary genes, *Starship* elements can contain hundreds of protein-coding genes encoding a diverse array of traits³⁹. The majority of these genes have no recorded COG (Clusters of Orthologous Genes) annotations and their function is thus currently unknown⁴². Some cargo genes have been studied more closely, such as the abovementioned plant-pathogenic *ToxA* virulence factor and metal resistance genes, as well as formaldehyde-detoxifying gene clusters⁴¹, and biofilm-related virulence factors^{45,46}. Broad-scale bioinformatic investigations into *Starship* cargo found that they were generally enriched in some COG-categories such as “Cellular processes and signaling”, “Information storage and processing”, and “Metabolism”⁴². An in-depth study of the *Starships* present in opportunistic human pathogen *Aspergillus fumigatus* shows that cargo of some COG categories are enriched in clinical isolates while others are enriched in environmental strains, suggesting that *Starship* cargo could affect fungal lifestyle in clinically relevant ways⁴⁵. Some cargo

genes were found almost exclusively in *Starships*, while others could be found in both *Starship* and host genomic regions⁴⁵. The same article posits that *Starships* are a key driver of strain heterogeneity in *A. fumigatus*, which affects treatment options in clinical infections. Parallels can be drawn here to cargo-carrying mobile elements in bacteria, creating a pangenome of conditionally beneficial traits that can have clinical relevance^{13,16,45}.

A major player in *Starship* evolution and mobility is thought to be horizontal gene transfer, allowing reliable expansion into new genomic backgrounds⁴¹. Bioinformatic support for *Starships* being horizontally transferred has been recorded in multiple instances. The same *Starships*, with 95-100% sequence identity across >100bp, can be found in species that otherwise share only about 70-75% sequence identity, and the phylogeny of different *Starship* elements are inconsistent with the host genomes wherein they are found^{36,39}. A closer look into the *ssf* cluster, a formaldehyde-detoxifying biosynthetic cluster found as cargo in four different *Starships*, found signs of a total of 9 different HGT events where it had been transferred⁴¹. It also seems like cargo genes such as the *ssf* cluster can be exchanged between distantly related *Starship* elements, and get permanently integrated into the host genome upon *Starship* “death”, highlighting the significant impact that horizontal *Starship* transfer of cargo genes could have on fungal evolution⁴¹.

This support for *Starship* HGT was solely bioinformatic, until now. Recently, Urquhart et. al. managed to experimentally verify a horizontal transfer of *Hephaestus* from *Paecilomyces variotii* to another strain of the same species, another species within the same genus, and even another family within the same order⁴⁷. By inserting an antibiotic selection marker into the *Starship* of an auxotrophic *P. variotii* donor strain, the transfer of the *Starship* into a prototrophic acceptor strain could be easily selected for on antibiotic-containing minimal medium, and the complete transfer of the *Starship* was then confirmed via sequencing. To our knowledge, this is the first experimentally verified event of eukaryotic horizontal gene transfer of this size.

Mycoparasitism and *Starships*

The exact mechanism of *Starship* transfer is not yet elucidated. Possible mechanisms of transfer could involve, for example, hyphal fusion between donor and acceptor strains, or extracellular vesicles (EVs).

- Fungal hyphal fusion is tightly controlled and primarily occurs between strains of the same species; even within a species, if two strains differ significantly in the polymorphic HET (Heterokaryon Incompatibility) genes, hyphal fusion will quickly trigger apoptosis of the fused cells, forming a barrage between the two strains^{48,49}. Thus, hyphal fusion is unlikely to happen across larger phylogenetic distances. However, one study showed that mycovirus transmissions requiring hyphal fusion are more permissive during natural conditions over

longer periods of time, suggesting that short laboratory experiments on nutrient agar are not an accurate representation of the hyphal fusing conditions present in nature ⁵⁰. Furthermore, mycoviruses are seemingly able to interfere with this non-self recognition in fungi in order to facilitate horizontal transfer through hyphal fusion ⁵¹; it is possible that *Starships* could possess a similar mechanism.

- Extracellular vesicles are membranous particles released by cells in all domains of life, which can contain various types of molecular cargo and are suspected to be involved in various types of cellular interactions ^{52,53}. Until recently, it was thought that the fungal cell wall would inhibit EV transfer between cells, thus the information on fungal EVs is still in its early stages ⁵⁴. However, it has been shown that release and uptake of EVs and EV-like particles is indeed possible through the fungal cell wall ^{55,56}. Fungal EVs have now been implied to be involved in various intercellular communication processes such as biofilm formation and drug resistance ⁵⁷, pathogenicity ⁵⁸, and mycorrhizal interaction ⁵⁹.

It may be that that HGT rate, whether through hyphal fusion, EV transfer, or other unknown mechanisms, could be influenced by the amount of physical contact between donor and acceptor. One study found that the rate of prokaryote-derived HGT in fungi is significantly affected by lifestyle, with parasites having the highest rate of transfer, likely due to their dynamic interactions with their hosts ⁶⁰. Furthermore, one of the furthest known horizontal transfers of mycoviruses happened between two species that have a mycoparasitic lifestyle ⁶¹. *Trichoderma* is a genus consisting mostly of fungal mycoparasites whose mechanism of action involves physically entangling the host's hyphae with its own, as well as penetrating into them, acting as a necrotroph- and hemibiotroph on other fungal species ⁶². The ability for *Trichoderma* to display a plant-decomposing lifestyle in addition to its parasitism has also been suggested to be a result of HGT from its plant associated host fungi, where the Eurotiales (including *Aspergillus*) were shown to be the main genetic donor ⁶³. One could thus theorize that the close physical contact between mycoparasite and host hyphae might be a factor that can facilitate HGT, including the transfer of *Starships*.

While *Trichoderma* is commercially available and commonly used as an agricultural biocontrol agent against plant-pathogenic fungal infections ⁶⁴, recent reports have shown *Trichoderma* itself acting as a primary pathogen on corn in Europe ^{65,66}. Experiments have shown that several *Trichoderma* species from different clades are capable of causing significant disease; within a species however, some isolates did not cause any disease while others caused high disease severity ⁶⁷. Phylogenetic analyses of *Trichoderma* polymorphic markers *tef1-α* and *rpb2* (standard for *Trichoderma* species identification ⁶⁸) was unable to differentiate between pathogenic and non-pathogenic strains. A possible explanation for this phylogenetically uneven distribution could be horizontal *Starship* transfer of the pathogenicity factors between strains. No *Starships* have previously been recorded in

Trichoderma, partly due to the fact that most long-read assemblies lack annotation, which is needed for high-throughput bioinformatic identification of *Starships* ⁴².

Objective of the study

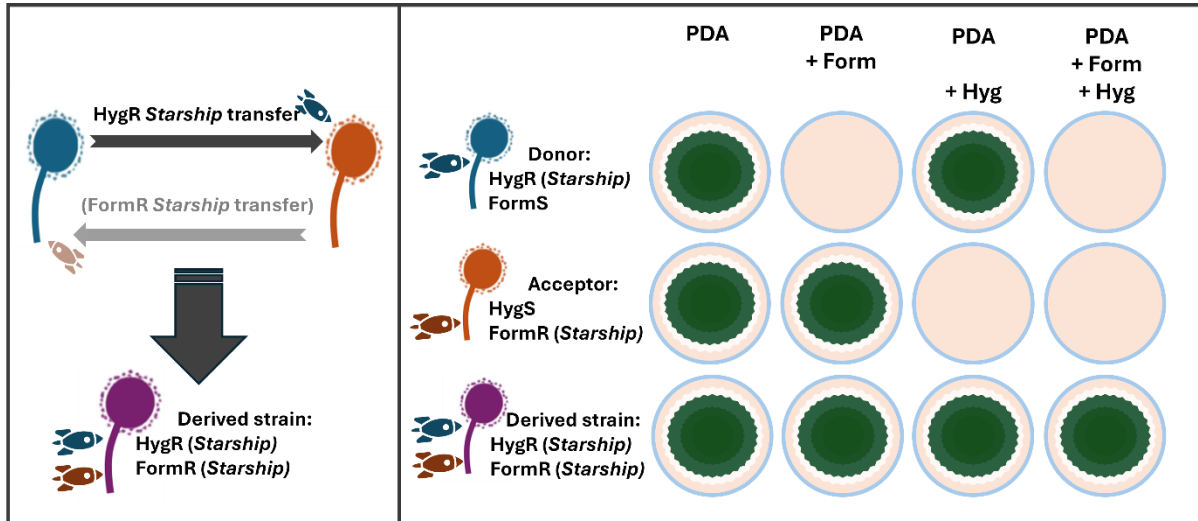


Figure 1. Graphical summary of project design for *Starship* HGT confrontation experiments. Hyg = hygromycin, Form = formaldehyde. A donor strain contains a *Starship* conferring hygromycin resistance, while the acceptor strain contains a *Starship* conferring formaldehyde resistance. If a *Starship* is horizontally transferred between the strains, the resulting derived strain becomes resistant to both formaldehyde and hygromycin, and is able to grow on media that contains both.

As part of the same large-scale project as described by Urquhart et.al ⁴⁷, the aim of my project was to experimentally verify horizontal *Starship* transfer between two different fungi in a laboratory setting. This was to be achieved by creating a leucine-auxotrophic *A. fumigatus* strain, and tagging a *Starship* (*Logos_h1*) in this strain with a hygromycin resistance tag, resulting in an auxotrophic and hygromycin-resistant “donor” strain. After exposing this donor strain to a prototrophic acceptor strain, a horizontal transfer of the *Logos_h1* *Starship* from donor to acceptor would lead to a derived strain able to grow on minimal media with hygromycin. Thus, the results of successful HGT events could be selected for and identified. Due to obstacles during the transformation process the experimental setup was later changed, instead exposing the donor strain to formaldehyde-resistant acceptor strains, and using hygromycin-formaldehyde double selection for the derived strains (see Figure 1). In a separate experiment, donor strains were also exposed to mycoparasitic *Trichoderma*, in order to investigate whether the close hyphal contact between mycoparasite and host could facilitate a higher rate of *Starship* horizontal transfer. Bioinformatic analysis was also performed to identify *Starship* presence in *Trichoderma*.

The larger aim of the project is to establish a system for systematically tracking the frequency of *Starship* horizontal transfer in a laboratory setting, under what conditions and across what phylogenetic distances such a transfer is achievable. With a working experimental system for investigating *Starship* transfer, further investigations can be made as to the role of *Starships* as key players of fungal evolution. In particular, if *Starships* can mediate horizontal transfer of pathogenicity factors, understanding when, how, and how often this happens could be crucial for monitoring the spread of new diseases past conventional transfer barriers. With the increasing threat of fungal infections in both agricultural and clinical contexts, understanding fungal evolution and biology is crucial for developing strategies to prevent infections and mediate disease severity.

Results and discussion

Fungal transformation and troubleshooting

In the original experimental setup, the first step in creating a donor strain was to knock out the *LeuA* gene of *A. fumigatus* strain A1160. *LeuA* is part of the leucine biosynthesis pathway, and a knockout of this gene results in a leucine-auxotrophic strain, which can consequently be selected against using minimal media⁶⁹. It has previously been found that *A. fumigatus* strain A1160 contains 6 different *Starships*⁴⁵. The idea was to first create an auxotrophic version of A1160, which could eventually be utilized for separate HGT investigations for each of these 6 *Starship* elements. For this project I elected to target the *Logos_h1 Starship*; this element contains the *BafZ* gene, thought to be involved in biofilm formation during infection⁴⁶. By replacing *BafZ* with a hygromycin resistance gene, the movement of the *Logos_h1* element could be traced (the *BafZ* knockout can then also be used for separate future studies into *Baf* functions). First, I constructed knockout plasmids for both *LeuA* deletion and hygromycin insertion, to use homologous recombination to knock out the target genes and replace them with the antibiotic resistance markers. I designed PCR primers for the desired homology fragments flanking the target genes, and for the resistance markers. After PCR these fragments were transformed into *Saccharomyces cerevisiae* along with a restriction-enzyme-cleaved delivery plasmid. These components were then assembled into a finished knockout plasmid (resistance marker placed between the target gene flanking regions) by the homologous recombination machinery of *S. cerevisiae*. The assembled plasmid was then amplified in *E. coli*, and the sequence was verified via Oxford Nanopore sequencing, see methods for details.

These knockout plasmids were then to be transformed into *A. fumigatus* A1160 through protoplast transformation. Homologous recombination between the target gene flanking regions in the genome and on the plasmid would lead to the target gene being replaced by the antibiotic resistance marker. First, I attempted protoplast transformation of the *LeuA* knockout plasmid into *A. fumigatus* A1160. This proved unsuccessful, even after attempts at troubleshooting and fine-tuning the method (re-

calculating and remaking media components, control experiments of the antibiotic concentration, comparing similar protocols). The positive controls (no antibiotic selection) consistently showed growth, suggesting that viable protoplasts had been generated, but no successful *LeuA* knockout transformants were acquired.

Successful hygromycin insertion transformants were eventually acquired after using the following modifications to the base protocol: keeping the protoplasts on ice between centrifugation steps, mixing the protoplasts directly within the molten agar during plating, and pouring the selection agar on top after an overnight recovery period. The successful hygromycin-resistant transformant was single-spore isolated, and CTAB DNA extraction was performed. To ensure that the hygromycin resistance tag was inserted into the correct genomic location, PCR was performed (see methods), and the PCR fragment was sequenced with Sanger sequencing and then aligned to the reference to confirm successful integration into the *Logos_h1 Starship*.

However, the *LeuA* knockout transformation remained unsuccessful even with the abovementioned modifications. Eventually, I decided to reinvestigate the target of the knockout plasmid, using NCBI blast of the construct flanks against the A1160 genome, followed by a nucleotide-to-protein blastx search of the targeted sequence. The targeted sequence encodes the protein 2-isopropylmalate synthase. In many organisms, this protein is called *LeuA*; however, in *A. fumigatus*, 2-isopropylmalate synthase is named *LeuC*, and *LeuA* is the name of the 2-isopropylmalate isomerase. The knockout plasmid had thus targeted *LeuC* instead of the desired *LeuA*. *LeuC* is also involved in the leucine biosynthesis process, and previous studies in *A. fumigatus* show that *LeuC* knockout will also produce a leucine-auxotrophic phenotype, but a leucine concentration of about 9 mM is needed to restore its growth⁶⁹. As the protoplast recovery media contained 2.8 mM leucine (intended for *LeuA* knockouts), any *LeuC* knockout cells resulting from the transformation would not have been able to germinate.

Formaldehyde co-selection confrontations

Due to time constraints, the abovementioned troubleshooting left little time left to perform, verify, and purify an auxotroph donor and perform the confrontations in the intended way as described above. Instead, confrontations were set up using the abovementioned transformant strain containing the hygromycin-tagged *Logos_h1 Starship* as a donor. Chosen as acceptors were one *A. fumigatus* strain and one *P. variotii* strain, both containing the formaldehyde-resistance-providing *Chrysaor Starship*⁴¹. Thus instead of using minimal media, formaldehyde could be used to select against the donor, as depicted in Figure 1. Additionally, this setup could allow for bidirectional *Starship* transfer, as *Chrysaor* could also be transferred from acceptor to donor. (However, note that bidirectional transfer would not be detected on the *P. variotii* confrontations, as the selective levels of formaldehyde for this species is too high for *A. fumigatus* to survive even with an acquired *Chrysaor* element⁴¹.)

After co-culturing donor and acceptor together on the same plate, selection was to be performed on media containing both formaldehyde and hygromycin, where only a recombinant strain following a *Starship* transfer would be able to grow. First, pilot experiments were conducted to ensure that the formaldehyde and hygromycin were not interacting in the media. Based on previous formaldehyde resistance level experiments ⁴¹, Potato Dextrose Agar (PDA) plates were made with 1) non-lethal levels of formaldehyde, 2) with hygromycin, and 3) with hygromycin + formaldehyde. Hygromycin-resistant *A. fumigatus* had similar levels of growth arrest on formaldehyde with and without hygromycin, while hygromycin-sensitive *A. fumigatus* did not grow on either.

Confrontations were performed in the same manner as described by Urquhart et. al. ⁴⁷. Spores from donor- and acceptor strains were streaked in a horizontal line on top of each other. This was performed on both PDA (complete media) and on Aspergillus Minimal Media (AMM). Table 1 shows the number of plates per acceptor strain for each media and incubation condition. Growth at 30°C was assessed after 2 weeks and after 1 month (31 days), while growth at 20°C was assessed after 1 month. In total, 72 confrontations were made per acceptor strain, making 144 confrontations in total see Table 1. After incubation, spores from the confrontation plates were scraped with a flame-sterilized scalpel and placed on PDA plates with selective levels of hygromycin and formaldehyde, and then incubated at 37°C. Growth was assessed after 2-3 days, and potential transformants were re-streaked on the same selective media.

Table 1: Amount of confrontation plates - per acceptor strain - made for each temperature, media, and incubation time condition.

	PDA- 1 month	AMM- 1 month	PDA- 2 weeks	AMM- 2 weeks
20°C	20	20	-	-
30°C	10	6	10	6

None of these 144 confrontations resulted in a detectible *Starship* transfer. After 3 days of incubation, many plates had small amounts of *A. fumigatus* growth radiating out from the clumps resulting from the scraping technique (Figure 2), but when these spores were re-streaked onto new selective media, they were unable to germinate. This phenomenon was present on the *P. variotii* selection plates as well despite the higher concentration of formaldehyde for *P. variotii* selection, which should have inhibited *A. fumigatus* growth regardless of *Chrysaor* presence.

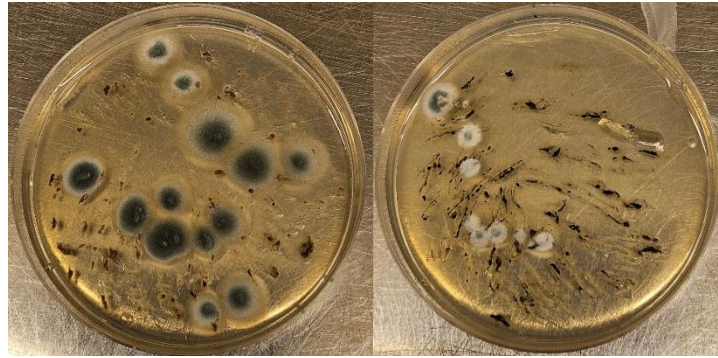


Figure 2. False positives for *Starship* transfer after 3 days of incubation on selective media, likely due to formaldehyde breakdown. Note the compact radial growth. Did not germinate when re-streaked onto new selective media.

Despite the initial pilot test showing no interaction between the formaldehyde and the hygromycin, it appears that the formaldehyde could be breaking down after a few days, allowing these false positives. Additionally, the large volume of spores gathered by the scraping-and-replating technique used lead to a massive number of spores present on the plate. It is possible that a subset of spores would germinate, absorb formaldehyde, and then perish, lowering the formaldehyde concentration in that specific area. This could thus have allowed other spores to germinate and grow slightly further before repeating the same process, a pattern that would gradually facilitate slow radial growth.

It should be noted that the different growth temperatures induced different morphologies in the confrontation plates: on the 30°C plates, *A. fumigatus* formed more areal hyphae, with some turning to a white “fluffy” morphology with a large hyphal body and no visible spores, possibly as a competition strategy (see Figure 3). In contrast, at 20°C, all plates retained typical *A. fumigatus* morphology, with pale green spores. If the mechanism of *Starship* HGT indeed works through the hyphal fusion theory, these alternate morphologies could affect the hyphal interactions and thus the rate of HGT.

Temperature might thus be an important factor in inducing *Starship* transfer, and is a key element for further investigation in the area.

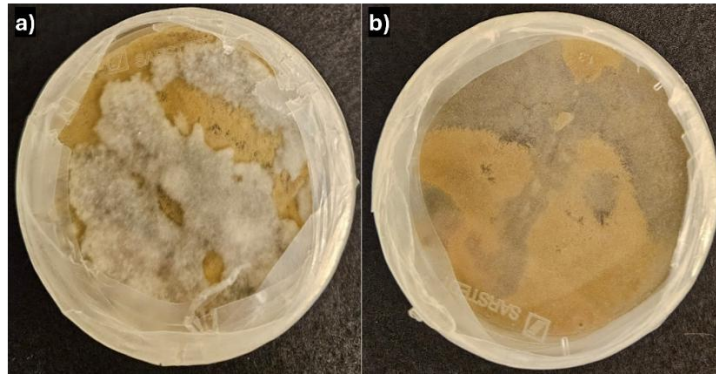


Figure 3. Varying *A. fumigatus* morphology after confrontation experiments at 30°C. a) Atypical *A. fumigatus* morphology only observed in 30°C confrontations, exhibiting tall white aerial hyphae with no visible spores. *A. fumigatus* in white, *P. variotii* in yellow. b) Typical *A. fumigatus* morphology, pale green spores and no tall hyphae. *A. fumigatus* in dark green, *P. variotii* in yellow.

There could be many potential explanations for why, unlike the successful *Starship* HGT previously performed by Urquhart et. al. ⁴⁷, none of the confrontations in this experiment resulted in a detectable HGT event. The simplest explanation would be that the *Logos_h1 Starship* used in my experiment is simply not capable of horizontal transfer. While HGT has been detected in various instances across *Starships* as a superfamily ^{36,39-41,47}, this specific *Starship* element might have an incomplete HGT machinery. Another explanation could be that that *Logos_h1* has a lower transposition frequency, and that HGT could be potentially possible, but was not detected in the 144 confrontations performed. While the same logic could be applied the inability of *Chrysaor* to be transferred from *A. fumigatus* acceptor to donor under these laboratory conditions, note that there is bioinformatic evidence showing *Chrysaor* HGT events have occurred in nature ⁴¹. However, this does not rule out the possibility of low transposition frequency or loss of element HGT machinery in the specific strain used. It is also possible that horizontal *Starship* transfer might require other interactions *in trans* that we are not yet aware of: for example, the recorded transfer of *Hephaestus* is suspected to have been aided by the co-transfer of *Pegasus*, another *Starship* ⁴⁷. One could even theorize that *Starship* elements are involved in their own networks of inter-element interactions, and perhaps *Logos_h1* and *Chrysaor* could have some sort of antagonistic effect on each other. However, this is not something that has been noted previously, and many genomes contain multiple *Starships* from different families without any observed antagonism between elements.

Different stress factors could also be required to induce HGT: Urquhart et. al used *P. variotii* as a donor strain, grown at 20°C ⁴⁷. As this is a thermophilic strain, perhaps the growth temperature induced a stress response facilitating HGT. While this experiment did induce stress in the form of nutrient starvation by extended incubation on minimal media, *A. fumigatus* might require other stress conditions to induce horizontal transfer. Another possible explanation for the lack of HGT in this

experiment could be that *A. fumigatus* itself might lack some inherent HGT machinery that is present in *P. variotii*. While *Starship* HGT has been indicated in *A. fumigatus*^{36,41}, the A1160 strain used is a lab strain that has been modified for improved homologous recombination rates, such as through the deletion of the *ku80* gene⁷⁰. As this gene is crucial for non-homologous end joining (NHEJ), removing this mechanism forces homologous recombination instead⁷¹. Although there is no immediate connection between NHEJ and HGT activity, perhaps there is some deeper mechanism involved which could be impacted by this modification. Furthermore, *A. fumigatus* is a heterogeneous species⁴⁵, and HGT competence could theoretically be governed by a strain-specific machinery that was already absent in the environmental progenitor of A1160.

We currently do not know to what degree the machinery of *Starship* HGT is governed by the elements themselves, versus by the host species they reside within. Perhaps *Starships* work analogous to mycoviruses, interfering with host defense systems such as against hyphal fusion⁵¹, or perhaps they simply turn themselves into cargo for some type of innate EV transfer system encoded by their fungal hosts. There could also be completely different mechanisms at play; could parallels be drawn to bacterial conjugative plasmids, that encode their own molecular machinery for horizontal transfer⁷²? It is clear that further research is needed to elucidate when, under what conditions, and through what kind of mechanism *Starships* are transferred. For future steps of this project, I would investigate and compare 1) the inherent transposition rates of different *Starships*, similar to previous experiments⁴³, 2) the effect of temperature and other stress factors on the HGT of the element previously achieved by Urquhart et. al.⁴⁷, and 3) investigate the differences between using different donor species.

***Trichoderma* confrontations**

To investigate if mycoparasitic interactions facilitate the rate of *Starship* HGT, donor strains with hygromycin-tagged *Starships* were exposed to mycoparasitic *Trichoderma* as an acceptor strain. First, as the *Trichoderma* strain acquired by our lab was an environmental isolate that had not been characterized previously, pilot experiments were required. To determine hygromycin resistance level, spores were streaked on PDA with different levels of hygromycin, and 80 µg/ml was found to effectively hinder growth. Then, mycoparasitism experiments (Figure 4) were performed to ensure that *Trichoderma* was able to successfully infect our donor strains: the abovementioned *A. fumigatus* donor strain with the hygromycin-tagged *Logos_h1*, as well as a previously constructed *P. variotii* strain with a hygromycin-tagged *Hephaestus Starship*⁴⁷. As a first experiment, *Trichoderma* was inoculated at a 2.8 cm distance from each respective donor strain on a PDA plate, and left to incubate at room temperature. This led to *Trichoderma* quickly colonizing the entire plate. To allow the donor strains to reach proper growth before being parasitized, strains were grown at 37°C for 3 days until they covered the entire plate, whereupon *Trichoderma* spores were inoculated in the corner of the

plate, which was left at room temperature. *Trichoderma* was able to effectively colonize both strains in these conditions as well.

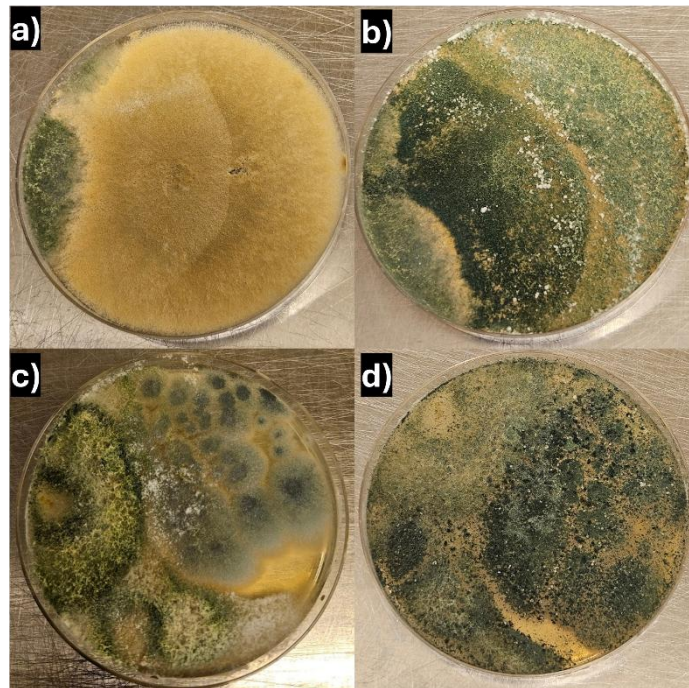


Figure 4. Mycoparasitic *Trichoderma* and host fungi, in different stages of infection. a) *P. variotii* (yellow) in intermediate stage of infection, 6 days after *Trichoderma* (green) inoculation. Note the hypha radiating out from the sporulating *Trichoderma* inoculation point. b) The same plate with *P. variotii* in late stage of infection, 12 days after *Trichoderma* inoculation. c) *A. fumigatus* (pale green) in intermediate stage of infection, 7 days after *Trichoderma* (bright green) inoculation. d) The same plate with *A. fumigatus* in late stage of infection, 1 month after *Trichoderma* inoculation.

The *P. variotii* strain used was a $\Delta LeuA$ leucine auxotroph and showed heavily impeded growth on plain PDA, as the inherent leucine concentration of PDA was too low to fully restore its growth. However, the experimental setup for the *Trichoderma* confrontations had no selection against *A. fumigatus*. Instead, I intended to utilize the necrotrophic abilities of *Trichoderma* itself, coining an “eat-the-donor” mechanism instead of using negative selection. Pilot experiments were performed where *Trichoderma*-infected *A. fumigatus* was scraped with a scalpel and streaked onto PDA with 80 $\mu\text{g/ml}$ hygromycin, followed by incubation at room temperature. Even though *Trichoderma* was unable to grow on the plate, *A. fumigatus* initially showed no growth at all. After a week, faint traces of pale green could be seen on the plate, which is the color of *A. fumigatus* conidia, but no hyphal growth extended from this even after an additional week of incubation (Figure 5). This is likely due to *Trichoderma* secreting a wide variety of secondary metabolites that inhibit and poison the host fungus⁶², meaning that any surviving conidia had a delayed recovery period and were unable to restore

normal growth. Thus, it seems like the *Trichoderma* “eat-the-donor” approach is effective enough to not require any additional selection against the donor, at least in the case of *A. fumigatus*.

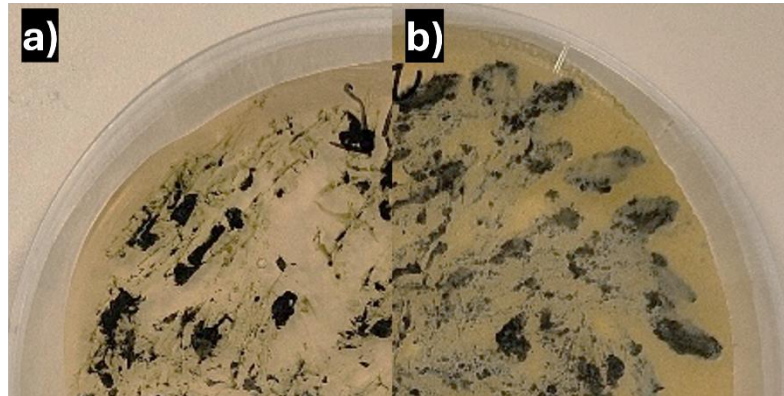


Figure 5. Harvested spores after *Trichoderma* growth on hygromycin-resistant *A. fumigatus*, plated on hygromycin media. a) After 1 day, neither species shows any growth, despite there being no added selection against *A. fumigatus*. b) After 1 week, a faint pale/dusty green color is visible, indicative of *A. fumigatus*, but not accompanied by any visible hyphal growth. Image contrast of adjusted for visibility.

For the confrontation experiments, the abovementioned donor strains of *A. fumigatus* and *P. variotii* were grown on PDA media at 37°C overnight. The next day, *Trichoderma* spores were placed at the edge of the plate. After this, three incubation conditions were used, with 10 plates of each donor strain in each condition: room temperature with exposure to light, darkness at 30°C, or darkness at 20°C. The plates grown at room temperature were harvested after 1 month; due to time constraints the 20°C and 30°C plates were harvested after 25 days. After incubation the plates were scraped with a flame-sterilized scalpel in the same fashion as the previous experiments described above, and streaked onto PDA containing 80 µg/ml hygromycin.

The ability for *Trichoderma* to parasitize on *A. fumigatus* was significantly impacted by incubation temperature, as incubation at 20°C led to complete colonization of *Trichoderma* on the plate, while incubation at 30°C led only to partial coverage of the *A. fumigatus* mycelia, see Figure 6. This is likely a result of *Trichoderma* having an ideal growth temperature of 25-30 °C⁷³ while *A. fumigatus* has a growth maximum of 37-42 °C⁷⁴. A similar effect could be observed in *P. variotii*, but less distinct and with more variation between individual plates.

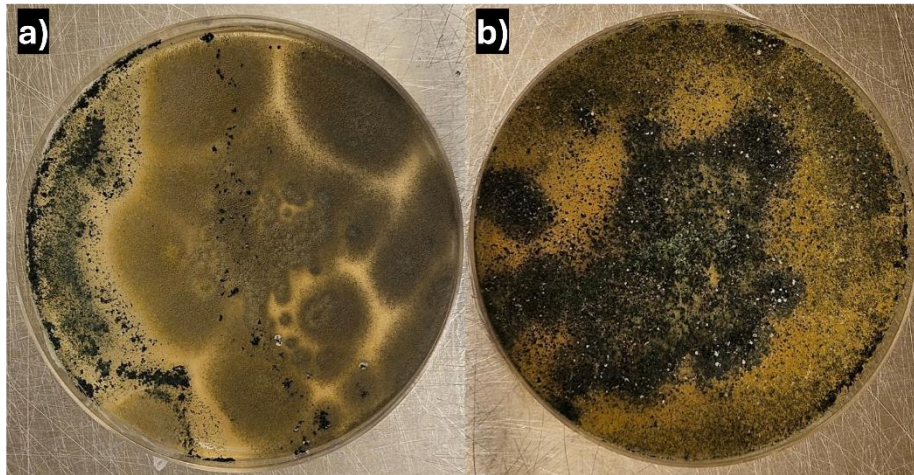


Figure 6. *Trichoderma* growth on *A. fumigatus* after 25 days at 30 °C (a) and 20 °C (b). *A. fumigatus* in pale/dusty green, *Trichoderma* in dark green. a) At 30 °C (a), *Trichoderma* is in the intermediate stage of infection, with the edge of sporulation at the midpoint of the plate. b) After the same incubation time at 20 °C, *Trichoderma* has completely colonized the entire plate.

No *Starship* transfer was detected in any of the 60 *Trichoderma*-infected plates used in this experiment. As stated above, it is technically possible that my *A. fumigatus* donor strain with the *Logos_h1 Starship* was somehow incapable of HGT. On the other hand, the *P. variotii* strain with a hygromycin-tagged *Hephaestus Starship* had successfully been used as a HGT donor previously⁴⁷. However, the furthest phylogenetic transfer in that experiment was a trans-family transfer, between species of the same order. *Trichoderma* is not only of a different order, but of a different phylogenetic class: *Trichoderma* is Sordariomycetes, while *A. fumigatus* and *P. variotii* are both Eurotiomycetes. While trans-class transfers like this have been indicated to happen in *Trichoderma*⁶³, the rate of transfer is likely lower across such a phylogenetic distance, and *Starship* transfers across such a distance have not been verified. If such transfers are possible, especially in a laboratory setting, a much larger sample size might be required to detect them. Future studies could try similar experiments with other *Starship*-containing Sordariomycetes, such as *Podospora* or *Fusarium*. After conditions for HGT have been optimized, transfer across further taxonomic distances could be investigated. It is worth noting here that the hyphal fusion theory of *Starship* transfer would likely be more sensitive to taxonomic distance between donor and acceptor, as opposed to the extracellular vesicle theory, and thus these results could indicate more support for the former.

Furthermore, a longer incubation period could be required, to ensure that *Trichoderma* has had extended time for *Starship* uptake. This is especially true for the plates grown in 30 °C, where *Trichoderma* had not even had time to spread across the entire *A. fumigatus* plate, and thus did not make contact with the entire hyphal body. It is also possible that the digestion of the host fungus is too effective, breaking down the donor before transfer is achieved. Perhaps a different *Trichoderma* strain

that has a longer biotrophic phase before breaking down the host, or extended time at growth conditions (such as 30 °C) where *Trichoderma* colonization is slower, would allow more time for mechanisms such as hyphal contact or EV transfer while the donor is still alive.

Bioinformatic identification of *Starships* in *Trichoderma*

To identify *Starships* natively present in the *Trichoderma* genus, I used the NCBI database of publicly available genomes. Starfish is a bioinformatic toolkit developed for *Starship* identification ⁴²; for this project, I used the “Nextflow” pipeline for this toolkit currently being developed by Adrian Forsythe in my research group. This requires genomes that are high quality assemblies, and that have GFF annotation files. For this run, I thus filtered the NCBI database for annotated genomes only, with an assembly level of “chromosome level” or higher. This resulted in 8 assemblies: *Trichoderma asperellum*, *Trichoderma atroviride*, *Trichoderma simmonsii*, *Trichoderma semiorbis*, *Trichoderma breve*, *Trichoderma cornu-damae*, as well as two different isolates of *Trichoderma virens*, see methods. I also attempted using Liftoff ⁷⁵ to annotate additional genomes of *T. harzianum*, but was unsuccessful. Running the abovementioned 8 genomes through the Nextflow Starfish pipeline resulted in the identification of three different *Starships*: all of them were in *T. virens* (Table 2). A homology-based search against Starbase, our database for identified *Starships*, returned no close matches, nor could it classify them into any existing families, suggesting that these might be previously unidentified *Starships*.

Table 2. Accession numbers, chromosomal locations, and lengths of three bioinformatically identified putative *Starships* in *T. virens*.

Nr.	Source strain	Genome accession	Chromosome	<i>Starship</i> start	<i>Starship</i> end	<i>Starship</i> length
1	<i>T. virens</i> Gv29-8	GCA020647635.1	4 (GenBank CP071110.1)	<i>CP071110.1</i> : 1033259	<i>CP071110.1</i> : 1325755	292 kbp
2	<i>T. virens</i> FT-333	GCA020647705.1	1 (GenBank CP071115.1)	<i>CP071115.1</i> : 254453	<i>CP071115.1</i> : 467305	213 kbp
3	<i>T. virens</i> FT-333	GCA020647705.1	4 (GenBank CP071118.1)	<i>CP071118.1</i> : 1258647	<i>CP071118.1</i> : 1353292	94.6 kbp

To investigate the cargo genes present in these putative *Starships*, I searched for conserved protein domains using NCBI conserved domain search. All of the *Starships* contained some expected auxiliary genes such as ankyrin-repeats (present in NLRs), PLPs, and DUF3723. Many of the predicted domains were broad gene families from which I could not identify specific functions. Some domains found, like GH18 chitinase found multiple times in *Starship nr 1* and 2, are found in large numbers in mycoparasitic *Trichoderma* species, thought to be used to break down host cell walls ⁷⁶.

Starship nr 2 had several domains identified as “FxSxx-COOH system tetratricopeptide repeat protein”, which belongs to a *Streptomyces* biosynthetic system. However, a blastx search of the sequence revealed, in addition to several hypothetical proteins predicted in fungi, a 61% identity match to a NLR in *Podospora bellae-mahoneyi* (XP_062733979.1). I performed an AlphaFold structure prediction on both proteins and aligned the structures using PyMol. The structural similarity suggests that these proteins are thus also a type of fungal NLR, see Figure 7. NLRs are common fungal proteins, and thus there is no strong evidence pointing to this gene being the result of a *Streptomyces* HGT.

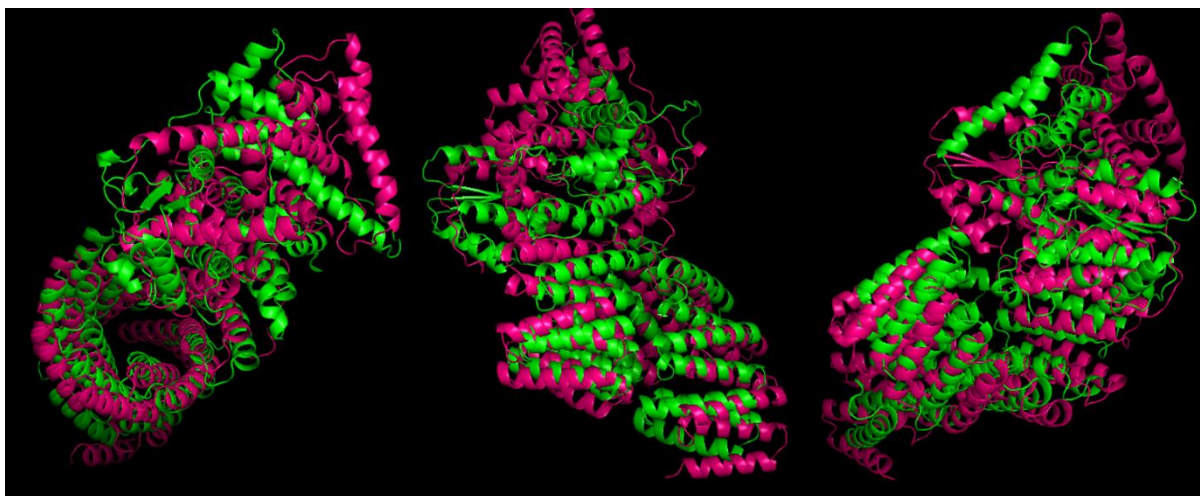


Figure 7. Multiple angles of aligned AlphaFold structures of two predicted proteins. Green: Cargo protein in newly identified *T. virens* Starship nr 2, with a predicted “FxSxx-COOH system” domain, normally present in *Streptomyces*. Pink: Nod-like receptor (NLR) from *P. bellae-mahoneyi*. The structural similarity between the proteins suggests that the predicted cargo protein (green) is also a fungal NLR.

In *Starship nr 1*, a domain was predicted as “antiviral radical SAM protein viperin”. Viperin is an antiviral protein present across all domains of life, and has been the subject of various structural studies⁷⁷. There are two viperin-like genes in *T. virens* Gv29-8: one in chromosome 2, and one inside the newly identified *Starship nr 1* residing in chromosome 4. Previous *in vitro* studies of these two proteins found that one of them (uniprot entry G9MQB8) performed the catalytic activities expected of a viperin-like protein, while the second one (uniprot G9N0G3) did not⁷⁸. A tblastn alignment of the G9N0G3 sequence against the Gv29-8 genome confirms a 100% identity with the viperin-like sequence in *Starship nr 1*, and only a 66% identity with the viperin-like gene in chromosome 2. Thus, it appears that the “native” viperin gene of *T. virens* is the one located on chromosome 2, and that the *Starship* is encoding the additional “non-canonical” viperin-like protein.

The abovementioned study concluded that the function of G9N0G3 remains unknown; they found that homologues of this protein are found mainly in *Trichoderma*, and importantly: genomes that contain this gene also contain a copy of the “canonical” viperin-like protein, leading to speculation of additional functions in the divergent version⁷⁸. This is especially interesting in light of my discovery that the “non-canonical” viperin-like protein is actually cargo within a *Starship*. Gene duplication and differentiation is a powerful driver of evolution, as mentioned in the introduction, and the balance between deletion/pseudogenization and neofunctionalization is complicated. *Starships* frequently contain genes also found in the genome, which could suggest that the host genome is used as a source to acquire cargo genes³⁷. Additionally, their cargo can become permanently incorporated into the host genome upon *Starship* “death”. This has some interesting implications: if *Starships* are able to acquire copies of genomic DNA, these duplicate genes would then fall under different evolutionary pressures than they would in the host genome. *Starship* mobilization of gene copies could thus add an additional layer of complexity to the differentiation and neofunctionalization of duplicated genes, and their capacity for HGT could lead to spread between, and eventual fixation in, different species and populations. The dynamics of *Starship* cargo acquisition, differentiation, and mobilization could have an interesting impact on fungal evolution and is an area of great interest for further study.

While interesting, this discovery is not able to provide support for the initial hypothesis, that the lifestyle of mycoparasitic genera such as *Trichoderma* leads to a higher frequency of *Starship* uptake. As *Starfish* works by finding presence/absence gaps between assemblies, at least two annotated high-quality assemblies are needed for *Starship* discovery. The fact that I only found *Starships* in *T. virens* is likely due to this being the only species with two annotated high-quality genomes available, and my attempt at Liftoff-annotating additional genomes did not work. The difference between different *Trichoderma* species is likely too great for a between-species comparison to result in *Starship* discovery. There are now over 40 long read *Trichoderma* genomes available on NCBI, with multiple entries for most species. A future project could perform proper annotations on these genomes, and thus run Nextflow on a broad array of *Trichoderma* species. In particular, comparing mycoparasitic species to the non-mycoparasitic *T. reesei* could provide insights into lifestyle impact on *Starship* presence.

Conclusions

The mechanisms of *Starship* transfer are not yet fully elucidated, and the methods that work for one element in one species might not be ideal for another. Further laboratory work will be necessary to construct a system to induce HGT of different *Starship* elements, in different host species, in a consistent way. The formaldehyde-hygromycin double-selection method I used is less practical due to the possibility for false positives to grow, a problem that is not present if using the originally planned

setup with an auxotrophic donor strain. On the other hand, *Trichoderma* seems to effectively hinder donor strain growth even in the absence of selection, and could thus be of continued interest as an acceptor strain as it provides a simple experimental setup. However, having a large phylogenetic distance between donor and acceptor presumably impedes the likelihood of successful element transfer and integration, and as such a more closely related donor strain would be recommended. Bioinformatic comparisons of *Starship* frequency in mycoparasitic *Trichoderma* as compared to non-parasitic fungi was impeded by the lack of annotated high-quality genomes, something that could be subject for a future project. I did however identify three new *Starships* in *T. virens*, one of which carries a viperin-derived protein of unknown function, highlighting the interplay between genomic content and *Starship* cargo, and its implications for fungal evolution.

Material and methods

Strains and media

PDA, Potato Dextrose Agar: Potato peptone 4 g/l, glucose 20 g/l, agar 15 g/l.

PDB, Potato Dextrose Broth: Potato peptone 4 g/l, glucose 20 g/l.

AMM, Aspergillus Minimal Media: NaNO₃ 6 g/l, KCl 0.52 g/l, MgSO₄*7H₂O 0.52 g/l, KH₂PO₄ 1.52 g/l. NaOH adjust pH to 6.5. Glucose 10 g/l, Hunters trace elements 2 ml/l, agar 15 g/l.

Protoplast recovery media: AMM (agar concentration 7 g/l), sucrose 342 g/l, leucine 340 g/l.

YPD, Yeast extract Peptone Dextrose media: Yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l, agar 15 g/l.

LB, Luria-Bertani: Casein peptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, agar 15 g/l

SOC, Super Optimal broth with Catabolite repression: Tryptone 20 g/l, yeast extract 5 g/l, NaCl 10 mM (0.58 g/l), KCl 2.5 mM (0.19 g/l), MgCl₂ 10 mM (0.95 g/l), MgSO₄ 10 mM (1.2 g/l), glucose 20 mM (3.6 g/l).

Uracil-free Yeast Selection Media: Y1501-20G [Sigma-Aldrich] 1.92 g/l, Yeast Nitrogen Base Y1250-250G [Sigma-Aldrich] 6.7 g/l, glucose 20 g/l, agar 20 g/l

SAB, Sabouraud media: Dextrose 40 g/l, peptone 10 g/L, pH 5.6.

TRAF01: KCl 0.6 M (44.7 g/l), CaCl₂ 50 mM (5.5 g/l), 5 mM Tris-HCl (pH 7.5)

TRAF02: TRAF01 solution + 40% PEG4000

Donor strains:

The *Aspergillus fumigatus* donor strain was constructed from lab strain A1160. After inserting the HygR gene into the *Logos_h1 Starship*, the resulting donor strain was named **STLH**.

For mycoparasitism experiments a previously constructed donor strain was used⁴⁷: *Paecilomyces variotii* strain **HhpjKO**, constructed from the strain CBS 144490, auxotrophic for leucine and with a HygR-tagged *Hepheastus Starship*.

Acceptor strains:

For the formaldehyde- selection experiments, environmental *A. fumigatus* strain **C-1-80s-1**, and *P. variotii* strain **CBS 101075** were used, both possessing the *Chrysaor Starship* conferring formaldehyde resistance ⁴¹.

For the *Trichoderma* experiments, a recent environmental isolate was used, which had been identified as *Trichoderma harzianum* via ITS (Internal Transcribed Spacer) sequencing. However, no full-genome sequencing has been performed. As the *harzianum* clade of *Trichoderma* actually contains multiple different species that cannot be reliably distinguished via ITS ⁶⁸, this isolate is referred to simply as *Trichoderma*.

Trichoderma pilot experiments

Trichoderma spores were acquired from a previous environmental isolate acquired from soil in Sweden, and grown on PDA plates at room temperature. To investigate hygromycin resistance levels, spores of *Trichoderma* were picked with a flame-sterilized loop and placed on PDA plates containing 20, 40, 60, 80, and 100 µg/ml hygromycin respectively, incubated at room temperature, and assessed for growth after 3 days. To assess competitive ability at room temperature, spores from *Trichoderma* and *A. fumigatus*/*P. variotii* respectively were placed at equal distances of 2.8 cm from each other on a PDA plate. Growth was assessed after 3 days. To assess mycoparasitism of established cultures, *Trichoderma* spores were picked with a loop and placed at the edge of sporulating *A. fumigatus* and *P. variotii* respectively, and left to incubate at room temperature. Growth was monitored over the course of two weeks.

Construction of *LeuC* and *BafZ* gene knockout plasmids

To generate the gene knockout plasmid constructs, PCR primers were designed to amplify a ~1500 pb fragment on the 5' and 3' flanks of the target gene. For the *LeuA/LeuC* knockout, the target gene was *LeuC*, and which was to be replaced with the *PtrA* gene for pyrithiamine resistance. For the hygromycin resistance insertion, the target gene was the native *BafZ* gene present in the *Logos_h1 Starship*, which was to be replaced with the *HygR* gene. Primers were designed to amplify these antibiotic resistance genes as well. At the tail end of each primer, a 20 bp overlap with the neighboring fragment was added, in order to allow for correct assembly into a knockout construct, see Figure 8. Previously extracted genomic A1160 DNA was used for the amplification of gene flank fragments, and the *PtrA* and *HygR* genes were amplified from the PSK275 and pAN 7.1 plasmids respectively.

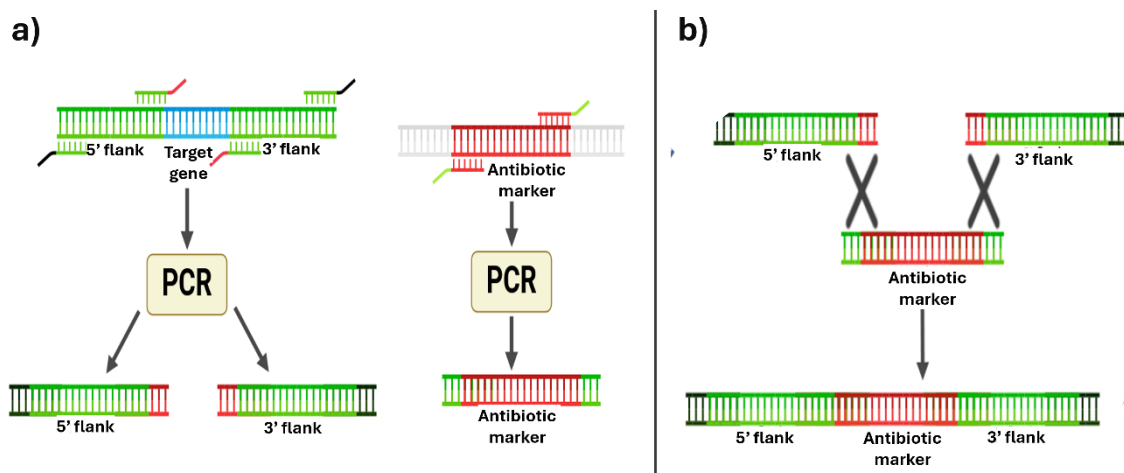


Figure 8. Graphical summary of generating knockout constructs. a) PCR primers were constructed for the 5' and 3' flanking region of the target gene, as well as for the antibiotic resistance marker. At the edge of each primer, 20 bp of overlap with the neighboring fragment was added. b) the overlapping regions of the constructed fragments allow homologous recombination into one contiguous sequence. Not shown: knockout fragment being inserted into the pYES2 vector plasmid via the same method of homologous recombination, using sequence overlap here represented in black. Created with BioRender.com.

Primers used (5'-3'), overlap sites underlined:

LeuC knockout:

5' flank fragment:

F: CTACTAGCAGCTGTAATACGACTCACTATAGGGAATATTAAGCTTACTGAA
CTAATCGGCACTGC

R: CCAATGGGATCCCGTAATCAATTTAGGCATGATGTCGGTATCG

3' flank fragment:

F: ATACAAACAAAGATGCAAGAGCCAAAAGTTGATCTGGACG

R: TGCATGCTCGAGCGGCCCGCCAGTGTGATGGATATCTGCAGAATTCAACCTC
TTTGCAGAAGCTCC

PtrA gene:

F: CGATACCGACATCATGCCTAAATTGATTACGGGATCCCATTGG

R: CGTCCAGATCAACTTTTGGCTCTTGCATCTTTGTTTGTATTATACTG

PCR program using Q5 polymerase [New England Biolabs]: 35 cycles of: 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Finish by 72°C for 2 min, followed by 4°C.

Hygromycin insert:

5' flank fragment:

F: TAGCAGCTGTAATACGACTCACTATAGGGAATATTAAGCTACATGTTTAGCC
CTCCACAG

R: GTGACTTTTGGTTACGCCGTGTTAAGCCACGCAGTGATTG

3' flank fragment:

F: GGACACACATTCATCGTAGGGAGATCGAGAGGTTTCATGG

R: TGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTACATGCTACTC
ACAGGATGC

Hygromycin resistance gene:

F: CAATCACTGCGTGGCTTAACACGGCGTAACCAAAAAGTCAC

R: CCATGAAACCTCTCGATCTCCCTACGATGAATGTGTGTCC

PCR program, using Q5 polymerase [New England Biolabs]: 35 cycles of: 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Finish by 72°C for 2 min, followed by 4°C.

The vector pYES2 plasmid was cleaved using HindIII and EcoRI restriction enzymes. The construct component DNA (5' flanking region, 3' flanking region, antibiotic selection marker, and pYES2 plasmid) were then transformed into *Saccharomyces cerevisiae* strain BY4742 using standard PEG+LiAc transformation method⁷⁹ with minor modifications. Briefly, *S. cerevisiae* was grown in liquid YPD media, pelleted in a benchtop centrifuge at 13 000 g and washed with H₂O. The pellet was then mixed with 240 µl PEG4000 (50% W/V), 12 µl LiAc (1.0 M), 50 µl heat-shocked single-stranded carrier DNA, 1 µg transformation plasmid, and H₂O up to 34 µl. The mixture was vortexed and then left to incubate for 1h at 42 degrees. The mixture was then pelleted by centrifugation, resuspended in H₂O, and plated on uracil-free media; BY4742 is auxotrophic for uracil, but the URA3 gene is present on the pYES2 plasmid. The plates were then incubated at 30 °C.

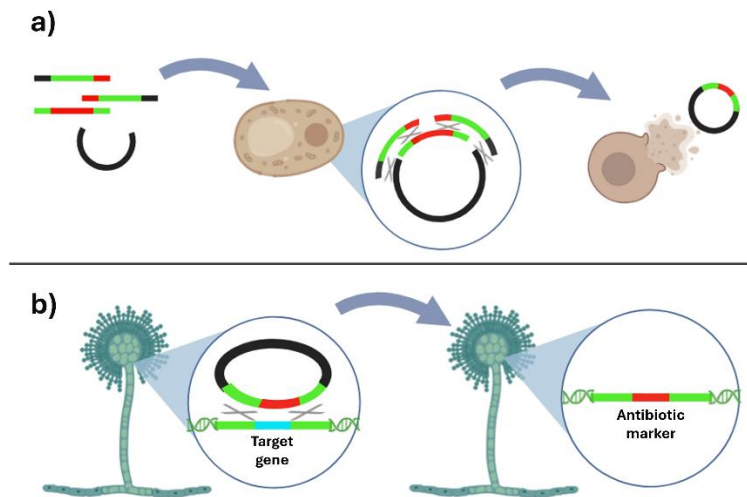


Figure 9. Graphical summary of plasmid gene knockout procedure. a) PCR fragments of target gene flanks (green), antibiotic resistance marker (red), and the vector plasmid (black) are transformed into *S. cerevisiae*. Due to the overlapping sequences, the fragments are homologously recombined into an assembled knockout plasmid. b) Knockout plasmid is transformed into *A. fumigatus*, wherein homologous recombination between the flanks of the target gene and the knockout plasmid leads to the target gene being replaced with the antibiotic resistance marker. Created with BioRender.com.

The intrinsic homologous recombination machinery of *S. cerevisiae* assembles the overlapping DNA fragments into the finished knockout plasmid, where the 5'- and 3'- flanking fragments are now flanking the antibiotic resistance marker, see Figure 9. This plasmid was then extracted from the yeast cells using Zymoprep™ Yeast Plasmid Miniprep II kit [Zymo Research] according to manufacturer's instructions.

To amplify the plasmid, it was transformed into previously prepared competent *Escherichia coli* using standard heat shock protocol. Briefly: 2 µl of plasmid suspension is added to 1ml pre-prepared competent cell solution. Incubate on ice for 30 minutes, heat shock at 42 °C for 30 seconds, then leave 2 minutes on ice. Add 950 µl of SOC recovery media, shake at 37 degrees for 1h, plate on selective LB media (100 µg/ml ampicillin, as pYES2 contains ampicillin resistance gene). After overnight incubation, the amplified plasmid was extracted using QIAGEN® Plasmid Midi Kit [QIAGEN] according to manufacturer's instructions. Plasmid sequence was then confirmed via Oxford Nanopore sequencing.

Transformations

A. fumigatus transformations were performed using a modified version of a protoplast transformation protocol described previously⁸⁰. Briefly, 10⁶ spores were suspended in 50 ml SAB liquid media, and incubated with shaking overnight at 37 °C. The mycelia was then filtered with a sterile miracloth,

washed with water, and digested in a solution of 10 ml TRAF0 1 with 0.5 g VinoTaste® Pro enzymes [Novozymes], shaking for 1-2 hours at 30 °C. After verifying protoplast formation via microscopy, the protoplasts were filtered through a 40 µm cell strainer, and pelleted at 1600 g for 10 minutes at 4 degrees, keeping the solution on ice when not centrifuged. The supernatant was then removed, pellet resuspended in 10 ml TRAF0 1, and centrifuged at the same settings again. Then it was resuspended in 0.5 ml TRAF0 1, protoplasts were counted in a hemocytometer and adjusted to $0.5-1 \times 10^7$ protoplasts/ml using TRAF0 1. 105 µl of protoplast suspension was then added to a microcentrifuge tube along with 1 ng of the knockout plasmid construct diluted to 20 µl H₂O, and 25 µl TRAF0 2. To make a positive and negative control, two additional mixtures were made in the same way, but with 20 µl H₂O with no plasmid added. The tubes were then left to incubate on ice for 25 minutes. Then 250 µl of TRAF0 2 was added to each tube, and left to incubate for 5 minutes at RT. A thin layer of protoplast recovery agar was poured and allowed to solidify on a plate. Then the protoplast mixture was added to 6 ml molten protoplast recovery agar, mixed, and poured on top of the plates. To ensure that the temperature of the agar had not killed the protoplasts, the positive control was performed first. The plates were left at room temperature for an hour, then left to incubate overnight at 37 °C. After incubation, 25 ml of protoplast recovery agar with selection (0.1 or 1 µg/ml pyrithiamine, or 200 µg/ml hygromycin) was carefully poured on top of the transformation plate as well as the negative control. For the positive control, the same amount of agar was poured on top, with no selection. The plates were then incubated at 37 °C for another 2 days.

To verify successful transformants, spores from growing colonies were single-spore isolated. Conidia were streaked onto selective PDA and incubated at 37 °C overnight, whereupon germinating single spores were identified under a stereo microscope, cut out from the agar and transferred to a new plate using a flame-sterilized scalpel, then incubated at 37 °C for two days. Then, CTAB DNA extraction was performed as described previously⁴⁷. Briefly, mycelia was grown overnight in PDB, followed by freeze drying for 24 hours. Samples were then homogenized by bead beating, suspended in 1 ml of CTAB buffer (100 mM Tris-HCl pH 7.5, 0.7 M NaCl, 10 mM EDTA pH 8, 1% CTAB, 1% β-mercaptoethanol). After incubation at 65°C for 30 minutes, 500 µl was added to an equal volume of chloroform and centrifuged at maximum speed in a benchtop centrifuge (~16000 x g) for 10 min. The aqueous phase was mixed with an equal volume of isopropanol to precipitate the DNA which was subsequently pelleted, washed with 70% ethanol and air dried before being resuspended in 50 µl H₂O.

After DNA extraction, PCR followed by Sanger sequencing was performed to confirm that the insert was in the correct genomic location. One primer (CTTCTACAAGGATTCGGTGG) was designed corresponding to a genetic region 225 bp upstream of the 5' flanking region used in the transformation construct (see Figure 10, green arrow). A second primer (ACATCGAAGCTGAAAGCACG)

corresponded to the middle of the hygromycin resistance gene, which after a successful integration should reside 3 kbp downstream of the first primer (see Figure 10, red arrow). A third primer (AACACCCATAGCACGAAGAC) corresponded to the start of the native *BafZ*: if still natively present, this would reside 1.5 kbp downstream of the first primer (Figure 10, blue arrow). After PCR with DreamTaq polymerase [Thermo Scientific] using these three primers (*Denaturation: 95°C for 3 min. 30 cycles of: 95°C for 30 s, 55.3°C for 30 s, 72°C for 3 min. Finish by 72°C for 5 min, followed by 4°C*), a successful recombination of the hygromycin gene into the *Starship* would yield a 3 kbp PCR product, while the presence of a native *BafZ* gene would lead to a 1.5 kbp PCR product. After a 3 kbp product was acquired after successful transformation, its sequence was confirmed via Sanger sequencing.

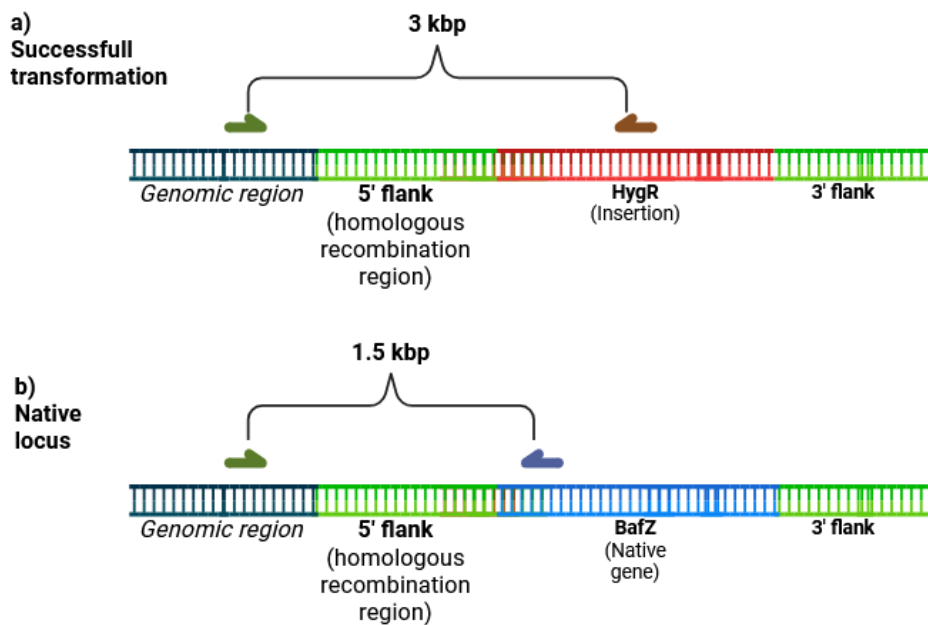


Figure 10: Graphical summary of gene knockout verification procedure. First primer (green) corresponds to a genomic location outside of the recombined region. The second primer (red) binds to the *HygR* gene, which in the case of successful knockout replacement will bind to a location 3 kbp downstream of the first primer. The third primer (blue) binds to the native *BafZ* gene. If the native locus is still intact (after unsuccessful gene knockout), this will bind at a position 1.5 kbp downstream of the first primer. If transformation succeeded, PCR will result in 3 kbp fragment: if native gene is still present, PCR will result in 1.5 kbp fragment. Figures not to scale. Created with BioRender.com.

Horizontal gene transfer experiments

For the horizontal transfer confrontation experiments, conidia from the donor (*A. fumigatus* STLH) and acceptor strains (*A. fumigatus* C-1-80s-1 and *P. variotii* CBS 101075) were streaked in a horizontal line across PDA- or AMM agar plates, directly on top of each other. Plates were wrapped in

parafilm and left to incubate for 2 weeks or 1 month, at 30 °C or 20 °C. After incubation, conidia from the confrontation plate were scraped with a flame-sterilized scalpel and streaked onto a selective PDA agar plate containing the appropriate concentration of hygromycin and formalin. For C-1-80s-1: 250 µg/ml hygromycin and 0.5 µl/ml formalin, for CBS 101075: 100 µg/ml hygromycin and 2 µl/ml formalin. Selection plates were then left to incubate for 2-3 days at 37 °C. Any significant growth was then restreaked onto selective media.

For the *Trichoderma* experiments, conidia from the donor strain (*A. fumigatus* STLH or *P. variotii* HhpjKO) were plated on PDA (with added 2.8 mM of leucine in the case of HhpjKO) and incubated overnight at 37 °C. After incubation, conidia from *Trichoderma spp.* were placed at the corner of the plate. Plates were incubated in the following conditions: 1 month at room temperature (22 °C) in light, 25 days at 20 °C in darkness, or 25 days at 30 °C in darkness. 10 plates of each strain were made for each incubation condition, resulting in 60 plates in total. After incubation, the conidia were scraped with a flame-sterilized scalpel and streaked onto PDA agar with 80 µg/ml hygromycin.

Starship identification in *Trichoderma*

To identify *Starships* in *Trichoderma*, the “Nextflow” version of *Starfish*-identifying pipeline “Starfish” was used ⁴². Annotated genomes of *Trichoderma* species were downloaded from NCBI, with an assembly level of “chromosome” or “complete”, leaving 8 genomes with the following accession numbers:

GCA_020647865.1 *Trichoderma asperellum*
GCA_020647795.1 *Trichoderma atroviride*
GCA_019565615.1 *Trichoderma simmonsii*
GCA_020045945.2 *Trichoderma semiorbis*
GCA_028502605.1 *Trichoderma breve*
GCA_020631695.1 *Trichoderma cornu-damae*
GCA_020647635.1 *Trichoderma virens*
GCA_020647705.1 *Trichoderma virens* FT-333

These sequences were downloaded and ran through Nextflow, a pipeline currently in development for *Starfish* automation. Identified *Starship* sequences were then ran through NCBI CD search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). NCBI blast, blastx, and tblastn was used to compare both nucleotide and amino acid sequences.

For structural comparisons, amino-acid sequences were submitted to AlphaFold for structural prediction ⁸¹, and aligned using PyMol ⁸².

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