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PERSPECTIVE

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That's no moon, it's a *Starship*: Giant transposons driving fungal horizontal gene transfer

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Abstract

To date, most reports of horizontal gene transfer (HGT) in fungi rely on genome sequence data and are therefore an indirect measure of HGT after the event has occurred. However, a novel group of class II-like transposons known as *Starships* may soon alter this *status quo*. *Starships* are giant transposable elements that carry dozens of genes, some of which are host-beneficial, and are linked to many recent HGT events in the fungal kingdom. These transposons remain active and mobile in many fungal genomes and their transposition has recently been shown to be driven by a conserved tyrosine-recombinase called 'Captain'. This perspective explores some of the remaining unanswered questions about how these *Starship* transposons move, both within a genome and between different species. We seek to outline several experimental approaches that can be used to identify the genes essential for *Starship*-mediated HGT and draw links to other recently discovered giant transposons outside of the fungal kingdom.

KEYWORDS
fungi, horizontal gene transfer, Starship, transposon, tyrosine recombinase

1 | INTRODUCTION

Horizontal gene transfer (HGT) is the movement of DNA between individuals outside of the vertical inheritance pathway (Keeling & Palmer, 2008). HGT occurs extensively within prokaryotes and is accepted as a major mechanism driving rapid adaptive evolution (Gogarten & Townsend, 2005; Woods et al., 2020). Within prokaryotes, we have an in-depth understanding of mechanisms such as transduction, conjugation and phage-mediated HGT. Reports of HGT between eukaryotes emerged swiftly after the advent of PCR in the early 1990s (Andersson, 2005; Kidwell, 1993; Rosewich & Kistler, 2000). Most of these early reports were primarily HGT of transposon sequences. However as more whole genomes became available, many studies showed strong evidence for the horizontal transfer of nuclear genes (Richards et al., 2009; Wisecaver & Rokas, 2015). Like prokaryotes, the genes identified as moving horizontally between eukaryotes often had functional domains associated with survival in a particular environment or could confer defences against parasites. For example, Richards et al. (2011) reported several high confidence HGT events between fungi and oomycetes of genes hypothesised to facilitate the colonisation of plants (Savory et al., 2015). Other more distant HGT events between fungi and plants have also been described, such as the transfer of the *fhb7* gene from an unknown *Epichlöe* sp. to the wheat relative *Thinopyrum elongatum* (Wang et al., 2020). This HGT event is considered impactful as the *fhb7* gene is known to confer resistance to fusarium head blight disease (Wang et al., 2020). In most cases, high sequence similarity between these distantly related organisms is the primary, but indirect, evidence of horizontal exchange of DNA (Rosewich & Kistler, 2000).

There are multiple documented examples of horizontally transferred transposons within fungi (Casacuberta & González, 2013; Rosewich & Kistler, 2000). Furthermore, work on plant genomes has strengthened the link between HGT and transposons, and shown that horizontal transfer could be an important part of the transposon lifecycle to avoid silencing (Baidouri et al., 2014). More recent reports of HGT events in fungi have noted the proximity of the horizontally transferred DNA to repeat-rich regions of the genome or known transposable elements. This led many to speculate that transposons might play a role in either moving or acting as a 'safe-landing' zone for the horizontally transferred DNA (Cheeseman et al., 2014; Friesen et al., 2006).

Repetitive genomic regions are often difficult to assemble with short-reads alone, so despite a plethora of available genomes, most assemblies have been unable to place these horizontally transferred DNA segments into better context within the whole genome. The advent of more accessible long-read sequencing technology has increased the number of chromosome scale assemblies, thereby enabling us to place many 'orphan' HGT events into fully assembled chromosomes (McDonald et al., 2019). This has led to the discovery of a novel group of giant transposons known as *Starships* (Gluck-Thaler et al., 2022). Over 100 *Starship* transposons have now been identified in dozens of Pezizomycetes species, a fungal class within the filamentous Ascomycete phylum (Gluck-Thaler et al., 2022). Many of these *Starships* have been observed with exceptionally high sequence identity in very distantly related fungi (Gluck-Thaler et al., 2022; Urquhart et al., 2023, McDonald, unpublished data). The observation of highly identical transposons within distantly related species is suggestive that *Starship* transposons encode all the genetic machinery required to move themselves between different species. This perspective article will focus on potential mechanisms that might drive *Starship*-mediated HGT and discuss future experiments which test these hypotheses.

2 | OVERVIEW OF THE DISCOVERY OF *STARSHIPS*

Transposons are mobile genetic elements which can excise and reintegrate throughout the genome. Transposons are split into class I retrotransposons and class II DNA-based transposons. Herein, we focus only on the DNA class II transposons. Class II transposons use a 'cut-and-paste' model, where the transposon moves between different genomic loci as a DNA intermediate (Wicker et al., 2007). Class II transposons often leave evidence of transposition events called target site duplications (TSDs). TSDs are created when a transposase makes a staggered cut in double-stranded DNA creating two single-stranded overhangs of the target sequence. The transposon then inserts into this cut-site, between the two single-stranded overhangs. DNA repair machinery native to the host cell fixes these short stretches of single-stranded DNA resulting in a duplication of the target sequence (thus TSD) on either side of the transposon insertion. The size and sequence of these TSDs is often used to classify transposons into different superfamilies. Other characteristics used to classify transposons into smaller families are terminal inverted repeat (TIR) sequences and nucleotide and protein similarity, especially of the transposase enzyme (Wicker et al., 2007).

Starships have not been formally grouped according to the Wicker classification system for transposons; however, due to their putatively unique replication strategy and definitively unique size, we suggest they be included as a novel transposon order, alongside Cryptons. Cryptons are an order of class II transposons that differ from other DNA-based transposons due to their hypothesised YR-mediated, recombination-driven transposition. Despite moving as a DNA intermediate, the YR domain of Cryptons shares strong amino acid similarity to the YR domain found in class I *DIRS*-retrotransposons (Goodwin et al., 2003). *Starships* are defined by the presence of a DUF3435-containing tyrosine recombinase (YR) gene, colloquially known as the 'Captain'. This gene is always the first gene in the transposon and is used to orient all *Starships* discovered thus far in the same direction, with the Captain taking the first 5′ gene position. The most notable difference between *Starships* and other class II transposons is their size. *Starships* are much larger than most known transposons with many exceeding 100 kbp (Gluck-Thaler et al., 2022). There are several conserved gene families found in most but not all Starships thus far, known as 'accessory' or 'auxiliary' genes (Figure 1a) (Gluck-Thaler et al., 2022; Urquhart et al., 2023). These genes are highly diverse, both in putative function and sequence, and include patatin-like phosphatases, ferric reductases and NOD-like receptors (NLR). As genes with these domains are found in most, but not all, *Starships*, it is hypothesised that they confer a fitness benefit to the transposon itself, perhaps related to transmission through horizontal transposon transfer or retention in a novel host genome (Urquhart et al., 2023).

3 | EXAMPLES OF HORIZONTALLY TRANSFERRED *STARSHIPS*

Starships also carry 'cargo' genes, which have been shown to increase the fitness of the fungal host and presumably then survival of transposon itself (Gluck-Thaler et al., 2022; Vogan et al., 2021). These cargo genes differ to auxiliary genes as they are not conserved between different *Starship* elements. *Hephaestus* is an example of a *Starship* which benefits its host fungi by carrying cargo that confers metal tolerance. Research by Urquhart et al. (2022) demonstrates that *Hephaestus* provides resistance to lead, cadmium, zinc, copper and arsenic in *Paecilomyces variotii*. Heavy metal resistance in fungi is known to be a complex trait, often with significant amounts of redundancy (Bazzicalupo et al., 2020) and in *Hephaestus* metal tolerance is attributed to four loci (Urquhart et al., 2020). To what extent metal tolerance contributes of the fitness benefit *Hephaestus* provides to its host is unclear. *Hephaestus* has since been discovered in *Paecilomyces lecythidis* and *Penicillium fuscoglaucum* (Urquhart et al., 2022, 2023). The conserved *Hephaestus* element in *P. lecythidis* differs by only one nucleotide from *P. variotii* indicating an extremely recent HGT event. The high level of sequence conservation of the element within multiple species lends support to the idea that this element does confer a strong host fitness benefit.

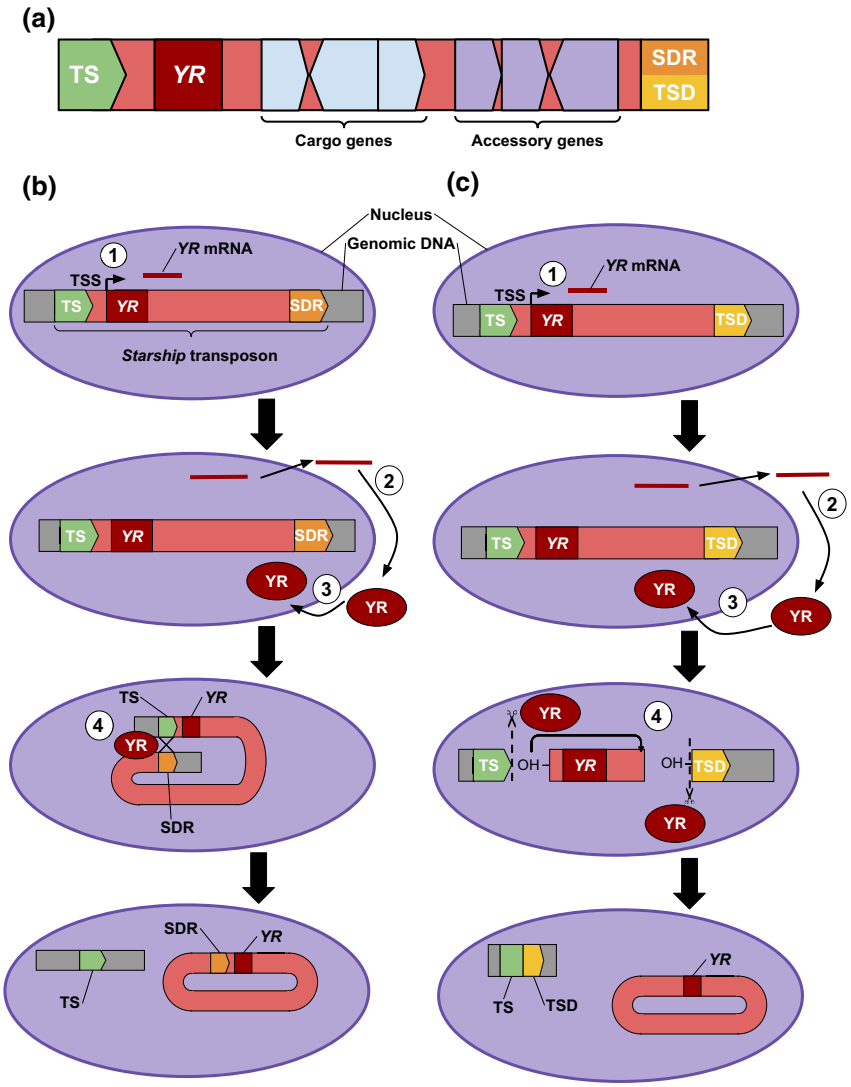

FIGURE 1 A schematic overview of Starship composition and two models for movement via extra-chromosomal circular DNA (eccDNA). (a) Overview of the architecture of a generic *Starship*, adapted from Gluck-Thaler et al. (2022). Starships have a tyrosine recombinase transposase gene (YR, red) at their 5′ end. 'Cargo' genes (light blue) are genes that confer putative fitness benefits to the fungal host, while 'accessory' genes (light purple) are hypothesised to facilitate horizontal Starship movement. (b, c) A comparison between excision of a Starship via homologous recombination (b) versus nuclease activity (c). The top panel in both models show transcription from the transcription start site (TSS, white circle 1) of the tyrosine recombinase (YR) gene (red) into mRNA. Next, the Captain mRNA is translated in the cytoplasm (white circle 2) and re-imported into the nucleus (white circle 3). In (b), a Captain-driven homologous recombination occurs between the TS and SDR (white circle 4), which results in a circular *Starship* excised from the genomic DNA. Shown in the bottom panel is the result of the recombination, which is a circularised *Starship* that contains a short, directed repeat (SDR) (orange) which is used as the homologous sequence to target the next insertion site. The genomic DNA (grey box) has a 'clean' excision site with only the TS (green) remaining. In (c), the YR-captain acts as a nuclease cutting at or near the target site (white circle 4). This leaves exposed 3′-OH groups which attack the same strand leaving a circular *Starship* molecule. In this model, the excised *Starship* leaves behind the target site (TS) (green) and target site duplication (TSD) (yellow) within the chromosomal DNA (grey).

Another example of horizontally transferred cargo is the *ToxA* gene now found in three plant pathogenic fungi; *Bipolaris sorokiniana*, *Pyrenophora tritici-repentis* and *Parastagonospora nodorum*. ToxA is a well-characterised, secreted, effector protein that induces cell death in wheat lines carrying a specific susceptibility gene, *Tsn1* (Friesen et al., 2006; McDonald et al., 2017). In this example, the *Starship* cargo is a small class II transposon called toxhAT which carries *ToxA*, (McDonald et al., 2019). While the evolutionary journey of *ToxA* between these three species is unknown, what is clear from the current genome assemblies is that toxhAT (carrying *ToxA*) has been independently captured by two different *Starship* transposons, *Sanctuary* and *Horizon* (Gourlie et al., 2022; McDonald, unpublished data). While the cargo toxhAT is conserved to a high degree (>92% identical across 14 kbp), *Sanctuary* and *Horizon* share no sequence similarity to each other, and their YR-captains are extremely phylogenetically distant (McDonald, unpublished). The high percentage identity and presence within two separate *Starships* does suggest that toxhAT is capable of independent movement outside of either *Sanctuary*

or *Horizon*. *Sanctuary* has been identified only within *B. sorokiniana*, while *Horizon* has been identified in *P. tritici-repentis* and *P. nodorum*, though degraded in the latter (Gourlie et al., 2022; McDonald, unpublished data). Based on the degraded state of *Horizon* in *P. nodorum*, it is difficult to conclude if this species could have been the donor of the active *Horizon* now found in *P. tritici-repentis*. What remains unclear is how toxhAT has moved between *Horizon* and *Sanctuary*. Current analysis is ongoing to determine if these two *Starships* can be found together in one species, thereby providing the opportunity for toxhAT to jump from one *Starship* to the other.

The recent discovery of *Starships* had led to a retrospective look of previously identified large HGT elements within fungi for reclassification. One such element is *Wallaby* which is found in multiple *Penicillium* spp. Originally discovered by Cheeseman et al. (2014) alongside other HGT regions now recognised as *Starships*, *Wallaby* contains all the characteristics of a traditional *Starship* transposon. Furthermore, *Wallaby* and another HGT element, now known as *Aristeus*, contain genes with Hce2 domains (Urquhart et al., 2023), which confer an advantage in microbial competition and plant virulence (Stergiopoulos et al., 2012). *Wallaby* also carries *paf* as a cargo-gene, which is a known cytotoxic antifungal effector (Ropars et al., 2015). Another identified *Starship* element originally discovered by Cheeseman et al. (2014) and designated a *Starship* by Urquhart et al. (2023) is *CheesyTer*. This element conferred a fitness advantage to growth within lactose (Ropars et al., 2015).

To summarise, *Starships* have a wide array of host-beneficial cargo which is hypothesised to allow these large elements to persist in their host genomes. While *Starships* have only recently been described, the case study of *Wallaby* and *CheesyTer* demonstrate that a retrospective look on past large, mobile genomic regions can also identify further evidence of *Starship*-HGT.

4 | HOW DO STARSHIPS MOVE?

The discovery of *Starships* and their links to multiple large HGT events in fungi leads to the question of how they move both within a genome and between species. Due to the relatedness between the 'Captain' and YR-genes found in Cryptons (Gluck-Thaler et al., 2022; Urquhart et al., 2023), it is hypothesised that these transposons could share a mechanism for transposition. However, the extent of the relatedness between 'Captain' and Crypton YR-genes remains unclear. Gluck-Thaler et al. (2022) argues that the phylogenetic relatedness of the 'Captain' to a Crypton YR-gene is equal to that between 'Captain' and bacterial YR-genes. Despite their phylogenetic distance, both Crypton and *Starship* YR-genes have a similar predicted 3D conformation that shows the highly conserved tyrosine recombinase R-Y-R-Y tetrad at the predicated active site (Goodwin et al., 2003; Vogan et al., 2021). This link between *Starships* and Cryptons is exciting because of the way in which Cryptons are predicted to move, as an extra-chromosomal circular DNA (eccDNA) (Goodwin et al., 2003). The transposition method used by Cryptons has never been experimentally proven, but it is hypothesised to involve a site-specific recombination at the termini of the transposon resulting in an eccDNA molecule. Recent research has associated eccDNA formation with Ty1 and Ty3 retrotransposons within *Magnaporthe oryzae* (Joubert & Krasileva, 2022). These findings corroborate previous research into eccDNA formation by Ty1 retrotransposons within yeast (Møller et al., 2015). These data add to a growing body of evidence that eccDNAs are involved in transposition of many transposon orders. They also provide an exciting, but tentative, link between *Starships* and Cryptons, and Cryptons and LTR-retrotransposons regarding eccDNA formation. Under this model, once the eccDNA element has formed, it travels to a new genome site where the YR targets a sequence similar or identical to the terminal sequence and integrates (Goodwin et al., 2003). The recombination-driven insertion leads to the same short target sequence flaking both ends of the element, which Goodwin et al. (2003) define as a 'short-direct repeat' (SDR). While not stated explicitly by the authors who coined this term, SDRs are distinct from TSDs because they are created as a result of this recombination event by the YR-gene. The other major difference between SDRs and TSDs is what remains in the empty transposon site after the transposon moves to a new location.

In Figure 1b,c, we outline two possible models for *Starship* YR-driven excision from the genome. In these models, we highlight the difference between a recombinase-driven excision versus nuclease-driven excision as described for other class II transposases. In a recombinase-driven mechanism, the target sequence and the 3′ termini of the *Starship* are nearly identical. A YR-driven recombination between these two sequences results in the excision of the 3′ termini with the *Starship* as it moves as a circular extrachromosomal element to the next target sequence. In this model, excision leaves a single 'clean' excision site (Figure 1b, bottom panel). Alternatively, *Starships* could move in a way similar to other class II transposons that generate TSDs upon transposon insertion (Figure 1c). In this model, excision by the YR-gene does not involve a recombination between the 3′ termini and the transposon, but instead a simple cut at either end of the transposon. Here, the TSDs remain after excision of the transposon, leaving 'scars' within the genome (Figure 1c, bottom panel). Herein, we propose that *Starships*' Captains' are recombinases and the putative 'target site duplications' (TSDs) reported in most studies may be instead 'short direct repeats' (SDRs). This is supported by recent data from Urquhart et al. (2023), who showed that *Hephaestus* left a clean excision site after moving to a novel genome location.

In both models of excision, we propose that *Starships* may move between fungal cells as a stable eccDNA (Figure 1b,c, bottom panel), similar to what has been proposed for Cryptons (Goodwin et al., 2003). To test this hypothesis, we propose a simple PCR screen with primers pointing outwards towards the edges of the linear *Starship* when integrated in the genome. Upon excision and circularisation, these primers would face towards each other, thereby allowing PCR amplification. If transposition occurs through a different mechanism, then amplification would not occur. An alternative, but much more expensive approach, is to sequence the entire eccDNA population from the cell to look for *Starship* eccDNAs. This approach requires significantly more work to optimise

and control for sequencing artefacts, however, a DNA extraction that specifically targets circular DNAs may be required even for the PCR experiment described above. Both experiments require a control to ensure the transposon is active. Measuring transposition of Starships has been done previously using a split-reporter mechanism (Urquhart et al., 2023). However a more simple method that quantifies transcription of the 'Captain' through qPCR, as used previously to quantify the activity of large transposases in cyanobacteria, could also be an effective strategy (Hackl et al., 2023). Together, these data would provide evidence to support or refute the hypothesis that *Starships* transpose as eccDNAs.

5 | HOW DO *STARSHIPS* MOVE HORIZONTALLY?

Despite strong genomic evidence that *Starships* are active transposons, this does not elucidate how they move from one fungal species to another. Successful integration of horizontally transferred DNA between eukaryotic species requires solutions to multiple problems. These issues have been well described by Fitzpatrick (2011) and Huang (2013) but, to summarise, they can be broken into three main categories: movement, processing and genome defence. Firstly, the transposon DNA must exit the nucleus and pass though the cell membrane and cell wall of both the donor and recipient individuals before integrating into the new genome. Secondly, once integrated, the coding sequences must handle differences in intron processing as well as incompatible promoters. During processing and transcription, the transferred DNA must avoid genome defence mechanisms which could induce repression or inactivation of the transposon. This defence could occur through RNA interference, or, in fungi, by a process called repeat induced point mutation (Buchon & Vaury, 2006; Hood et al., 2005). Given these barriers, the observation of multiple examples of horizontally transferred *Starships* suggests that these elements have specific properties or genes that enable them to move horizontally. The conserved 'auxiliary' genes found within *Starships* may hold the key to horizontal movement, as many of these genes contain domains already associated with DNA translocation in bacteria or hyphal fusion in Actinomycetes and fungi (Gluck-Thaler et al., 2022; te Poele et al., 2008).

The transfer of genetic material between two separate cells requires a physical connection. Fungi lack any structure analogous to bacterial pili which could be used as this 'bridge' between the cells. Instead, cellular fusion could be the necessary link between the cells allowing the transfer of genetic material, this process is called anastomosis.

The concept of eukaryotic HGT through fungal anastomosis is not new (Fitzpatrick, 2011; Mehrabi et al., 2011), but these experiments are technically difficult to conduct as fungi have sophisticated and understudied self/nonself-recognition pathways. Whether *Starships*, through their auxiliary genes, can promote the active formation and stabilisation of anastomoses between hyphae of different fungal species, is one of the most exciting and challenging areas for future research.

Anastomosis of nonself in fungi has been observed in both hyphae and early germinating conidia, forming conidial anastomosis tubes (CATs) (Mehrabi et al., 2011). Both conidial and hyphal anastomosis forms heterokaryotic cells, which contain nuclei from two genetically distinct individuals. The formation of heterokaryons could provide the necessary 'bridge' allowing the transfer of genetic material between the two nuclei (Figure 2a).

Such a transfer has been reported under laboratory conditions before, the non-*Starship* transposon *Tad* has been observed to move horizontally between forcibly induced heterokaryons of *Neurospora crassa* (Kinsey, 1990). Currently, one of the most distantly related observations of hyphal fusion was described by Ishitani and Sakaguchi (1956) between auxotrophic mutants of *Aspergillus oryzae* and *Monascus* sp., that both fall within the family *Aspergillaceae* (Houbraken et al., 2020). While the majority of CAT fusion research has focused on intra-specific fusions of *N. crassa*, the mechanism has also been observed between species of *Colletotrichum* (Gabriela Roca et al., 2004, 2005). The reporting of CAT fusion and heterokaryosis between *C. gossypii* and *C. lindemuthianum* was especially exciting as it involved two common plant pathogens and produced 'hybrid' strains with clear phenotypic differences from their parents (Gabriela Roca et al., 2004). However, these experiments also required selection with hygromycin to form the heterokaryons. Without auxotrophy or strong selection, there are very few reports of hyphal or CAT fusion above the genus level, which makes some of the putative Starship HGT events reported between different genera especially interesting to explore further.

Heterokaryon incompatibility (HI) is one reason why CAT or hyphal fusion, and subsequent successful heterokaryosis, between distantly related fungi is not easily observed. In fungi, HI induces programmed cell death of the heterokaryon in response to nonself-recognition (Mehrabi et al., 2011). While this signalling pathway is not well defined, there are examples where HI can be circumvented. For example, Ishikawa et al. (2012) showed that HI is supressed in heterokaryons derived from CAT fusion and mature fungal colonies derived from these CAT fusions also showed evidence of non-meiotic genetic recombination. HI is also not activated if the two fungal individuals are recognised as 'self'. This process is known to be partly controlled by the expression of different NLR proteins between the nuclei within the heterokaryon (Dyrka et al., 2014). Many *Starships* auxiliary genes can be classified as NLRs, which leads to the speculative hypothesis that these genes could play a role in 'tricking' the self-/nonself-recognition pathways in these fungi to facilitate *Starship* transfer. *Starships* also carry other immunity-related genes which contain NUDIX domains (Gluck-Thaler et al., 2022). Typically, fungal proteins containing NUDIX domains act as effectors, but these domains play a known role in hyphal regulation within Actinomycete bacteria (te Poele et al., 2008). We know too little about the NLRs and other auxiliary protein families found in *Starships to* speculate

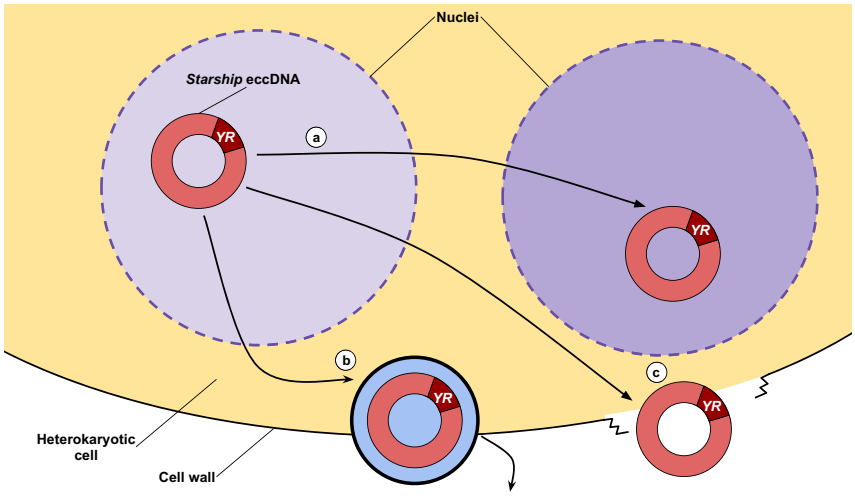

FIGURE 2 Schematic of possible pathways for interspecific Starship movement. (a) Within a interspecific heterokaryon (yellow), the Starship eccDNA (red) could move between two genetically distinct nuclei (light and dark purple representing two different species). (b) Alternatively, the eccDNA Starship is encapsulated within an extracellular vesicle (blue) to pass through the cell and undergo exocytosis. (c) The eccDNA Starship escape through a cell wall breakage. This mechanism would not require encapsulation but assumes the eccDNA could survive unencapsulated within the extracellular environment. Not shown in pathways (b, c) is uptake by the second species and incorporation into the novel host genome. The formation of a heterokaryon is also not required for these two pathways.

further about the how these genes could be involved in facilitating the establishment of stable inter-species heterokaryons, but if supported by further experiments, it is exciting to consider how these transposons might be used in the future to further our understanding of fungal immunity.

Similar to the active method proposed above, another novel area in fungal biology that could be linked to *Starship* transfer is extracellular vesicles (EVs). Here, *Starships* would move from a cell through vesicular exocytosis, where after leaving the nucleus, the transposon is encapsuled within a vesicle and exported from the cell (Figure 2b). Fungal EVs have been a growing field in recent years especially in yeasts and human pathogenic fungi; however, there remains much to discover about the process and what molecules EVs can or cannot transport (Liebana-Jordan et al., 2021; Rizzo et al., 2020). While it is known that fungal EVs can carry DNA, much more work has been done to explore RNA cargo or other biological molecules (Yáñez-Mó et al., 2015). Currently, there is no known upper size limit of DNA which could be carried within a fungal EV. Work with human cell-derived vesicles, showed that linear DNA was loaded more efficiently into EVs when compared to plasmid DNA, but this was only tested up to a few kilobases (Lamichhane et al., 2015). With *Starships* often exceeding 200 kbp in length (Gluck-Thaler et al., 2022), it remains unknown whether these EVs could accommodate these long DNA molecules without pre-processing or even at all.

Finally, the third proposed mechanism of HGT is through cell wall escape. This mechanism requires the transposon to pass through a breakage within the cell wall (Figure 2c). Unlike the previous two proposed mechanisms, this is a passive mechanism requiring no active process by the transposon. While there is little research into unencapsulated extracellular fungal DNA, its release has been observed under laboratory conditions within *Aspergillus fumigatus* and *Candida albicans*. However, neither of these examples occurred due to cell wall breakages (Martins et al., 2010; Rajendran et al., 2013). Testing such a hypothesis would first require the ability to induce transposition events of a *Starship*. If this can be achieved, then cell wall disruption could be done artificially using detergents, inhibiting the cell wall integrity pathway within fungi, or through sonication. The circular DNA hypothesis for *Starships* could be beneficial under this model, as the structure would most likely make it more stable than linear DNA due to it being shielded from exonuclease activity (Sin et al., 2020).

6 | THE NEXT FRONTIER: FROM SEQUENCES TO WORKING EXPERIMENTAL SYSTEMS

While we have proposed three ways in which *Starships* could move between species, our ability to test these different hypotheses is limited as we do not have a working experimental model to observe horizontal *Starship* transfer in real time. We propose that the next frontier in *Starship* exploration must be to establish an experimental workflow, whereby *Starships* can be tracked as they move from one cell to another. Ideally, this workflow would incorporate fluorescent markers, which would allow for sub-cellular localisation of the *Starship* as it moves between fungal cells. However, in order to achieve this, a larger screen that first identifies the environmental conditions that activate *Starships and* encourage them to 'hop' to new locations is required. Such an experimental system has already been partially developed by Urquhart et al. (2023) who used a split-marker approach to identify transposition events of *Hephaestus* in *P. variotii*. In this system, the hygromycin phosphotransferase gene is separated from an actin promoter by the *Hephaestus* element. Upon transposition of *Hephaestus*, the actin promoter and hygromycin

phosphotransferase gene are brought together, allowing expression and conferring resistance to hygromycin. Using this strain, the authors calculated the rate of *Hephaestus* hopping by recovering isolates that were able to grow on media with hygromycin and high levels of both zinc and cadmium (Urquhart et al., 2023). Expanding this work to a wide variety of developmental growth stages and environments could be used to systematically identify conditions that activate the *Starship*. Once the activity of a *Starship* can be induced, the next stage is to facilitate an interspecific transfer. For this experiment, we propose the creation of 'donor' strain that contains a *Starship* tagged with a selectable marker and 'recipient' *Starship-less* strains tagged with a different selectable marker. To observe interspecific *Starship* transfer, both strains would be grown together, under a wide variety of environmental conditions. These conditions would vary with the *Starships* being experimented on and should focus on the conditions under which the cargo gene can confer a strong fitness benefit. For example, *in-planta* conditions would be suitable for either *Sanctuary* or *Horizon* as their *cargo ToxA* confers pathogenesis benefits on susceptible wheat varieties. However, *in vitro* conditions would be more appropriate for other *Starships*, such as heavy metal exposure for *Hephaestus* or lactose exposure for the *CheesyTer* element. These co-cultures could then be filtered and screened for HGT of the Starship by simply selecting for cells that grow on both the tagged *Starship* and 'recipient' selectable markers. If successful, this system could be used to define the minimal *Starship* unit capable of transposition and HGT, like the minified *Hephaestus* element used by Urquhart et al. (2023). Defining the minimal unit still capable of HGT would then enable systematic testing of some of the hypotheses proposed above. For example, are the NLR genes required for HGT and are they required for successful cell fusion? Knocking out or swapping NLR genes present in a minified model *Starship* could then provide insight into whether these NLRs determine the 'host-range' of *Starships* (i.e. does the auxiliary gene sequence determine which fungal species a Starship can invade).

The discovery of *Starships* is exciting as these elements add to a growing body of evidence, from all kingdoms of life, that large horizontal DNA transfers (>100 kbp) are actively contributing to adaptive evolution. This suggests that HGT driven by super large DNA transposons is an ancient and evolutionarily successful mechanism for moving adaptive traits between species. Some examples of these elements include bacterial genomic islands (Dobrindt et al., 2004), DNA 'Borgs' in Archaea (Al-Shayeb et al., 2021) and the giant 'Tycheposons' in cyanobacteria (Hackl et al., 2023) most of which have been described within the last year. Interestingly, both *Starships* and Tycheposons encode putative transposases with YR-domains, which in *Starships* has been shown to be essential for transposition. Outside of the YR-domain, these proteins show very little sequence similarity, which suggests that YR-driven transposition has evolved independently in both cyanobacteria and fungi. Many questions still remain: To what extent do the mechanisms that facilitate these giant horizontal transfers overlap? What tools do these transposons use to facilitate their uptake by different species and when does this exchange occur? Why do these transposons maintain their large size? *Starships* are just one piece in this larger puzzle, which hold great promise to further our understanding the mechanistic basis underpinning adaptive HGT.

AUTHOR CONTRIBUTIONS

Megan C. McDonald: Conceptualization; writing – original draft; supervision; resources. **Angus H. Bucknell:** Conceptualization; writing – original draft.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ETHICS STATEMENT

The funders played no role in the conception, design or drafting of this perspective.

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