Review

Gene transfer into the fungi

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ABSTRACT

A growing body of data suggests that fungi have gained genes by horizontal gene transfer (HGT). This is an exciting result because fungi at first glance represent the most recalcitrant of all organisms to gene transfer, possessing robust cell walls and having lost phagotrophic capacities because they feed exclusively by osmotrophy. Nonetheless, a number of mechanisms have been implicated in gene transfer including: anastomosis of cellular structures, conjugation-like transfer between bacteria and yeasts, and exchange of supernumerary chromosomes. Despite absence of clearly identified mechanisms driving gene transfer in fungi, genome analysis has provided evidence for a number of fungal genes derived from foreign genomes by HGT. We briefly summarise current approaches to identifying HGT using genome data and make the case that phylogenetic analysis is the best approach to find and test potential examples of HGT. By applying this approach we have collected as many datasets as we could find for which phylogenetic analyses have been used as evidence of HGT and re-tested all 340 examples using updated taxon sampling. This approach enabled us to provide further supporting evidence for 323 examples of HGT, representing a significant pattern of transfer from both prokaryotes (mainly bacteria) and fungi into fungal genomes. Annotation of the HGTs suggests that these transfers have added to the core nutrient-processing metabolic network of many fungi, expanding the sugar, nitrogen, amino acid, nucleobase, and macromolecule metabolism of fungal microbes. Furthermore, these transfers appear to have added a significant number of new genes to the secretome and transporter repertoire of fungi, implying that gene transfer has added to the osmotrophic capacity of many fungal species.

1. Introduction

Horizontal gene transfer (HGT), or lateral gene transfer, describes the transmission of genetic material between organisms, specifically across species boundaries (Andersson, 2009; Doolittle et al., 2003; Keeling and Palmer, 2008; Ochman et al., 2000; Richards et al., 2003). The term species is of course a difficult concept to apply to asexual microbes (Cohan, 2002; Doolittle, 2008; Konstantinidis and Tiedje, 2005) so HGT is best thought of as transmission between distinct, reproductively isolated genomes. HGT therefore leads to patterns of gene ancestry that contradict typical vertical transmission of genetic material from parent to offspring.

HGT in prokaryotes

HGT represents an important factor in shaping the genomes of prokaryotes and has provided a key source of evolutionary

innovations (e.g. Doolittle, 1999a; Jain *et al*., 2003; Ochman *et al*., 2000). Several routes for transfer have been identified including gene transfer agents, transduction, transformation and conjugation (Lang and Beatty, 2007; Thomas and Nielsen, 2005). Phylogenomic analyses of prokaryotic genomes have demonstrated that HGT has also occurred at a high frequency between prokaryotes (Bapteste *et al*., 2005; Bapteste *et al*., 2008; Doolittle, 1999b; Kloesges *et al*., 2011). This has led some researchers to suggest that HGT represents such a significant force that a single bifurcating phylogenetic tree and unified taxonomic hierarchy cannot accurately describe the tree of life (Bapteste and Boucher, 2008; Doolittle, 1999b). In short, the tree of life may instead be best represented as a complex net of gene ancestries. In contrast, others have argued that careful targeting of specific gene markers, combined with sophisticated phylogenetic methods can identify a skeleton tree of life, upon which hangs an extensive web of gene transfers (Cox *et al*., 2008; Gribaldo *et al*., 2010; Kelly *et al*., 2010).

HGT in eukaryotes

In comparison to prokaryotes, HGT is thought to occur at a lower frequency in eukaryotes, for several reasons discussed below. Nonetheless, a growing body of evidence suggests HGT has proved to be a factor in the evolution of eukaryotic genomes. Currently four broad categories of gene transfer have been identified in eukaryotes:

(1) Gene transfer as part of the process of primary endo-symbiosis (i.e. mitochondrial or plastid endosymbiosis) in which genes have been transferred from the endo-symbiont's genome to the host nuclear genome (often called endosymbiotic gene transfer or EGT – Esser *et al*., 2004; Martin *et al*., 2002). EGT has been an impor-tant source of foreign genes; for example, large-scale genome analysis suggests 18 % of the *Arabidopsis* genome was derived from the proto-plastid (Martin *et al*., 2002).

(2) Secondary, serial and tertiary endosymbiotic events, involving the engulfment of a photosynthetic plastid-bearing eukaryote by another eukaryote, have also lead to extensive gene transfer between eukaryotes where components of the mitochondrial, plastid and nuclear genome of the endosymbiont have been incorporated into the host (for review see: Archibald, 2009; Cavalier-Smith, 2000; Keeling and Palmer, 2008).

(3) Gene transfer from prokaryotic to eukaryotic genomes without endosymbiosis and often by an unidentified mechanism (e.g. Andersson *et al*., 2003; Archibald *et al*., 2003; Carlton *et al*., 2007; Loftus *et al*., 2005).

(4) Gene transfer between eukaryotes (e.g. Moran and Jarvik, 2010; Richards *et al*., 2006; Richards *et al*., 2009; Slot and Hibbett, 2007; Slot and Rokas, 2010, 2011).

This review will focus on mechanistic factors related to HGT into eukaryotic and fungal microbes. We discuss how to identify and test putative HGTs. We will then summarise currently identified cases of HGT events in fungi. Finally we will describe how this pattern of transfer has affected the gene/protein repertoire of fungal microbes.

2.   Mechanistic aspects and HGT in eukaryotes

Mechanisms driving gene transfer in eukaryotes

Several mechanisms have been implicated in gene transfer in eukaryotes beyond endosymbiosis. These include the activity of third party biological factors or vectors such as viruses (Liu *et al*., 2010), gene transfer agents (Richards and Archibald, 2011) and transposon-mediated transfer (Clark *et al*., 2002; Diao *et al*., 2006; Engels, 1992). For example, Taylor and Bruenn (2009) have provided evidence for integration of clus-tered non-retroviral RNA virus genes into distantly related fungal genomes: *Candida parapsilosis*, *Penicillium marneffei*, and *Uromyces appendiculatus*. These include totivirus-derived puta-tive RNA-dependant RNA polymerase and capsid-encoding genes (Taylor and Bruenn, 2009). No evidence of virus produc-tion could, however, be identified demonstrating that the genes have been integrated (co-opted) into the host genome and sug-gesting that virus infection can lead to gene transfer into fungal genomes.

Doolittle has suggested that phagocytosis represents a major route for HGT into eukaryotes. Specifically, eukaryotic microbes that feed by phagocytosis (phagotrophy) create a gene transfer ratchet, whereby the act of grazing on foreign cells followed by intracellular digestion, sets up the opportunity for repeat incidences of gene transfer from prey-to-predator; a gene transfer ratchet (Doolittle, 1998). Large-scale phylo-genomic analysis of phagotrophic protists has provided evidence for HGT from prokaryotes-to-eukaryotes, consistent with the 'you are what you eat' hypothesis, (Andersson *et al*., 2003; Archibald *et al*., 2003; Carlton *et al*., 2007; Loftus *et al*., 2005). This pattern is, for instance, apparent in the phagotrophic intestinal parasite *Entamoeba histolytica* that has acquired 96 genes from prokaryotic sources by HGT. The ancestry of these HGTs appears to be predominantly of Bacteroidetes origin. Interestingly, members of this bacterial phylum are known to share the oral and intestinal environments of vertebrates along with *Entamoeba* (Loftus *et al*., 2005) so presumably must repre-sent a source of prey for this phagotrophic amoeba. This last example highlights the importance of considering the ecolog-ical and biological circumstances for proposed cases of HGT.

Mechanisms retarding gene transfer in eukaryotes

Several characteristics of eukaryotic cells potentially act as barriers to HGT. These include the possession of a nuclear envelope and the storage of genetic material in chromatin. In addition, the RNA interference system of many eukaryotes with associated gene silencing systems such as Repeat induced Point Mutation (RIP), methylation induced pre-meiotically (MIP) and quelling as observed in many filamen-tous fungi (Irelan and Selker, 1996), must also act to protect, or purge genomes of foreign nucleotide sequences.

Many eukaryotes are multi-cellular organisms and there-fore separate their reproductive and somatic cell lines. Such a physical segregation of somatic and germline cells also theo-retically limits the opportunity for transfer into gamete cells; minimizing the rate at which transferred genes could become fixed within an evolutionary lineage (Richards *et al*., 2003).

Furthermore, incompatibility of gene promotor and intron-splicing systems may also limit gene transfer between eukaryotes and from eukaryotic-to-prokaryotic genomes (Keeling and Palmer, 2008).

Carl Woese has suggested that the maintenance of a universal genetic code across the tree of life has provided an important pre-requisite to enable gene transfer (Woese, 2000), effectively allowing distantly related organisms 'to trade in the same currency' as other species. However, several eukaryotic subgroups have different codons usage patterns (e.g. ciliate and oxymonad protists). Such changes can prevent the successful translation and/or functional activity of invading genes, limiting fixation of foreign genes (Silva *et al.*, 2007). In the fungi, such genetic code variations have been suggested to act as a barrier to HGT for *Candida albicans* and close relatives; all of which translate the CUG codon as a serine rather than a leucine (Silva *et al.*, 2007; Sugita and Nakase, 1999). Phylogenomic analyses specifically looking for examples of prokaryote-derived HGTs among the 'CTG' species has identified only two examples that post-date the change in genetic code (Fig. 1 – Marcet-Houben and Gabaldon, 2010). While Fitzpatrick *et al.* (2008) identified an additional two prokaryote-derived HGTs specific to the *C. parapsilosis* genome (not shown on Fig. 1). These data demonstrate that a very small proportion of the HGTs occurred after the point in the fungal phylogeny when the CTG genetic code alteration occurred, in contrast to equivalent branching depths on the fungal phylogeny (Fig. 1). When considered together, these data support the hypothesis that changes in genetic code can act as partial barriers to HGT.

3.   Mechanistic factors and HGT in the fungi

The fungal lifestyle and HGT

Fungi have several biological characteristics that putatively reduce their permeability to invasion by foreign genes. Fungi generally possess robust chitin-rich cell walls and obtain nutrients exclusively by osmotrophic feeding. These traits underpin the ecological success of the fungi and are mechanistically linked: the chitin wall reinforces the fungal cell and enables it to resist: (1) substantial osmotic pressure that is produced during osmotrophic feeding; (2) structural strains during growth (usually as polarized cells in the form of hyphae or rhizoids); and (3) the diverse and heterogeneous environments within which fungal filaments grow. These adaptations drive the high metabolic rate, fast growth, and ecological success of the fungi (Bartnicki-Garcia, 1987). However, as a consequence of this lifestyle, fungi have lost the ability to perform phagocytosis and therefore cannot engulf and digest prey cells in the same way as many other eukaryotes. Furthermore, fungal cells are surrounded by robust cell walls making them recalcitrant to the entry of foreign DNA. As a consequence the gene transfer ratchet of phagotrophic eukaryotes proposed by Doolittle (1998), must have ceased to function deep within the fungal radiation and therefore one would expect that HGT should have played a less significant role in the evolution of fungal genomes when compared to the genomes of phagotrophic protists.

Factors promoting HGT into fungi

Additional routes for horizontal transfer have been proposed for fungi, with many commentators suggesting that because fungi are often found in intimate ecological associations with both living and dead organisms (e.g. Garcia-Vallve *et al.*, 2000; Hogan and Kolter, 2002; James *et al.*, 2006; Wang and Qiu, 2006) there may have been ample opportunity for gene transfer to occur (Friesen *et al.*, 2006; Richards *et al.*, 2009; Slot and Rokas, 2011). These suggestions are of course based upon circumstantial observations of incongruent gene phylogenies and therefore lack direct evidence for transfer mechanisms. However, several reports have identified examples of inter-domain conjugation-like transfer between bacteria and Saccharomycotina yeast species (Heinemann and Sprague, 1989; Inomata *et al.*, 1994; Sawasaki *et al.*, 1996). Specifically, Heinemann and Sprague showed that conjugative plasmids of *Escherichia coli* could mobilize and transmit to *Saccharomyces cerevisiae* (Heinemann and Sprague, 1989). While in laboratory experiments *Agrobacterium tumefaciens* has been demonstrated to transform many species of filamentous fungi including *Aspergillus niger*, *Agaricus bisporus*, *Colletotrichum gloeosporioides*, *Fusarium venenatum*, and *Neurospora crassa* (de Groot *et al.*, 1998). These data demonstrate viable routes of transfer from bacteria to fungi, which is consistent with the fact that phylogenomic analyses have demonstrated that many such fungi possess genes of HGT ancestry from prokaryotic sources (Dujon *et al.*, 2004; Fitzpatrick *et al.*, 2008; Hall *et al.*, 2005; Hall and Dietrich, 2007; Marcet-Houben and Gabaldon, 2010; Rolland *et al.*, 2009; Whitaker *et al.*, 2009).

Gene clusters and HGT

Clustering of genes that encode linked metabolic functions has been suggested to be both an evolutionary consequence and driving force of HGT in fungal genomes (Walton, 2000). Specifically, genes that function in sequential steps of secondary metabolism and produce a range of novel metabolites and toxins are often found in gene clusters and acquisition of such gene clusters has often been implicated as being the result of HGT. Many of these clusters also contain genes responsible for regulating transcription of the genes within the cluster and conveying resistance to the toxic metabolites generated by the function of the same gene cluster (Walton, 2000). Walton (2000) therefore suggests that clustering of secondary metabolite genes conveys selective advantage to the cluster itself, specifically because it allows horizontal gene transfer of a gene network encoding linked functions in a single step. Selection would therefore lead to clustering because it would improve the chance of co-transfer, which in turn will select for maintenance of the cluster. This theory is analogous to the Selfish Operon Theory (Lawrence and Roth, 1996) and has also been invoked for acquisition of pathogenic functions in the fungi, where transfer of gene clusters has been suggested to be important for the evolution of virulence (van der Does and Rep, 2007). Considerable support for this hypothesis has emerged recently, with key examples of HGT of gene clusters generally (Slot and Hibbett, 2007; Slot and Rokas, 2010) and specific examples of transfer of gene clusters which function in secondary metabolism and toxin
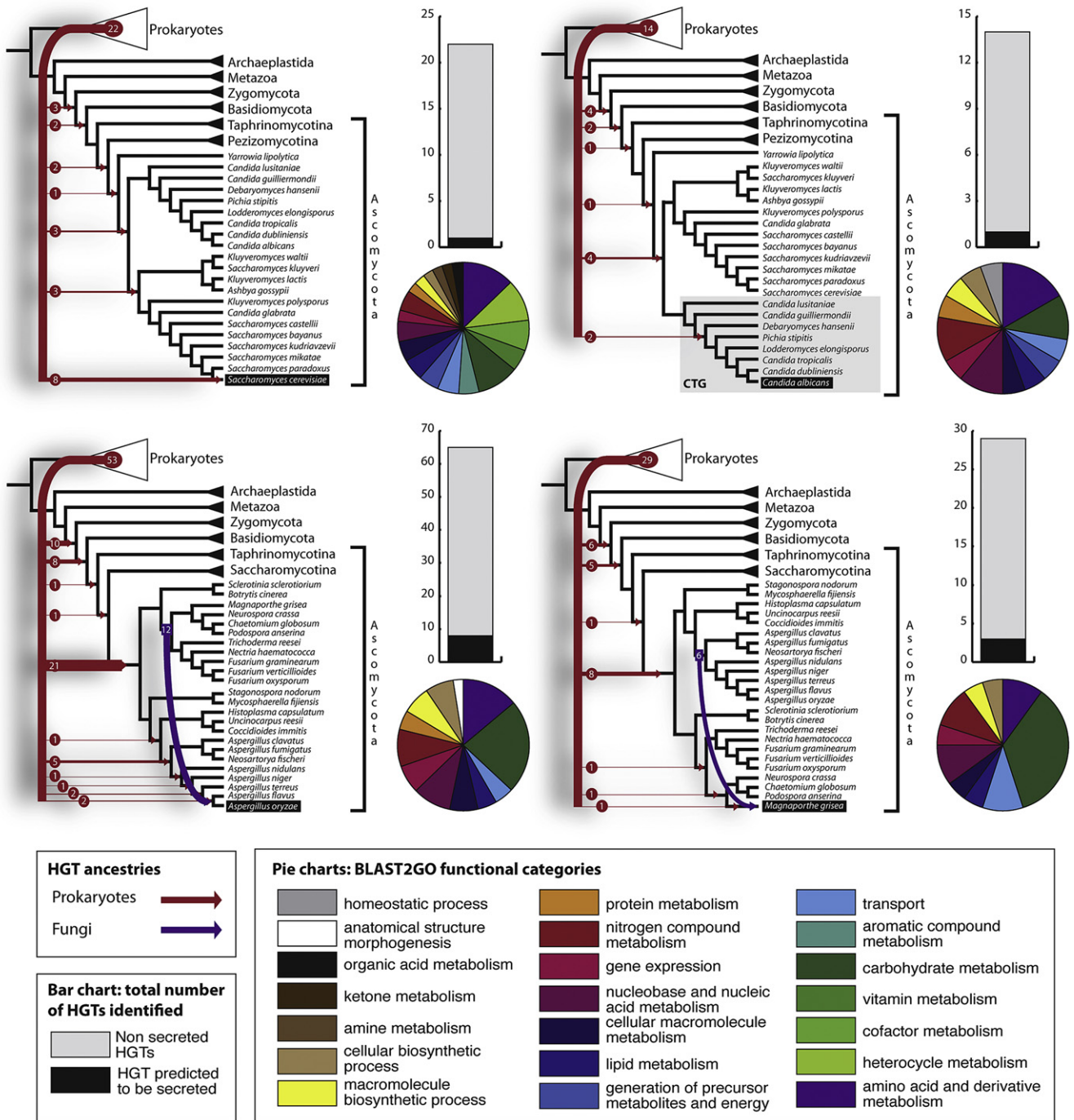
Fig. 1 — Ancestry and functional annotation of HGTs in four model fungal genomes (*Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus oryzae*, and *Magnaporthe grisea* species complex, which includes the rice blast fungus *M. oryzae*). Four phylogenies demonstrate ancestry of prokaryote (red) and fungal (purple) derived HGTs illustrated using coloured arrows. Black and grey bar charts demonstrate total number of HGTs confirmed by phylogenetic analyses, and the number of transferred genes that putatively encode proteins with N-terminal secretion signals. N-terminal secretion signals identified using both SignalP and WolfPSORT. The pie charts summarise the results of the Blast2GO functional classification (level 4 Biological process annotations). The HGTs in the two Saccharomycotina genomes distribute evenly across functional categories while the HGTs in the two Pezizomycotina are dominated by genes which encode components of: nitrogen compound, carbohydrate, and amino acid metabolism. Grey box illustrates the change in CTG codon usage in the ancestor of *Candida albicans* and is matched with relatively few transfers which post-date the change in codon-usage suggesting that this represents a partial barrier to HGT. The ancestry of each HGT is polarized based on the distribution of taxa in which the HGT was present (accounting for secondary loss) — this should be seen as the latest point of transfer because lack of taxon sampling and unidentified secondary loss may obscure an earlier point of transfer. The tree topologies are based upon the work of Fitzpatrick *et al.* (2006).

production (Khaldi *et al.*, 2008; Patron *et al.*, 2007; Slot and Rokas, 2011). An alternative hypothesis for clustering of secondary metabolic pathways concerns the potential toxicity of biosynthetic intermediates, providing selective pressure for all steps of a given biosynthetic pathway to be maintained together over evolutionary time (Slot and Rokas, 2011).

Supernumerary chromosomes and HGT

Gene transfer between fungi has also been connected with the role of supernumerary or b-like chromosomes. These dispensable chromosomes have been demonstrated to be non-critical for growth, but instead encode specific gene functions that may be associated with fungal virulence (Covert, 1998; Han *et al.*, 2001; Oliver and Solomon, 2008; Temporini and VanEtten, 2004; van der Does and Rep, 2007). For example, some pathogenicity genes of *Nectria haematococca* (*Fusarium solani*), including a phytoalexin detoxifying enzyme encoding gene are located on a 1.6 Mb supernumerary chromosome (Han *et al.*, 2001; Temporini and VanEtten, 2004). The chromosome also contains a large number of transposable elements and an aberrant GC composition compared to the rest of the genome, leading to the suggestion that HGT has altered this chromosome, or that the chromosome itself has been acquired by HGT (Temporini and VanEtten, 2004; van der Does and Rep, 2007).

He *et al.* (1998) further tested the hypothesis that supernumerary chromosomes could be transferred between two vegetatively incompatible strains. Using two isolates of *C. gloeosporioides*, one bearing a 2 Mb supernumerary chromosome with an introduced hygromycin resistance gene, and a second phleomycin-resistant strain that did not possess the supernumerary chromosome. The strains were co-cultivated and a doubly resistant hybrid strain was obtained demonstrating transfer of the 2 Mb supernumerary chromosome (He *et al.*, 1998). He and colleagues also re-ran the experiment this time introducing the hygromycin resistance gene onto a different chromosome, however in this scenario transfer was not achieved suggesting that mobility was restricted to the supernumerary chromosome (He *et al.*, 1998).

Chromosome transfer was also suggested as the ancestry of chromosome 14 of *Fusarium oxysporum*, which encodes several genes associated with virulence and for which presence/absence of the chromosome correlates with tomato wilt disease phenotypes (Ma *et al.*, 2010). Using a similar experimental approach to the *Colletotrichum* study above, Ma *et al.* (2010) confirmed mobility of this chromosome from a pathogenic strain to a non-pathogenic strain. PCR experiments confirmed transfer of chromosome 14 with the newly formed double drug resistance strains also acquiring the ability to infect tomato (Ma *et al.*, 2010). These data confirm that mobility chromosomes can play an important role in reconfiguring the genetic repertoire of fungal genomes and spread of pathogenicity traits, yet the mechanism of transfer, and the phylogenetic distribution of supernumerary chromosomes across the fungi remain unclear.

Cytoplasmic interconnection and HGT

Fungi are regularly found in large populations composed of multiple, genetically distinct strains. Anastomosis of conidia

and hyphae has also been suggested as an additional route for gene transfer between fungi (Friesen *et al.*, 2006; Roca *et al.*, 2005; van der Does and Rep, 2007). Furthermore, there is mixed evidence that fungi can maintain heterogeneous populations of nuclei within the same cytological network (Bever and Wang, 2005; Kuhn *et al.*, 2001; Pawlowska and Taylor, 2004). Such scenarios would serve to further facilitate gene exchange. Generation of interconnected cytoplasm between fungal cells involves cellular fusion (plasmogamy) between genetically distinct fungi. Yet most fungi have sophisticated genetic compatibility mechanisms to restrict gene flow *via* this route, which result in 'vegetative incompatibility'. Consequently, genetically distinct species/strains, which differ in specificity at one or more 'heterokaryon incompatibility' (het) loci, are unable to make viable fusion cells. Instead the fused cells are sealed from the rest of the mycelium and ultimately broken down by cell lysis (Glass *et al.*, 2000). These observations have led to the suggestion that gene transfer by anastomosis between genotypically distinct fungal lineages may be very rare in nature (Glass *et al.*, 2000). Yet like all biological processes this system of incompatibility is unlikely to operate with 100 % efficiency, especially as the process of vegetative incompatibility acts 'after the fact' once a cytoplasmic connection has been made. Therefore transient anastomosis between genetically distinct species/strains may provide a narrow window for cytoplasmic/organelle/DNA/RNA/nuclei exchange between genetically distinct hyphal networks. Given the large population densities of fungi in terrestrial environments, errors in vegetative incompatibility systems need only occur at a low frequency for anastomosis-mediated HGT to represent a viable mechanism for gene transfer. It is of course, also possible that some genes, such as those encoded on supernumerary chromosomes, may encourage transfer (van der Does and Rep, 2007) by anastomosis, for example: by encouraging a higher rate of cellular fusions or by obstructing genetic incompatibility systems.

Data supporting vegetative fusion as a pathway for gene transfer has been published by Croll *et al.* (2008). This study demonstrated viable cytoplasmic connections between genetically distinct field isolates of the arbuscular mycorrhizal fungus *Glomus intraradices*. They also demonstrated that these connections appear to lead to genetic exchange and recombination producing both genetically and phenotypically distinct 'progeny' (Croll *et al.*, 2008). This scenario provides a route for gene transfer side-stepping the putative barriers to HGT found in fungi discussed above (i.e. the robust cell wall and loss of phagotrophy), and provides evidence that HGT between fungi may represent a, as yet underappreciated, factor in fungal genome evolution (Richards, 2011).

Such interactions are driven, or blocked by, genetic systems that determine sexual interactions in fungi such as the mating type (*mat*) loci. However, some studies have now shown that HGT is implicated in the evolution of these very gene families. Inderbitzin *et al.* (2005) have demonstrated that self-fertile *Stemphylium* species contain both *mat*1-1 and *mat*1-2 loci as a fusion gene. Phylogenetic analyses demonstrated incongruence between the *mat* gene phylogeny and a four-gene 'species'-tree, suggesting that HGT has played a role in spreading self-fertility in the fungi by spreading the *mat*1-1/2 gene fusion (Inderbitzin *et al.*, 2005). Additional cases of HGT

of *mat* loci have been suggested for the Dutch Elm disease fungus *Ophiostoma novo-ulmi* (Paoletti *et al.*, 2006), demonstrating that vegetative incompatibility systems, may not lead to immunity from HGT.

4.   Identifying and testing HGTs

Phylogeny − the gold standard for testing HGT

The primary method for identifying HGT is the construction of a phylogenetic tree with appropriate taxon sampling and tree-building methodologies. Using this approach HGT can be identified when a gene ancestry contradicts the established species phylogeny by placing the gene of a species, or the genes from a group of species, *within* a clade of sequences from unrelated species (Fig. 2 − Andersson, 2009; Keeling and Palmer, 2008; Richards *et al.*, 2003). This method enables a researcher to test directly the HGT hypothesis, observe potential evidence of gene duplication and loss within the gene family, and use appropriate statistical methods to test support for the tree topology and, by implication, the HGT. This method also allows the researcher to identify the taxonomy of the donor group and investigate the ancestry of transmission relative to sampled taxa. It is for these reasons that phylogenetic assessment of HGT has been recognized
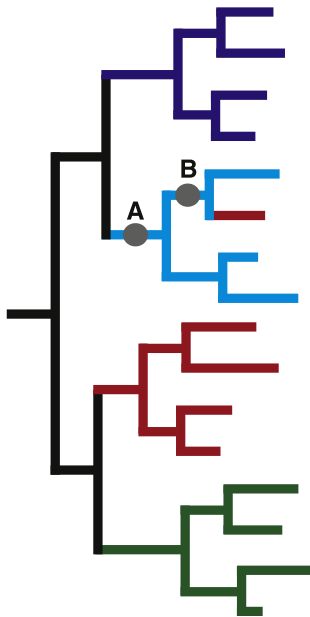

Fig. 2 − **Schematic phylogenetic tree showing the best possible phylogenetic support for a gene transfer hypothesis. The tree is a schematic presentation of a phylogeny showing four major taxonomic groups (colour coded: blue, light blue, red and green). The tree shows the gene of one 'red' species branching within a cluster of genes from 'light blue' species; an incongruent gene phylogeny that therefore suggests HGT. Key nodes are marked: A, strong statistical support is needed for recipient taxa branching *with* donor lineages, B, strong statistical support is needed for recipient taxa branching *within* donor lineages.**

as the highest standard of proof for the identification of HGT (see Keeling and Palmer, 2008 for different scenarios in which HGT can be inferred from phylogenies).

Surrogate methods

Three additional methodological approaches have been used to investigate HGT. These are often called 'surrogate methods' because they do not require calculation of phylogenetic trees (Ragan, 2001a). These methods include:

(1) **The identification of a mosaic distribution of a gene family across the tree of life** (e.g. Keeling and Inagaki, 2004; Richards *et al.*, 2009). This approach depends on accurately assessing homology and identifying the distribution of gene families across taxa, and furthermore accurately accounting for patterns of gene duplication and loss. This is especially difficult without using a phylogeny to directly infer HGT (Fig. 2), because it is impossible to identify patterns of orthology and paralogy unambiguously (Keeling and Inagaki, 2004; Ragan, 2001a). Furthermore, gene/taxon distribution analyses are often based on sequence similarity searches such as BLAST (Altschul *et al.*, 1997). Yet, BLAST has been demonstrated to perform poorly for directly inferring evolutionary relationships. For example, large-scale comparisons have demonstrated that up to 40 % of BLASTp best matches do not represent the nearest neighbour in subsequent phylogenetic analyses (Koski and Golding, 2001). Consequently, gene/taxon distribution approaches are only useful for inferring HGT when genome sampling is high and gene family distribution is very patchy. In such scenarios phylogenetic trees become limited in their use because there are not enough taxonomic groups to use phylogenetic methods to test an HGT hypothesis directly, therefore gene/taxon distribution data can become the principal tool for inferring HGT (Richards *et al.*, 2009). Under these circumstances it is therefore extremely important to use sophisticated homology searching tools such as hidden Markov models (HMM) and/or PSI-BLAST (Altschul *et al.*, 1997; Bateman *et al.*, 2004) to sample divergent forms of the target gene family across genomes and therefore fully test the gene/taxon distribution.

(2) **Gene content comparisons between syntenic blocks and closely related genomes.** This approach has proved successful when whole genome sampling among the target group/genus is high (Friesen *et al.*, 2006) and the approach is followed up with phylogenetic analysis (Dujon *et al.*, 2004; Fitzpatrick *et al.*, 2008; Rolland *et al.*, 2009).

(3) **Identification of open reading frames with atypical composition.** Such analyses have included: nucleotide composition, dinucleotide frequencies, codon usage, or composition patterns identified by Markov chain analyses (Lawrence and Ochman, 2002; Ragan *et al.*, 2006). These approaches have recently been applied to the genome of the opportunistic human pathogenic fungus *Aspergillus fumigatus* identifying 189 compositionally apparent regions encompassing 214 putative genes, 40 % of which were suggested to be of prokaryotic origin, while

22 % were suggested to be of viral ancestry (Mallet *et al.*, 2010).

To examine the viability of composition analyses for identifying cases of HGT, Koski and co-workers performed phylogenetic analyses to test the support for HGT for 80 *E. coli* K12 genes. Of these, 24 genes demonstrated phylogenies consistent with vertical transmission, while 15 of the sub-sample of vertically transmitted genes had previously been classified as HGTs based upon composition data (Koski *et al.*, 2001; Lawrence and Ochman, 1998). In contrast, phylogenetic analyses demonstrated that of the 25 genes for which phylogeny suggests HGT, 12 genes were previously identified as vertically derived based on composition data (Koski *et al.*, 2001; Lawrence and Ochman, 1998). Although the authors were careful to state that this is an incomplete sample and may be affected by methodological artifacts, these analyses provide evidence that compositional analyses are unreliable indicators of HGT (Koski *et al.*, 2001). This is especially the case because transferred genes can quickly become ameliorated to the host genome, acquiring similar GC composition and codon-usage patterns, for instance, (Lawrence and Ochman, 1997) and suggesting that these methods are only really useful for assessing patterns of very recent gene transfer (Ragan *et al.*, 2006). Furthermore, eukaryotic genomes have compositionally definable regions or isochores, that are not thought to be the product of HGT, but

which possess nucleotide frequencies that can depart from the mean for the genome (Ragan, 2001a), suggesting that this approach can suffer from both false positive and false negative results.

Limitations of surrogate methods

Surrogate methods have been suggested to perform poorly – or at least inconsistently (Ragan, 2001b). For example, Ragan used a range of surrogate methods to identify putative HGTs in the genome of *E. coli* K12 and demonstrated that the majority of the methods used did not detect the same examples of putative HGT at a higher frequency than chance (Ragan, 2001b). The authors concluded that the surrogate methods used are therefore unreliable or depend too much on the relative phylogenetic age of the HGTs being investigated (Lawrence and Ochman, 2002; Ragan *et al.*, 2006). We would therefore argue that gene-by-gene phylogenetic analyses of a whole genome is the best method for identifying HGTs and that use of surrogate methods must always be accompanied by phylogenetic analyses. We would underline, however, that phylogenetic data is only as good as the taxon sampling and methods used, with many genome projects still containing substantial contamination (Longo *et al.*, 2011). Furthermore, with the best will in the world all phylogenetic analyses are hostages to progress, with genome/taxon sampling and phylogenetic methods
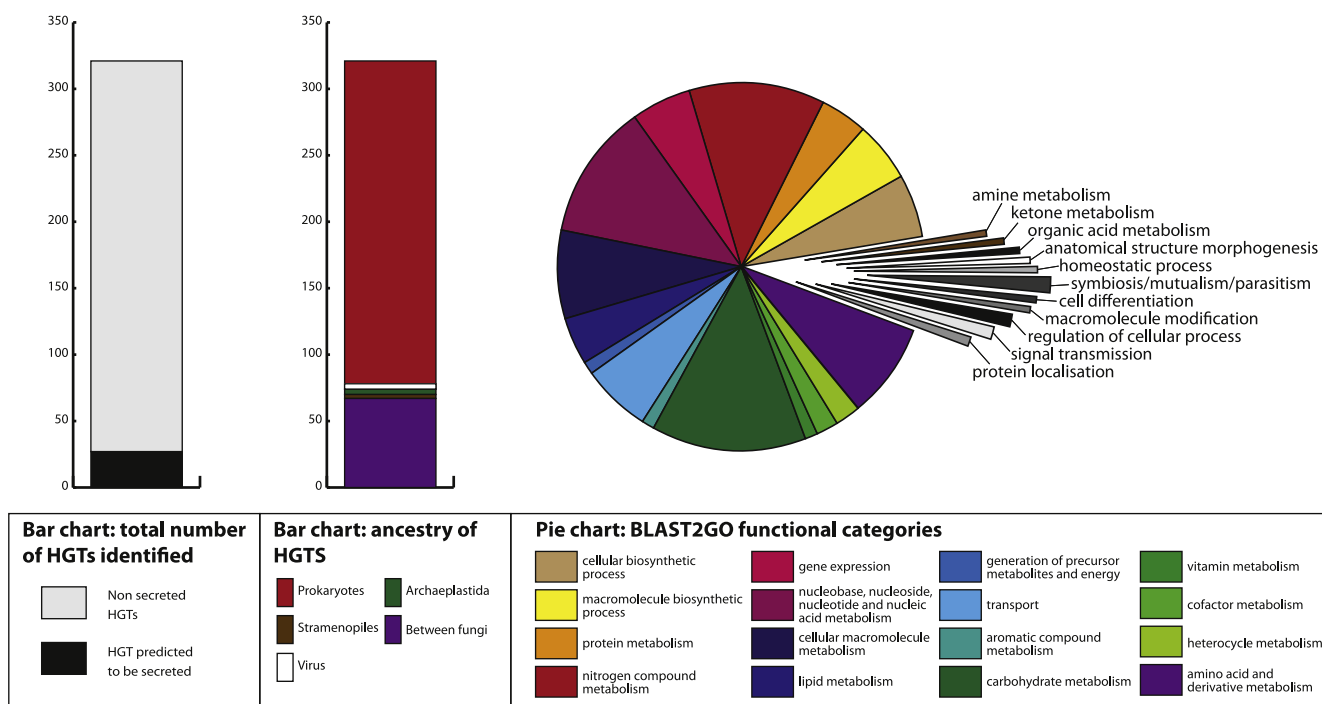

Fig. 3 – **Evidence of 323 transfers into the fungi re-tested using updated phylogenetic analyses. For evidence supporting the HGTs see Supplementary Dataset 1. Black and grey bar charts demonstrate total number of HGTs confirmed by phylogenetic analyses, and the number of transferred genes that putatively encode proteins with N-terminal secretion signals (27) identified using both SignalP and WolfPSORT. Coloured bar chart shows the ancestry of the 323-gene transfers and demonstrates the majority of the genes transferred are derived from prokaryotes (mainly bacteria) and to a lesser extent fungi. The pie chart summarises the results of the BLAST2GO functional classification analyses (level 4 Biological process annotations) and suggests the majority of the HGTs operate in: nitrogen compound, nucleobase, lipid, carbohydrate, macromolecule and amino acid metabolic pathways and transportation.**

constantly improving such that putative HGTs are continually subject to revision (Horner and Embley, 2001).

Contrasting alternative hypotheses: complex gene loss or HGT

At this point it is important to note that both the surrogate approaches (1 and 2 above), and to some degree the use of phylogenetic methods, are also compromised in their ability to demonstrate HGT by an alternative explanation of incongruent evolutionary histories, namely gene duplication and differential gene loss (hidden paralogy). Gene loss is difficult to identify, especially without phylogenetic analysis, but theoretically could be used to explain all complex patterns of gene/taxon distribution. Therefore, many HGT hypotheses rely on using parsimony arguments to distinguish between these alternative evolutionary explanations. Consequently, we would argue the best cases of HGT are those supported by a phylogeny that shows a recipient taxa branching both *with* and *within* the donor group with strong statistical support (Fig. 2). In such cases the alternative hypothesis of differential gene loss becomes extremely complicated so that the HGT hypothesis is more likely to be favoured over the alternative hypothesis of hidden paralogy on the grounds of parsimony.

5.   Re-analysis of 340 published examples of HGT into fungi

To begin to assess the broad nature and extent of HGT into fungal genomes we collected a comprehensive selection of published examples of putative HGT. Focussing on cases where phylogenetic methods had previously been used we collected 340 putative examples (listed in Supplementary Table 1). These data encompass a broad survey of prokaryote-derived HGTs in fungi (Marcet-Houben and Gabaldon, 2010) and several overlapping analyses of key genomes (e.g. S. cerevisiae − Dujon et al., 2004; Rolland et al., 2009; Whitaker et al., 2009) but together represent an incomplete and inconsistent sample of transfer with most published studies focussing on *ad hoc* examples of bacterial derived HGTs. To re-check each example we ran the HGT sequence through our custom built gene-by-gene phylogenetic reconstruction pipeline to recalculate a PhyML tree with approximate likelihood ratio tests (aLRT) on each branching relationship (briefly described in Supplementary Dataset 1 and Richards et al., 2009). Our pipeline, which benefits from an updated collection of genome sequences and therefore increased taxonomic coverage, identified 323 phylogenies with a tree topology consistent with the original proposed HGT hypothesis and the data used to infer it (see Supplementary Dataset 1). The remaining 17 phylogenies identified a tree topology that was not consistent with the original HGT hypothesis (see Supplementary Dataset 1). These 'rejections' were based on updated taxon sampling, suggesting either vertical inheritance with some gene loss or transfer in the opposite direction i.e. from a fungus to bacteria. This analysis identified a predominance of both fungal and prokaryote-derived HGTs, although this may be a sampling artefact, arising from the fact that these taxonomic groups are currently the best sampled among genome sequencing projects (Fig. 3).

HGT and the acquisition of osmotrophic capacity

The HGT summary statistics shown in Fig. 3 demonstrate that 27 of the 323 transferred genes putatively encode an N-terminal secretion motif (identified by both WoLFPSORT (Horton et al., 2007) and SignalP (Bendtsen et al., 2004)), suggesting HGT has played a significant role in adding to the secretome of many fungi. The Blast2GO (Conesa and Götz, 2008) annotation analyses also demonstrated that 11 of the HGTs are involved in transportation, specifically including six transfers of transporter encoding genes (e.g. Slot and Hibbett, 2007). Osmotrophic function is dependant upon both secreted proteins and transport systems, therefore these results are consistent with the hypothesis that HGT has played a role in providing genes that further equip fungal microbes for osmotrophy. We had previously hypothesized HGT from fungi-to-oomycetes had been a factor in reconfiguring or expanding the osmotrophic capabilities of the oomycetes (Richards et al., 2006). The preliminary data reported here provides support for a similar scenario for the fungi, with HGT amending and expanding the osmotrophic functions of many fungi.

HGT and metabolic functions

The Blast2GO annotations also demonstrate that the functional classification of HGTs is dominated by genes that putatively function in sugar, nitrogen, amino acid, nucleotide, and secondary metabolism, covering a wide diversity of metabolic functions and suggesting that HGT has played a significant role in expanding and reconfiguring the core metabolic network and nutrient-processing capacity of many fungi (Fig. 3). For example, Slot and Hibbett (2007) have identified a pattern of serial transfer of a three gene cluster which putatively functions in nitrate uptake and assimilation and includes a: high affinity nitrate transporter, nitrate reductase and a ferredoxin independent assimilatory nitrate reductase; required to reduce nitrate to ammonium (Slot and Hibbett, 2007). This gene cluster transfer therefore theoretically equips the recipient taxa with improved nitrate scavenging capabilities.

Fig. 1 demonstrates the ancestry of re-confirmed HGTs in four model fungi, two of which are Saccharomycotina yeasts, while two are plant-associated filamentous fungi of the Pezizomycotina. These analyses again confirm that prokaryote-derived HGTs have played a role in the ancestry of all four fungal genomes, but we note that this preliminary data shows that selective retentions of horizontally acquired genes seem to have been especially important in the reconfiguration of carbohydrate and amino acid metabolism in the two filamentous plant-associated fungi.

HGT and adaptation to different environments

Gene transfers have been linked to colonization of 'new' environments in several fungi. For example, phylogenetic analysis of dihydro-orotate dehydrogenase has demonstrated that S. cerevisiae possess a distinct version of this gene which branches with *Lactococcus* with 100 % bootstrap support, suggesting a HGT ancestry (Gojkovic et al., 2004; Hall et al., 2005). This transfer is hypothesized to have led to acquisition of

HGT ancestries
- Slot and Hibbett 2007
- Slot and Rokas 2010
- Khaldi *et al.* 2008
- Patron *et al.* 2007
- Khaldi and Wolfe 2008
- Slot and Rokas 2011
- Novo *et al.* 2009

Pie chart: BLAST2GO functional categories
- signal transmission
- regulation of cellular process
- cell differentiation
- cellular biosynthetic process
- macromolecule biosynthetic process
- protein metabolism
- nitrogen compound metabolism
- gene expression
- nucleobase, nucleoside, nucleotide and nucleic acid metabolism
- lipid metabolism
- generation of precursor metabolites and energy
- transport
- carbohydrate metabolism
- amino acid and derivative metabolism

Bar chart: total number of HGTs identified
- Non secreted HGTs
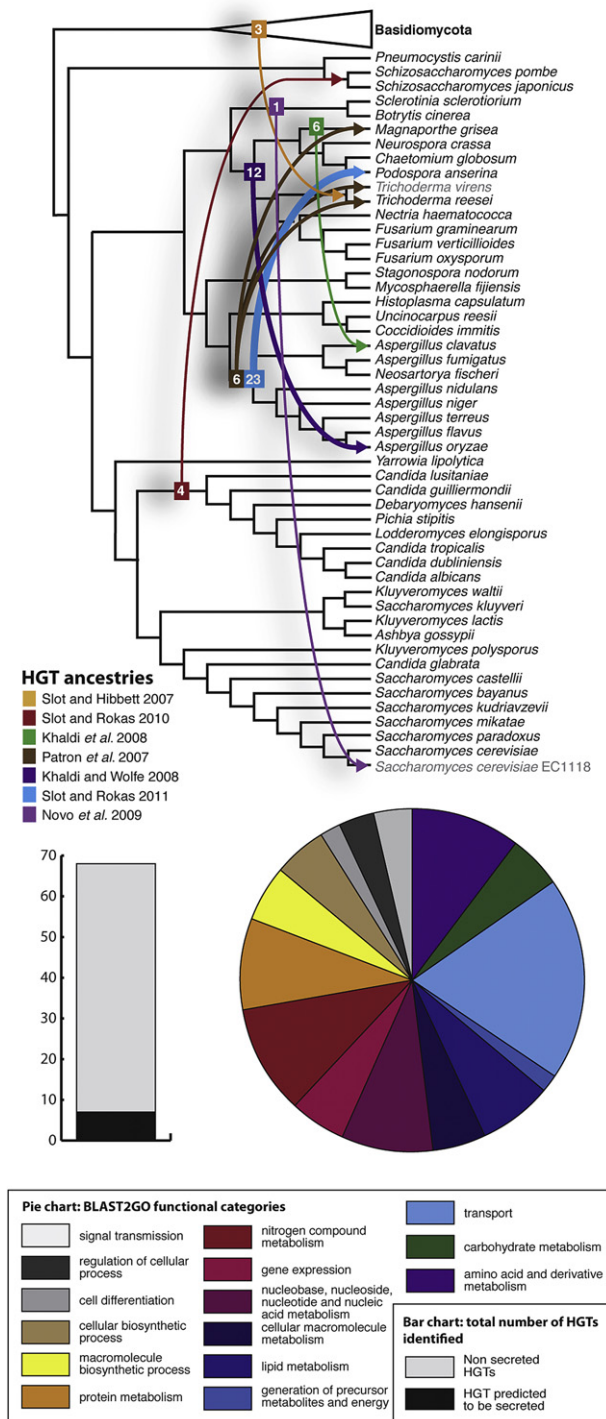- HGT predicted to be secreted

Fig. 4 — Evidence of 66 HGTs between fungi. The phylogeny demonstrates ancestry of HGT between fungi marked as coloured arrows with the source publications listed in the key (Khaldi *et al.*, 2008; Khaldi and Wolfe, 2008; Novo *et al.*, 2009; Patron *et al.*, 2007; Slot and Hibbett, 2007; Slot and Rokas, 2010, 2011). For evidence supporting the HGTs see Supplementary Dataset 1. The cases of HGT reported so far are dominated by transfers between and into Pezizomycotina. Black and grey bar charts demonstrate total number of HGTs confirmed by phylogenetic analyses and the number of transferred genes that putatively encode proteins with N-

a non-mitochondrial dihydro-orotate dehydrogenase (the fourth step in *de novo* pyrimidine biosynthesis) and which can function independent of oxygen. The authors therefore suggest that this gene transfer has facilitated colonization of anaerobic environments, enabling pyrimidine synthesis in anoxic conditions. Interestingly, additional examples of HGT in anaerobic protists have also been linked to adaptation to anoxic environments (Andersson *et al.*, 2003).

Garcia-Vallve *et al.* (2000) provided evidence that the glycosyl hydrolases of rumen 'chytrid' fungi have been acquired by HGT from bacteria and are suggested to represent important adaptations to breakdown and metabolism of complex sugars in the intestinal environment (Garcia-Vallve *et al.*, 2000).

The hypothesis that HGT can equip fungal microbes with new capacities and enable them to colonize additional environments can, however, also be turned on its head and used to help identify cases of HGT. For example, fungi theoretically lack the beta-glucuronidase (GUS) gene and therefore the metabolic capacity to utilize glucuronides as a carbon source. Wenzl *et al.* (2005) screened for fungi in vertebrate urine using culture enrichment with selection for fungi with glucuronide metabolic capabilities. Using this approach they identified GUS genes in *Penicillium canescens* and *Scopulariopsis sp*. Subsequent phylogenetic analysis demonstrated that these genes, along with GUS genes of *Aspergillus* and *Gibberella*, were derived by HGT from bacteria (Wenzl *et al.*, 2005), again demonstrating cases of HGT which have expanded the metabolism of fungi and enabled them to adapt to new environments.

6. Emerging pattern of fungi-to-fungi gene transfers

The number of fungal genome sequences is increasing rapidly, meaning that it is now possible to test for HGT between fungal species. Fig. 4 summarises nine published cases of HGT between fungi based on phylogenetic analysis that were re-tested using our pipeline (see Supplementary Dataset 1 and Supplementary Table 1). These nine transfers encompass 66 individual genes. The Blast2GO annotation analysis again confirms that HGT between fungi has been dominated by genes that function in core metabolism i.e. protein, nucleic acid, nitrogen, lipid and amino acid metabolism. However, cellular

terminal secretion signals. N-terminal secretion signals identified using both SignalP and WolfPSORT. The pie chart summarises the results of the Blast2GO functional classification analyses (level 4 Biological process annotations) and suggests the majority of the HGTs between fungi operate in: nitrogen compound, nucleobase, lipid, protein, macromolecule and amino acid metabolism and transportation. The ancestry of each HGT is polarized based on the distribution of taxa in which the HGT was present (accounting for secondary loss) — this should be seen as the latest point of transfer because lack of taxon sampling and unidentified secondary loss may obscure an earlier point of transfer. The tree topology is based upon (Fitzpatrick *et al.*, 2006) with some additional taxa marked in grey.

transport is the most prevalent putative function among the 66 identified fungal HGTs, consistent with the idea that HGT between fungi has been important in putatively reconfiguring the transporter repertoire and therefore potentially expanding or modifying the osmotrophic capacity of fungal species.

The fungi-to-fungi transfers identified encompass five gene clusters representing a total of 53 individual gene phylogenies and seven gene cluster transfer events (Khaldi et al., 2008; Patron et al., 2007; Slot and Hibbett, 2007; Slot and Rokas, 2010, 2011). These observations are consistent with the hypothesis that gene-clustering favours HGT discussed above (Lawrence and Roth, 1996; Walton, 2000). Perhaps the most striking example of a gene cluster transfer was the transfer of a 23-gene cluster from the *Aspergillus* lineage to *Podospora* (Slot and Rokas, 2011). These two genera are distantly related members of the Pezizomycotina − a subphylum within the Ascomycota (Fig. 4 − Fitzpatrick et al., 2006; James et al., 2006). Gene-by-gene phylogenetic analysis demonstrated all 23 *Podospora* genes branched with genes from *Aspergillus*. The 23-gene cluster includes genes that putatively encode the entire sterigmatocystin synthesis pathway, a toxic and mutagenic compound, a precursor of aflatoxins, and a potentially important adaptation for colonizing overlapping niches. Similar transfers of toxin encoding genes have also been reported and furthermore implicated in fungal pathogenesis and determining host range (Friesen et al., 2006; Khaldi et al., 2008; Patron et al., 2007), suggesting that sharing of toxic capacity has been important for both colonization of plant derived ecosystems but also co-occupation of environments.

7.   Conclusion

Using phylogenetic methods as the principal tool, this review has collected together and re-confirmed 323 examples of HGT into fungal genomes. This result provide evidence that the fungi have gained a significant number of genes by HGT, although a very small proportion when compared to the total coding capacity of any one fungal genome. The majority of identified HGTs appear to have originated from prokaryotes (mainly bacteria) or fungi, although this trend may be a product of the current genome data available for comparison, which is biased to both bacteria and fungi. The majority of gene transfers between fungi (53 of 66 genes) were located in gene clusters, providing direct support for the hypothesis that gene clustering has played a role in facilitating gene transfer in fungi (Walton, 2000). Functional annotation of the 323 HGTs demonstrates that transfer has played a role in reconfiguring the core nutrient metabolism of many fungi, added to osmotrophic capacity of fungi, and allowed fungi to colonize 'new' environments.

Mechanisms for gene transfer in fungi remain unclear, but the patterns of transfer (i.e. originating from bacteria and fungi) provide circumstantial evidence that supports two previously hypothesized routes of transfer: inter-domain transformation and conjugation-like transfer (for genes of bacterial origin) and anastomosis of cells (for those of fungal origin). However, further research is needed to test this pattern both experimentally and across a wide diversity of fungi.

Although the list of HGTs and associated publications is growing rapidly, with one notable exception (Marcet-Houben and Gabaldon, 2010), the majority of reported HGTs remain *ad hoc* discoveries or the results of genome specific analysis. Therefore, the census of gene transfer into the fungi is both incomplete and biased towards analyses of ascomycetes of the Saccharomycotina and Pezizomycotina. Future research focussing on the remaining fungal phyla and whole genome gene-by-gene phylogenetic analysis will therefore be extremely interesting. Such approaches will soon allow us to generate a model of how and when HGT shaped the evolutionary history of the fungi and how these transfers relate to the biological capabilities of different fungal lineages.

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