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ROLE OF HORIZONTAL GENE TRANSFER IN THE EVOLUTION OF FUNGI[1]

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■ **Abstract** Although evidence for horizontal gene transfer (HGT) in eukaryotes remains largely anecdotal, literature on HGT in fungi suggests that it may have been more important in the evolution of fungi than in other eukaryotes. Still, HGT in fungi has not been widely accepted because the mechanisms by which it may occur are unknown, because it is usually not directly observed but rather implied as an outcome, and because there are often equally plausible alternative explanations. Despite these reservations, HGT has been justifiably invoked for a variety of sequences including plasmids, introns, transposons, genes, gene clusters, and even whole chromosomes. In some instances HGT has also been confirmed under experimental conditions. It is this ability to address the phenomenon in an experimental setting that makes fungi well suited as model systems in which to study the mechanisms and consequences of HGT in eukaryotic organisms.

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INTRODUCTION

With the completion of total DNA sequencing of several archeal and bacterial genomes, comparative and intraspecific sequence analysis has revealed horizontal gene transfer (HGT) as a major force in the history and continuous evolution of prokaryotes (87, 115). Currently absent from much of the discussion is the importance of HGT in the evolution of eukaryotic organisms. Although at least one review has addressed this issue (98), HGT is generally assumed not to be common or to play only a minor role in the evolution of multicellular eukaryotes (44). Accordingly, instances of HGT in eukaryotes remain largely anecdotal (98, 155).

In this review article we summarize reports of genetic exchanges between fungal populations and between fungi and other organisms that have been attributed to horizontal transfer mechanisms. Horizontal transfer of genetic material (or lateral transfer) is generally defined as the mobilization and nonsexual transmission of DNA between genomes of different species (98, 116), therefore specifically excluding the transfer of genetic information among conspecific strains (116). However, as many fungal species can be differentiated into reproductively distinct lineages (6, 161), we propose that, conceptually, the definition of HGT involving fungi can be more comprehensive. We define HGT as the stable transfer of genetic material between individuals, not directly attributable to vertical, i.e. meiotic or mitotic, processes or, in other words, the mobilization of genetic material if it is not intrinsic to the transfer from a parent to a daughter cell at cell division. Our use of the term "individual" also requires a definition on what exactly constitutes an individual in fungal populations. The term and the definition of genet is probably the most accurate, as it encompasses all asexual descendants from an identical genetic source (149). In ascomycetes, genets are predominantly homokaryotic, whereas in basidiomycete populations, an individual would consist of heterokaryotic mycelium (149).

This definition of HGT is somewhat unsatisfactory, as it circumscribes an outcome, not a specific biological process (45). In fact, mechanisms for HGT may be diverse. For bacteria, HGT can occur by such assorted processes as conjugation, transformation, or transduction (39). For eukaryotes, transfer mechanisms are generally unknown. Regardless of mechanism, the observed or postulated outcome of HGT is that a small amount of a donor genome is discovered as a small, incongruent component of a larger recipient genome. The asexual hybridization and recombination process of fungi known as parasexuality thus also would not be included in this definition since it would largely result in introgression and vertical transmission of genetic material by mitotic mechanisms.

Evidence for HGT

As mechanisms for HGT in eukaryotes are generally unknown, evidence for HGT in fungi and other eukaryotes is indirect. In most cases, HGT is invoked after unusual features of genetic elements have become evident, including (*a*) an

inconsistency between the phylogeny of the genetic element in question and the traditionally accepted phylogeny of the organisms ("character-state discordance"); (*b*) a particularly high DNA or derived amino acid similarity between elements found in phylogenetically distant organisms; (*c*) irregular distribution of a genetic element in a variety of species or lineages; (*d*) similar genes shared among species within a geographic area or specific habitat independent of their phylogenetic relationship; and (*e*) characteristics of a gene (G+C content, codon usage, introns, etc) inconsistent with the resident genome.

HGT may indeed explain any or all of these features, but alternative explanations may also be fully consistent with these observations and therefore should be carefully considered before invoking HGT. Avise (8) outlined additional explanations for the irregularities mentioned above (Figure 1, see color plate). These alternative hypotheses include use of erroneous species phylogenies, inappropriate comparison of paralogous sequences, sporadic retention of a shared ancestral character, uneven rates of character change in different lineages, or introgressive hybridization. Discriminating between HGT and alternatives can be a daunting task, as will become more apparent in the following sections.

Phylogenetic approaches to detect HGT are often a spin-off of methods originally developed to assess congruence or homogeneity among data sets in the reconstruction of phylogenies and have been summarized by Bull et al (18). Examples are nonparametric bootstrapping (151), Felsenstein's (52) and Kishino-Hasegawa's (105) maximum likelihood tests, and Lake's (110) method of maximum parsimony. Other tests include the T-PTP tests (49), incongruence length differences (51), and Templeton's nonparametric test (163). A likelihood ratio test has recently been developed to determine whether the same phylogeny underlies all data partitions (82, 84). The resulting statistics are then compared to a simulated null distribution using parametric bootstrapping (84). Rejection of the null hypothesis indicates data heterogeneity, though without revealing its specific cause. Heterogeneity of data sets may be caused by intrinsic properties of the data sets, including HGT, but also may be caused by the use of paralogous genes or uneven rates of character change (83, 84, 157). Rejection of the null hypothesis may also indicate method failure, especially when assumptions are severely violated (18, 83, 84). All phylogenetic methods make assumptions about the evolutionary process, and therefore robustness of specific models has to be taken into account (84).

Though the phylogenetic approaches mentioned above may be potentially useful to detect HGT for nuclear genes, they may not be appropriate for parasitic elements, e.g. transposable elements with evolutionary strategies different from the resident genome. For genetic elements, which are rare among the species examined and for which heterogeneity with other data sets is not observed, parsimony analysis can be used to estimate the number of independent gains and losses. As illustrated by Hibbett (74), HGT can be invoked if optimization procedures support gains versus losses.

The increasing availability of genome sequences and accompanying advances in bioinformatics will undoubtedly provide new avenues to test for HGT. Such

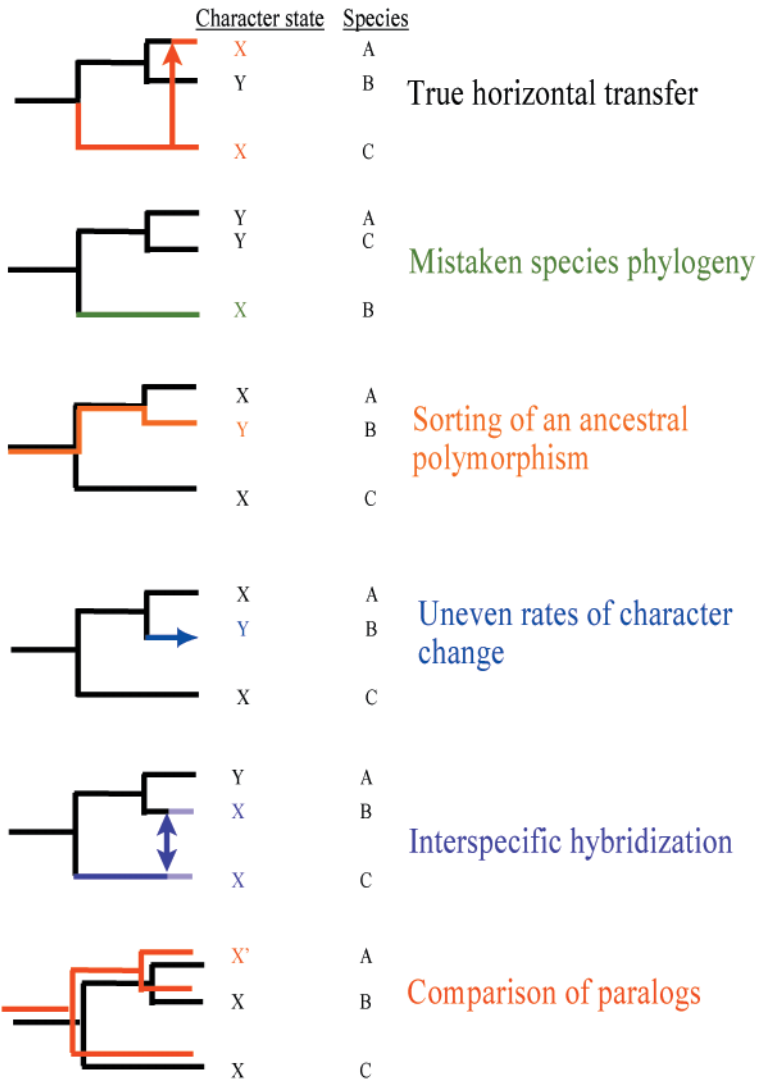

Figure 1 Explanations for character state discordance other than horizontal gene transfer. Adapted from Avise (8).

approaches may evaluate nucleotide frequencies or codon usage within a genome to determine segments that are significantly different from the rest of the genome. Analysis of these parameters revealed that the genome of *Escherichia coli* consists of circa 18% horizontally acquired sequences (115). HGT between phylogenetically distant species also may be affirmed if synteny is discovered for genes whose products do not physically interact, as gene order is rarely conserved in evolution (85).

POTENTIAL CASES OF HGT IN FUNGI

HGT in fungi has been implied in a surprisingly large number of publications. In summarizing this literature, we emphasize, as a disclaimer, that many of the examples mentioned should be considered anecdotal. It is hoped that advances brought by genomics, along with improved understanding of molecular phylogenetic relationships of fungal species and their reproductive and ecological interactions, will help to better evaluate these suspected cases and improve our understanding of the role of HGT in the evolution of fungi.

We have organized our discussion mainly according to the class of element and have included observations from both natural and experimental systems. As noted previously (98), suspected cases of HGT in eukaryotes are restricted largely to noninfective selfish genetic elements, e.g. plasmids, introns, transposons. This also predominantly holds true for fungi, with some notable exceptions.

Plasmids

Plasmids have been identified in many fungal species, but are only infrequently encountered in other eukaryotes (64, 92). In yeasts, plasmids are located in the cytoplasm, whereas in filamentous fungi, plasmids are ordinarily associated with mitochondria (64). Fungal plasmids range in size from circa 1 kb to 21 kb (usually between 2 and 10 kb), are usually linear, and show common structures [(64, 91) and references therein]. Open reading frame (ORFs) code predominantly for reverse transcriptase, DNA- and/or RNA-polymerase, and function in plasmid maintenance (91). Analysis of ORF sequences and other structural characteristics suggest an evolutionary link between plasmids and introns, retroelements, or viral genomes (91).

There are ongoing discussions on the origin of mitochondrial plasmids and how to explain their widespread distribution among filamentous fungi. Several non-mutually exclusive models have been proposed, all based on an ancient evolutionary origin. One model suggests plasmids may have arisen as bacteriophages of the endosymbiotic bacteria that became mitochondria (64, 92, 132). Similarly, it has been hypothesized that yeast plasmids have evolved from cytoplasmic viruses (92). Others have proposed that those mitochondrial plasmids with features of

retroelements may be "molecular fossils" that are remnants of the pre-DNA biotic world (171, 173). In addition, horizontal transfer has been proposed as a mechanism that allows for the spread of these plasmids between fungal strains and even between different species, implying that plasmids are highly transmissible molecular parasites (7, 129, 160, 162, 177). Three lines of evidence support the involvement of HGT in the evolution of fungal plasmids: (*a*) discordant phylogenetic relationships between plasmids and host genomes, (*b*) the geographic distribution of plasmids within and among host species, and (*c*) direct experimental demonstration of HGT.

Phylogenetic analysis of amino acid sequences of the DNA polymerase from 11 linear plasmids of fungal and plant origin as well as from 4 bacteriophages and 8 viruses indicated that plasmid groups of related host species were not necessarily the most closely related (92). For instance, the plasmid kalilo of *Neurospora intermedia* shared 49.8% sequence identity of aligned regions with the plasmid pAI2 of *Ascobolus immersus*, but only 34.7% identity with the maranhar plasmid of *Neurospora crassa* (92). Interestingly, the maize S1 plasmid appeared to be closely related to linear plasmids of ascomycetes, whereas a basidiomycete plasmid formed a branch of its own (92).

HGT was also invoked when global distribution patterns of 7 *Neurospora* plasmid groups among 225 *Neurospora* isolates from 5 species demonstrated that the plasmid groups were widely dispersed both within and among species in an almost random fashion (7). In addition, analysis of plasmid occurrence and distribution indicated that although many plasmids were globally dispersed, some regional clustering was evident. A statistically significant geographic clustering of three plasmid groups from *Neurospora* isolates from Hawaii was identified that was host independent.

The *Neurospora* strains used by Arganoza et al (7), which contained kalilo-like plasmids, were recently reexamined. Though He et al (71) found evidence for coevolution of kalilo with their respective host species, horizontal transfer could not be ruled out for LA-kalilo, which was present in *N. tetrasperma* from Louisiana and *N. crassa* from Haiti, and for kalilo found in *N. intermedia* from Hawaii and *N. tetrasperma* from Moorea-Tahiti. Introgression was suggested as an alternative to HGT to explain the presence of highly similar plasmids in independent species. The possibility of sexual introgression for plasmid transfer was experimentally demonstrated by developing hybrids, followed by repeated backcrossing (15). Both the kalilo and the LA-kalilo were transferred into different host species by this method.

Initial attempts to obtain direct experimental proof for horizontal transmission of mitochondrial plasmids used auxotrophic markers to force unstable heterokaryon formation between otherwise genetically isolated strains. Collins & Saville (32) showed independent transfer of the linear plasmids V and VS between vegetatively incompatible strains in *N. intermedia*. Using a similar approach, Griffiths et al (41, 65) demonstrated the horizontal transfer of the kalilo plasmid from *N. intermedia* to *N. crassa*. More recent experiments observed horizontal transfer of

plasmids between unrelated fungal strains without the use of heterokaryon-forcing markers (41, 90, 166). Using strains of *N. intermedia* and *N. crassa*, Debets et al (41) assessed the role of vegetative incompatibility on the potential for horizontal transfer of two mitochondrial plasmids. The authors first showed that both hanalei-2 and the linear kalilo (including Kalilo senescent phenotype, which is induced when kalilo inserts into mitochondrial DNA) were easily transferred between vegetatively compatible strains of *N. crassa*. In addition, hanalei-2 and kalilo (but not the Kalilo senescent phenotype) were in some cases transferred between strains that differed from each other by one or more polymorphisms in their *het*-genes. Similar results were obtained with isolates of *N. intermedia*.

Horizontal transfer of the linear mitochondrial plasmid pAI2 from the discomycete *A. immersus* to mitochondria of a plasmid-free strain of the pyrenomycete *Podospora anserina* was achieved experimentally by merely co-culturing these unrelated strains (90). Kempken (90) speculated that cytoplasmic components of the two fungi may have come in contact with each other during the vegetative incompatibility response, leading to horizontal transfer of the plasmid. Hybridization of an *A. immersus*–specific mitochondrial probe to *P. anserina* indicated that HGT was limited to the plasmid. Horizontal transfer of the longevity-inducing plasmid pAL2-1 from *Podospora anserina* also has been demonstrated experimentally. The linear plasmid was efficiently transferred between both vegetatively compatible and incompatible strains of *P. anserina* (166).

While the acquisition of fungal plasmids is an important issue in assessing the role of HGT in the evolution of these genetic elements, so is the issue of plasmid loss. The proportion of plasmid-containing strains in natural populations of fungi is often low (around 10%) and plasmid loss has been observed even in the laboratory, especially during the sexual cycle [see (166) for a full discussion]. In addition, it has been shown that some fungal host genomes are able to suppress plasmids (64). Since most plasmids would seem to confer no selective advantage to their host [but see (14)], indeed, encoding in most cases nothing more than genes responsible for their own replication, a likely explanation is that HGT assures their survival independent of their loss within a single lineage. This argument even can be taken one step farther. As plasmids are most certainly of ancient origin, one could speculate that other eukaryotes, which with few exceptions are devoid of plasmids, eliminated them from their populations over evolutionary time. Therefore, the widespread occurrence of plasmids in natural populations of fungi may be a sign that HGT in fungi is a more important evolutionary factor than in other eukaryotes.

Mycoviruses

Many fungal species contain cytoplasmic or mitochondrial viruses (59). Mycoviruses generally have double-stranded (ds) or, less commonly, single-stranded RNA genomes (130, 167). Though most mycoviruses can be classified into families, some mycoviruses do not fit those characteristics (130).

Mycoviruses differ from viruses of other organisms in that they lack an extracellular phase and therefore are not considered infective (59, 130). Vertical (or serial) transmission into asexual progeny is generally efficient (30, 167), whereas transmission into sexual progeny is often inefficient or lacking (28, 30, 167). Consequently, in some genera such as *Aspergillus* and *Penicillium*, asexual species often harbor virusus, whereas sexual species lack them. Horizontal transmission can occur by cytoplasmic mixing, and virus transfer is efficient between vegetatively compatible individuals (130, 133, 168).

The best known mycoviruses are those with a phenotypic effect, either debilitating the fungus, which includes a reduction in virulence (hypovirulence), or conferring a selective advantage over virus-free isolates (killer phenotype) [for reviews see (59, 130, 133, 138)]. However, the majority of mycoviruses are assumed to be neutral, as they are of no consequence to their hosts. This lack of phenotypic effect is thought to be the result of coevolution between viruses and fungi, which included selection against virulence [for a full discussion and additional arguments see (133)].

If we assume coevolution, sequence divergence of different viruses would presumably reflect divergence of their fungal hosts (79, 80). This hypothesis may also provide the basis for investigation of horizontal transfer of mycoviruses. Though few evolutionary studies have been conducted to address this question, some data may be consistent with horizontal transfer. For example, an RNA-dependent RNA polymerase (RdRp)-like protein encoded by a mitochondrial virus of the ascomycete *Ophiostoma novo-ulmi* had more sequence similarity to that of the basidiomycete *Rhizoctonia solani* than to that of the ascomycete *Cryphonectria parasitica* (79). Nevertheless, the authors argued against recent horizontal transfer, as codon usage in the respective dsRNAs was different between *R. solani* on one hand and *O. novo-ulmi* and *C. parasitica* on the other. Sequences similar to the above-mentioned RdRp-like proteins have also been identified in translated protein sequences from the mitochondrial genomes of *Arabidopsis thaliana* and *Vicia faba* (126). Marienfeld et al (126) therefore suggested horizontal transfer probably from fungi to those plant species, though Hong et al (79) also provided an alternative explanation for this phenomenon, i.e. evolution of these sequences from common ancestors. Another dsRNA virus from *R. solani* was characterized, whose RdRp showed extensive sequence similarity at the nucleotide and amino acid level with RdRps from the ascomycetes *Fusarium poae* and *Atkinsonella hypoxylon* (156). The RdRp of the dsRNA in a parasitic protozoan was also found to be more similar to polymerases from fungi than to those from other protozoa (97). Although these are isolated instances, they are most likely of significance as dsRNA viruses evolve and diverge rapidly as RdRps do not have a proofreading mechanism and therefore any detected sequence similarity should be meaningful. However, determining whether horizontal transfer is responsible for these similarities will require the comparison of RdRp sequences from a wider range of organisms (79).

Several experiments have been conducted to evaluate the role of vegetative incompatibility on virus transfer within and between fungal species. In some cases

vegetative incompatibility blocked virus transfer completely, e.g. in black *Aspergilli* (168), whereas in other fungi, virus transfer occasionally occurred across vegetative compatibility groups, e.g. in *C. parasitica* (5, 109). In the sexual species *Aspergillus nidulans*, where no viruses were found in 112 isolates (30), a virus was efficiently transferred by protoplast fusion from a virus-containing isolate of *Aspergillus niger*. In subsequent experiments, virus transmission was achieved in some cases by co-culturing the two species, implying cytoplasmic mixing (30). In another study, mitochondrial fusion was indicated as a mechanism by which mitochondrial dsRNA could spread between individuals within a vegetative compatibility group in *C. parasitica* (146).

Introns

Since their discovery, introns have been subject to speculation regarding their origin and evolution, culminating in a debate whether spliceosomal introns were instrumental in initial exon shuffling in the progenitor (introns-early theory) or were acquired much later (introns-late theory) (123). This discussion is pertinent to our horizontal transfer discussion insofar as the introns-early theory is based solely on the premise of substantial intron loss to explain their phylogenetically discontinuous distribution. In contrast, the introns-late theory per se invokes horizontal transfer mechanisms. Though the introns-early/introns-late issue is restricted largely to the spliceosome-dependent pre-mRNA (spliceosomal) introns of nuclear genes of eukaryotes (122), this debate is analogous to evolutionary questions raised by investigations into other types of introns. In contrast to other genomic regions discussed in this review, information is available on actual or potential mechanisms of intron movement.

Both group I and group II introns are characterized by the ability of their RNA to fold into characteristic secondary structures, thereby forming the active sites for splicing (46, 112). Group I introns have a wide phylogenetic distribution, can be nuclear or organellar, but are especially frequent in fungal mitochondrial DNAs and in nuclear rRNA genes. In eukaryotes, group II introns have been located to date only in organelles. The two intron groups differ in secondary RNA structure and in mechanism of splicing (112). In addition to their core sequence, many introns possess open reading frames (ORFs), which are thought to have been acquired separately (111, 112). It has been proposed that intron-encoded proteins of both groups initially promoted mobilization and that these proteins later acquired an ancillary role in RNA splicing (maturases) (112). As splicing requires specific base-pairing interactions between intron and exon sequences flanking the splice sites, introns of both groups are assumed to be functional only when inserted at sites where flanking exon sequences are compatible with splicing (111).

Intron homing is the insertion of the intron into the intronless allele of a homologous gene [for a review see (111)]. Mobility is facilitated by site-specific endonucleases in group I introns, and endonuclease/reverse transcriptase functions in group II introns (11, 35, 112). The target sites of endonucleases of group

I introns are characterized by long recognition sequences. Endonucleases target intronless sites, create a ds break, and thereby initiate intron mobility by a DNA-based method [reviewed in (112)]. Acquisition of the intron is thought to be accomplished by a ds-break repair pathway with the intron-containing donor allele serving as a template (25). Intron homing (retrohoming) in group II introns is thought to be achieved by a target DNA-primed reverse transcription mechanism in which the intron RNA reverse splices directly into the intronless allele and is then copied by the intron-encoded reverse transcriptase (35, 48). In both types of intron homing, acquisition is often accompanied by the permanent coconversion of flanking sequences during intron inheritance (48, 111, 112).

Intron transposition refers to the mobilization of an intron to an unrelated or ectopic site. Possible RNA-based pathways for intron insertion into a different RNA are based on reversal of the splicing reaction, followed by reverse transcription of the recombined RNA and integration into genomic DNA (111, 112). Transient integration of a *Tetrahymena* group I intron into bacterial rRNA by reverse splicing has been demonstrated in vivo (152). Unlike homing, this process can be independent of intron-encoded proteins and could provide a mechanism for intron integration into novel genome sites (152).

Both intron homing and intron transposition may be considered horizontal transfer events if the movement of the intron occurs between two different genomes/individuals during a nonmeiotic event. Finally, intron loss could result from the reverse transcription of spliced, and therefore intronless, RNA followed by homologous recombination (46).

Mitochondrial Group I Introns Fungal mitochondria generally are intron-rich, and only a few fungal species are known whose mitochondria are devoid of introns (141). The fact that mitochondrial and other introns are often located in identical gene positions (homologous introns) in different species facilitates investigations of intron movement, as intron phylogenies can be compared directly with the phylogeny of the gene they inhabit.

Probably the best substantiated case of intron horizontal transfer involves fungi as the potential donor. During a survey of plant mitochondrial cytochrome oxidase subunit 1 (*cox1*) genes, Vaughn et al (170) discovered a group I intron in the angiosperm *Peperomia polybotrya*. This was surprising, insofar as only group II introns had previously been found to be associated with plant mitochondria. Phylogenetic analysis revealed different evolutionary histories for intron and exon at that locus and clustered the intron together with group I mitochondrial introns from fungi. It was therefore hypothesized that *P. polybotrya* acquired the intron from a fungal donor. A follow-up study (1) determined the intron to be present in all *Peperomia* species tested, therefore dating the transfer event(s) before the divergence of the genus. The potential fungal donor was likely not an Ascomycete due to usage of a UGG tryptophan codon in the ORF, unlike the preferred UGA codon for tryptophan in mitochondria of Ascomycetes (1), except *Schizosaccharomyces pombe* (141). On the other hand, Basidiomycetes,

Zygomycetes, and Chytridiomycetes (*Allomyces macrogynus*) all use a UGG tryptophan codon (141).

Extensive Southern blot surveys testing for the presence of the *cox1* group I intron in 335 diverse genera of land plants (26) revealed its presence in 48 genera, albeit with an extremely patchy distribution. This was in stark contrast to a nearly universal presence of a *cox2* group II mitochondrial intron. High sequence identity (>92%) between copies of the group I intron and incongruencies between intron and organismal phylogenies pointed to extensive HGT during angiosperm evolution. Differential exonic coconversion tracts provided evidence for separate acquisitions. Absence of coconversion tracts further established that intronless species never had the intron, arguing against the alternative hypothesis of extensive intron loss during angiosperm evolution.

Analyses of fungal *cox1* group I introns have also led repeatedly to indications and claims of horizontal transmission. The ORFs of *cox1* intron i2 of *S. pombe* and the homologous intron i3 from *A. nidulans* were found to be similar with 70% amino acid identity, despite the phylogenetic distance between the two species (113). Also, a 60-bp exon region both up- and downstream from the intron (coconversion tracts) was highly homologous between the two fungi, displaying 82% and 70% identity, respectively (113). A potential horizontal transfer event involving these two species was suggested to have been recent in evolutionary terms.

The four *cox1* introns from the basidiomycete *Agrocybe aegerita* were compared to homologous introns from algae and Ascomycetes (61). Surprisingly, the ORF of one intron, called i4, displayed 73.5% nucleotide sequence identity and 90.5% amino acid identity with the ORF with the homologous intron, called i14, from the *cox1* gene of the ascomycete *P. anserina*. These values were the highest reported to date between introns of two phylogenetically distant species, which therefore strongly supports recent horizontal transmission. As the remaining three introns showed varied sequence similarities to other homologous ascomyceteous introns, all four introns were hypothesized to be of different ages and origins, again substantiating intermittent HGTs (61). In addition, a BLASTP (see Reference 4) search revealed higher amino acid similarity of the i2 ORF of *A. aegerita* with the previously mentioned ORFs of *A. nidulans* i3 (56% identity, 71% similarity) and ORF of *S. pombe* i2 (57% identity, 75% similarity) than with a recently submitted amino acid sequence (accession number: AB016791) of the ORF of a *cox1* intron from the basidiomycete *Flammulina velutipes* (36% identity, 58% similarity). Both *F. velutipes* and *A. aegerita* belong to the Agaricales. These incongruencies may point to horizontal transfer events.

The mitochondrial genome of the chytridiomycete *Allomyces macrogynus* differs from that of its close relative *Allomyces arbusculus* by having a DNA segment not present in *A. arbusculus* (142). The DNA segment consists of the C terminus of a foreign gene encoding a subunit of the ATP synthetase complex (*atp6*) and an ORF encoding an endonuclease. The foreign *atp6* part is in phase with the resident gene, which therefore results in a chimera. This strongly suggests that this insertion was acquired by HGT.

A claim by Colleaux et al (31) that "a horizontal transfer of introns must have occurred between *Neurospora* (*crassa*) and *Chlamydomonas* or their close ancestors" as introns located in the apocytochrome b gene shared sequence similarity in both primary and secondary structure could not be verified. Our comparison of the sequences revealed only a stretch of 80 bp of sequence overlap between the two introns, which we believe is too low to ascertain horizontal transfer.

Group I Introns in Nuclear and Mitochondrial rRNA Genes Introns within rRNA genes are valuable subjects for addressing questions of horizontal transfer because rRNA genes themselves have been extensively used to determine species phylogenies. The evolutionary history of introns in rRNA genes has been addressed repeatedly and common themes have been found. One theme is their highly irregular distribution even within closely related fungal species. This has been established at the class level for the Homobasidiomycetes (74); at the family level as exemplified by Holst-Jensen et al (78) for the Sclerotiniaceae and by Goddard & Burt (60) for the Saccharomycetaceae; and at the genus level for *Cordyceps* species (86) and even within a single species, as exemplified by Tan (158) in his study of varieties of *Gaeumannomyces graminis*. Another common feature of these introns is that they are inserted at few positions. For example, introns in the nSSU rDNA are predominantly inserted at two sites (site 943 and site 1506 relative to *E. coli*). Introns at the same sites form distinct lineages independent of host species and therefore have a common origin (143). Furthermore, intron phylogeny is in some cases incongruent with species phylogeny, which strongly implies HGT (78, 86, 137, 143). Even if species phylogenies are congruent with intron phylogenies, HGT sometimes cannot be ruled out, as argued by Hibbett (74). For example, a strictly vertical transmission of introns in the Homobasidiomycetes would assume that the rate of intron loss was several times higher than intron gain to explain patchy intron distribution. Additional support to infer horizontal intron movement in this case was provided when homologous introns from other fungi and green algae were included in the analysis. Neither algal nor fungal introns were supported as monophyletic. Therefore, Hibbett (74) speculated that host lineage switching via HGT must have occurred frequently.

The ω-homing endonuclease ORF and associated group I intron in the mitochondrial LSU rRNA gene in *Saccharomyces cerevisiae* have long been known and have been extensively analyzed (46). Recently, Goddard & Burt (60) examined 20 yeast species for presence or absence of the intron and ORF. Fourteen species contained the intron and of those, only five had the ORF. Two of the five ORF sequences had insertions disrupting the reading frame, which probably rendered them nonfunctional. The different intron/ORF states (presence, absence) and, more importantly, both intron and ORF phylogenies were not congruent with the species phylogeny, suggesting a succession of intron/ORF invasion, ORF degeneration, and intron/ORF loss (60). BLASTN (4) searches for both intron and

ORF sequences only gave significant scores with sequences from the Saccharomycetaceae. This suggested that horizontal transmission may be more common between closely related species (60).

Though they could not confirm movement of the ORF independent of the intron, as intron and ORF phylogenies were not incongruent, Goddard & Burt (60) nevertheless considered this possibility because of the independent distribution of the ORF among the species. In support of this conjecture, autonomous movement of the ORF of a mitochondrial intron (*nad1-i4-orf1*) between two strains of *Podospora comata* was proven to be independent of the core sequence of the intron (154), providing evidence that the ORF can function as an autonomously mobile element.

Group II Introns The only example we have found for possible HGT of fungal group II introns involves the yeasts *Kluyveromyces lactis* and *S. cerevisiae* (70). Both yeasts have a group II intron located in *cox1*. Sequence analysis of this pair of introns revealed 96% sequence identity (and 92% amino acid identity of the ORF) between *cox1* intron 1 in *K. lactis* and *cox1* intron 2 in *S. cerevisiae* over 2485 bp. This finding was in contrast to 88% sequence identity in the *cox1* exon regions. Also, two other homologous pairs of group I introns displayed only 72% and 38% sequence similarity.

Difficulties Working with Introns Examples for potential horizontal transfer of introns seem easier to find than examples for any other molecules discussed in this paper, as they are found often in homologous positions in various genomes. Substantiation for HGT may not be unequivocal, however. Conflicts among equally parsimonious intron trees may occur, and bootstrap values can be low, leading to errors or uncertainties in the estimate of the intron phylogeny (74). One likely error source includes saturation of variable sites by multiple substitutions (74). Another characteristic of introns is their patchy distribution among closely related species, as mentioned above. In addition to these examples, "optional" intronic sequences (i.e. an intron is not present in all strains) have also been identified in the mitochondrial genome of *P. anserina* (10). Of 15 strains, 9 contained optional intronic sequences and, on average, the strains of *P. anserina* differed from each other by 3.9 optional sequences. As it is not yet known whether intron loss is more frequent than intron gain, introns are generally optimized onto host phylogenies using equal weights. This practice may introduce serious errors (74). Detailed analysis of within-species distribution of introns or between closely related species or laboratory experiments may in future provide better estimates on rates of losses and gains. If feasible, coconversion tracts analysis should also be included as they provide information on whether an intron ever inhabited a particular site [e.g. see discussion in (25)]. Also, differences in coconversion tracts may unearth separate horizontal transfer events even if organismal and intron phylogenies are congruent (25).

Introns, maintained in regions of high homology, may well be much more easily distributed via HGT, because the exon regions adjacent to the intron would

provide the homology necessary for recombination events (113). Nevertheless, it is unclear how horizontal transfer of introns occurs at the cellular level (63). For intron homing to occur, both the intron-containing donor DNA and the intronless recipient DNA presumably must be in the same physical location, the intron-containing DNA must be transcribed, and the encoded endonuclease must be translated (63).

In summary, our compilation of potential intron horizontal transmissions in fungi indicates that horizontal transfer of introns among fungi may be quite common. Goddard & Burt (60) speculated that introns need to be occasionally horizontally transferred for long-term persistence and that their abundance in fungi and protists may be an indication that the germline of these eukaryotes may be more accessible than that of other eukaryotes. This is similar to the argument we have made for fungal plasmids.

Returning to our initial discussion of introns, a study of spliceosomal introns in fungi has also contributed to the introns-early/introns-late debate. The nuclear gene encoding triose-phosphate isomerase was examined for intron positions in protists, plants, fungi, and various members of the Metazoa (123). Twenty-one intron positions were identified and introns were classified as "old", "intermediate," and "recent." Considered to be recent additions were those positions, which were only found in 1 of the 17 intron-containing species. While 12 introns were considered recent acquisitions and therefore supporting the introns-late theory, over half of these (7) were found in 2 fungal species alone (4 in *Aspergillus*, 3 in *Coprinus*).

Transposable Elements

One could easily imagine that horizontal transfer may occur frequently for transposable elements owing to their autonomous mobility irrespective of host. Although most researchers agree that HGT has occurred in some instances, the extent to which it has contributed to the current distribution of transposable elements among organisms is vigorously debated. Among the many instances where circumstantial evidence of HGT for transposable elements among groups of eukaryotes has been invoked, definitive proof has been achieved in only a few cases (98).

Transposable elements of all organisms are subdivided into Class I and Class II elements (55). Class I elements transpose by a reverse transcription of an RNA intermediate and include retrotransposons with long terminal repeats (LTRs), long interspersed nuclear elements (LINEs or non-LTR retrotransposon) and short interspersed nuclear elements (SINEs). Class II elements transpose at the DNA level by excising from a donor site and reintegrating at another site (cut-and-paste). Elements in this class are characterized by short inverted terminal repeats (ITRs) and are grouped according to amino acid sequence similarities with other elements. Within a given host genome, Class II elements may increase in copy number, presumably by transposing during chromosomal DNA synthesis with a copy moving from replicated to unreplicated DNA, resulting in a net increase of the element

(36), or through ds gap repair of a target site when an element-containing DNA strand is used as template (34). Fungal transposable elements are known that are representative of all groups and subgroups mentioned above [for a comprehensive list see (95)].

HGT involving transposable elements is often invoked when inconsistencies with expected phylogenetic relationships are observed. This may happen if either the distribution of an element does not follow the phylogenetic pattern of the host fungus or when homologous sequences with high similarity are identified in phylogenetically unrelated organisms. Examples for both cases have been observed in fungi. Sporadic distribution of the LTR retrotransposon *grasshopper* was demonstrated in *Magnaporthe grisea* (43). A survey of 36 isolates of *M. grisea* from *Eleusine* host species and 8 isolates from other host species established that *grasshopper* was found exclusively in those strains infecting *Eleusine*. In addition, this element was present only in isolates from countries in Asia and western Africa. This sporadic distribution may point to an acquisition via HGT subsequent to the evolution of this host-specific form (43). In the most comprehensive study on the distribution of a transposable element in fungi, HGT was strongly suggested after sequences homologous to the transposon *Fot1* were found to be widespread in lineages of *F. oxysporum*, absent in the phylogenetically closely related *Fusarium* species aligned with a *Gibberella* sexual state, but present in five strains from species more distantly related, including those aligned with a sexual state in *Nectria haematococca* (37). In addition to this sporadic distribution, a low level of sequence divergence (around 2%) was found among elements from *F. oxysporum* and three of the five unrelated strains (*F. solani* var. *minus*, *F. javanicum* var. *radicola*, *F. caucasicum*). In contrast, the gene for nitrate reductase (*nia*) displayed $>25\%$ sequence divergence between representatives of *F. oxysporum* and isolates from the more distantly related *Fusarium* species (36–38). Moreover, the *nia* genes from *F. oxysporum* and the closely related *Gibberella fujikuroi* showed 95% sequence identity.

Future research will undoubtedly reveal other cases of phylogenetic inconsistencies between fungal transposable elements and their host genomes. Still, horizontal transfer is difficult to prove. Several authors have cautioned against invoking horizontal transfer as the most likely explanation for phylogenetic inconsistencies (19–21, 34). Though these authors do not deny that HGT may play a role in the evolution of some transposable elements, alternative interpretation of data may be possible without invoking HGT. If distribution patterns do not follow phylogenetic expectations, could it be that elements were lost in specific lineages through extinction, recombination, or genetic drift? A worldwide collection of 50 isolates belonging to 10 *formae speciales* of *F. oxysporum* were assessed for the presence or absence of *Fot1* and *impala*, both Class II transposable elements. Most of the isolates contained these elements, indicating that they were present in the common ancestor before the divergence of host-specific forms. In this case, stochastic loss was the best explanation to account for their absence in some lineages (38).

The appropriateness and sensitivity of experimental techniques also must be taken into account. Southern hybridization may not detect highly divergent copies of the same element (19, 34). Therefore, degenerate PCR primers of conserved areas may be a more sensitive method to establish distribution patterns of transposable elements (19, 34). A combination of both methods may be warranted in most cases.

Once copies of a transposable element from different host species have been identified and sequenced, the difficult task is to establish a molecular phylogeny for the element itself and especially to relate this with a phylogeny of its host species. Capy and coworkers speculated that within a genome, active copies of a transposable element are less divergent than nonautonomous copies, and within a copy, certain domains, especially those required for mobilization, may evolve more slowly than others (19–21). Thus, in establishing a phylogeny for a specific transposable element, copies to be analyzed should have a similar level of activity and potentially similar rates of evolution. Between host genomes, the rate of evolution of a transposable element may be influenced by a host-species effect (19–21). Phylogenetic inconsistencies may also reflect polymorphisms among multiple copies of an element in a common ancestor. Divergent ancestral copies could have been sorted out during the speciation process, each new species inheriting one or several polymorphic copies (19).

The identification of subfamilies of a transposable element within the same genome also presents a challenge for interpretation. In *F. oxysporum*, three highly divergent subfamilies of the *impala* element were discovered (81). The subfamilies differed by a high level of sequence divergence (around 20%). The coexistence of these subfamilies may indicate either ancestral polymorphisms among copies in the common ancestor of the species or the occurrence of multiple horizontal transfers. In the latter case, a given species might receive each subfamily from different sources and HGT can be inferred if copies in unrelated species are found that are more similar than copies of different subfamilies present in the same species (19).

How horizontal transmission might occur between unrelated species has always been an enigma that makes it difficult for the proponents of HGT to convince the skeptic. Several pathways have been proposed, but none has been proven (19, 20, 21). Current hypotheses mainly favor various symbionts or parasites of respective host species (e.g. mites, viruses, baculoviruses, ricketsia-like bacteria) as vectors for horizontal transfer in eukaryotes (19, 20, 98). If correct, one would expect similarities between transposable elements within organisms that share a specific parasite, independent of their phylogenetic relationships (19). Potential vectors do not necessarily need to integrate a transposable element into their own genome, as it could be transmitted directly as an excision product (20). Evidence for extrachromosomal copies of transposable elements comes from the work of Kempken & Kück (94) who found extrachromosomal circular transposition derivatives of a Class II transposon known as *Restless* from *Tolypocladium inflatum* (96). Using PCR, amplification products were identified that carried the joined end of

Restless transposon fused at its inverted repeats. In addition to the intact transposon ends, the eight sequenced products also contained a short insertion of between 1 bp and 93 bp of genomic DNA. Whether these are true transposition intermediates or rare transposition artifacts is less important than the fact that they are extrachromosomal plasmid-like sequences that may have the potential to cross species lines with or without further aid of a vector (94). Additional research on *Restless* (93) has revealed a discontinuous distribution of the element in 13 strains closely related to *T. inflatum*. Some strains contained only a single active or nonactive (truncated) copy. Since Class II elements are usually represented as multiple copies in a genome, horizontal transfer of this transposable element may have occurred in the recent past.

Sequence analysis of whole genomes should shed more light on the evolution of transposable elements. It has been suggested that after invasion of a specific host lineage, transposable elements display a "life cycle" consisting of active replication, inactivation, and degradation (20, 99). Transposition events of both classes of transposable elements are error-prone and over time produce nonautonomous and degenerated elements. If these assumptions are correct, genome-wide surveys for transposable elements could disclose differential ages of transposable elements within a specific genome. For recently invasive elements, one would expect a low number of mainly active copies, displaying little sequence variation among copies. For ancient elements, a high percentage of nonautonomous or degenerated copies within the genome should be observed. Such a study has been completed for *S. cerevisiae*. A genome-wide survey using the long terminal repeats (LTRs) of previously identified active copies of five families of retroelements (Ty1–Ty5) as query sequences yielded a total of 331 insertions, with 85% of insertions being solo LTRs or LTR fragments (101). Whereas the LTRs of Ty1, Ty2, and Ty5 displayed a broad range of sequence diversity, LTRs of Ty3 and Ty4 were highly similar within each family. Therefore, Ty3 and Ty4 appeared to be more recent additions to the genome (101). Another study developed a genomic demography model based on sequence data and estimated that the first elements of the Ty1, Ty2, and the hybrid Ty1/2 families entered the genome between approximately 50 million and 250 million generations ago (148).

Advances also have been made in the study of another major Class I element, the non-LTR retrotransposons (LINE-like elements) (124). A comprehensive phylogenetic analysis of LINE-like elements was conducted based on an extended sequence alignment of their reverse transcriptase domain (124). The authors detected 11 clades, including a "fungal clade," represented by the Tad1 family. Tad1 includes CgT1 described from *Colletotrichum gloeosporioides* (72), MGR 583 in *Magnaporthe grisea* (68), MARS1 from *A. immersus* (62), and Tad1 from *Neurospora crassa* (104). All 11 clades of non-LTR elements were determined to trace their origin to the pre-Cambrian period, indicating that LINE-like elements may not use HGT as an evolutionary strategy.

Improved scientific methodology and an understanding of the fundamental questions and approaches to judging HGT for transposable elements should now

move the field from anecdotal observation to provision of clear scientific evidence. If it can be shown that substantial interspecific gene transfer occurs, the concept of "genomic integrity" within species may also change (34). This is especially important with regard to transposable elements as they have been implied to have an effect on genes and genomes by promoting changes in gene expression, in gene sequence, and probably in chromosomal organization (36, 56, 99).

Nuclear Genes

Hydrolytic Enzymes of Anaerobic Fungi The digestive tract of herbivores is inhabited by a limited number of anaerobic pro- and eukaryotic microbial species. Besides protozoa, eubacteria, and archaebacteria, 17 anaerobic fungal species so far have been identified that inhabit the alimentary tract of ruminant and nonruminant herbivores (22). While gut eubacteria are highly diverse, anaerobic fungi are closely related to each other and have been placed into five genera of a monophyletic group of the Chytridiomycetes (57, 117, 118). Gut microorganisms are noted for their production of an array of highly active plant cell wall–degrading enzymes. Several genes coding for these polysaccharide hydrolases have been cloned and sequenced. Of the anaerobic fungi, hydrolytic enzymes of *Neocallimastix*, *Orpinomyces*, and *Piromyces* have been characterized.

One shared characteristic of many of the hydrolases isolated from anaerobic fungi is the presence of noncatalytic repeated peptide domains (NCRPDs). NCRPDs, which are repeats of 36 to 40 amino acids in unit length, have been postulated to function in protein docking during the interaction of enzymes in multienzyme complexes. Thus they are similar to the dockerin domain of multienzyme cellulosome complexes of anaerobic bacteria (9, 50, 119). A survey of NCRPDs of various enzymes from three anaerobic fungal species found them to be highly homologous (119). The similarity was present even if the catalytic domains showed substantial divergence. Therefore, it has been suggested that NCRPDs evolved from common ancestral genes and that they may have a separate evolutionary origin from the catalytic regions, implying horizontal transfer (119). Additional support that these noncatalytic domains may be horizontally transferred independent of the catalytic region was provided by Liu et al (121) who found similarity between two repeated sequences of *celA* from *Orpinomyces joyonii* and a noncatalytic region of the endonuclease EG3 from the anaerobic bacterium *Fibriobacter succinogenes*. As the catalytic regions themselves did not display any similarity, Liu et al (121) suggested evolutionary shuffling (mix and match) of the individual domains among anaerobic bacteria and fungi.

Horizontal transfer also has been suggested for the catalytic domains themselves. Homologous xylanases of *Neocallimastix partriciarum* (XYLA-A, XYLA-B) and *Orpinomyces* (XYNA) were similar (>88% amino acid identity of the catalytic domain) (119). In comparison, a xylanase isolated from *Piromyces*, the next highest match detected by a BLASTP (4) search, conducted by us, displayed only 33.5–34.5% identity with XYNA. This is in contrast to independent phylogenetic

studies which indicated a closer relationship between *Orpinomyces* and *Piromyces* (Nei's unbiased genetic identity = 0.469) than for *Orpinomyces* and *Neocallimastix* (Nei's unbiased genetic identity = 0.344) (75). All xylanases mentioned above are considered family G glycanases and presumably are homologs. A search for additional xylanases in *N. patriciarum* yielded XYLB, which belonged to family F, otherwise found predominantly in bacteria (12). This result not only provided evidence for independent evolutionary origins of xylanases in *N. patriciarum* (12), but also suggested a prokaryotic origin for XYLB.

Interkingdom gene transfer has also been postulated for other hydrolytic enzymes. The derived amino acid sequence of endoglucanase B (178) of the anaerobic fungus *N. patriciarum* is not only similar to CELB from *Orpinomyces*, with 83.1% amino acid identity (119), but also exhibited 36% to 45% identity with the catalytic domain of 7 different bacterial cellulases over 314 amino acid residues (178). Five of the endonucleases were derived from bacterial species that normally inhabit the rumen. The argument for HGT between bacteria and fungi is made even more convincing by the fact that percentage sequence identity between endogluconase CELB and the closest match of an endoglucanase from an aerobic fungus, endogluconase II from *Trichoderma reesei*, was only 26%.

When the cellulase CELA was isolated and characterized from *N. patriciarum*, no sequence homology with other rumen microbial enzymes was found. Rather, the highest match was to cellobiohydrolase CBHII of the aerobic *Trichoderma reesei* (identity of catalytic region: 37%, similarity: 53%) (42). Also, instead of NRCPDs, CELA had a type 2 family cellulose-binding domain (CBD), typical of aerobic fungal cellulases. Amino acid identity between the CBDs of *T. reesei* and *N. patriciarum* was 57%. From these data the authors concluded that this enzyme must have had an ancestral precursor common to both aerobic and anaerobic fungi. However, subsequent isolation of two cellulases (CELA and CELC) from *Orpinomyces* strain PC-2, which were homologous to CELA of *N. patriciarum* ($>$60% identity), did not have a CBD; instead, NRCPDs were identified, again being similar to NCRPDs of unrelated polysaccharide hydrolases from anaerobic fungi (120). This result is another example of NRCPDs and the catalytic domain having different evolutionary origins.

A similar case involves acetyl xylan esterase (AXEA) from *Orpinomyces* sp. PC-2. The only homologue so far to AXEA was identified in *Neocallimastix* and shared 64% identity and 80% similarity. The acetyl xylan esterases in *Orpinomyces* and *Neocallimastix* lacked and possessed NCRPDs, respectively. It was hypothesized that acetyl xylan esterase was transferred from one organism to the other with concurrent loss or gain of the NCRPDs (13).

Other hydrolytic enzymes are suspected to be of prokaryotic origin. The gene coding for 1,3-1,4-β-glucanase identified from *Orpinomyces* sp. PC-2 was devoid of introns and the enzyme displayed similarity to ß-glucanases of mesophilic and thermophilic bacteria, including ruminal bacteria (24). Although identity to some bacterial sequences was $>$50%, identity to the closest protein from *S. cerevisiae*

was only 29%. Three mannanases from *Piromyces* belonged to the "bacterial" glycosyl hydrolase family 26, implying a prokarotic origin (50, 135).

Other genes suggested to have been acquired by HGT include cyclophilin B and phosphoenolpyruvate carboxykinase (PEPck). Cyclophilin B from *Orpinomyces* sp. PC-2 displayed a higher similarity to vertebrate cyclophilin B (66%–70%) than to the homologous sequence from yeast (54%) (23). PEPck from *Neocallimastix frontalis* was found to be homologous to enzymes from animals, but did not show any similarity to PEPck from yeast (150). A recent database search of PEPck sequences has revealed a curiosity: Deposited sequences from animals, bacteria, and *N. frontalis* appear to be for GTP-dependent enzymes, whereas those from other fungi and plants appear to be ATP-dependent. There is no homology between ATP- and GTP-dependent PEPck enzymes. Since all other fungal PEPck described so far are ATP-dependent, one can conclude that the GTP-dependent enzyme from *N. frontalis* is likely of xenologous origin.

The digestive tract of herbivores constitutes a closed, spatially finite and specialized ecosystem and is densely inhabited by a phylogenetically diverse array of microorganisms (57). Therefore, this habitat may constitute a prime environment in which HGT may occur. Studies to date have been limited mainly to hydrolytic enzymes. To understand further the potential for HGT in the rumen, additional genes and whole genomes should be compared to determine if sequences under comparison are truly homologous (not parologous) to determine whether the genomes of gut fungi are indeed mosaics of eukaryotic and horizontally acquired prokaryotic genes.

Catalases Catalases are enzymes that facilitate the redox reaction converting two H_2O_2 molecules to two molecules of water and one molecule of O_2. Phylogenetic analysis of 70 catalase protein sequences from an array of pro- and eukaryotic organisms generally resulted in kingdom-specific branches, with one exception (107). Fungal catalases fell into two groups, whereby one of these groups (supported by 100% bootstrap confidence levels) contained a mixture of fungal and bacterial enzymes, i.e. catalases from *Aspergillus nidulans* and *A. niger*, in addition to six bacterial species representing high- and low-GC gram-positive bacteria and γ-proteobacteria. As this group was the only branch in the tree to contain sequences from more than one kingdom, HGT was assumed to be the most likely explanation.

Globins in Nonvertebrate Species Moens et al (136) evaluated a number of putative globins and globin-like heme-containing proteins from nonvertebrates. Based on a template that considered the structure-function relationship of 145 nonvertebrate globins, the sequences were differentiated into true globins and globin-like structures depending on a penalty score in relation to the template. The sequences of 25 nonvertebrate globins were aligned and analyzed using neighbor-joining and maximum parsimony. Both approaches resulted in three clusters: One comprised metazoan species and plants; a second cililates, *Chlamydomonas* and *Nostoc*; and a third combined two yeast species (*S. cerevisiae*, *Candida norvegesis*)

with sequences from three proteobacteria. In two cases, significant deviation from the phylogenetic tree (based on SSU rRNA sequences) was observed, suggestive of HGT. The most compelling case was seen in the yeast/proteobacteria cluster. The authors suggest that a common ancestor of these bacteria may have acquired the gene from an ancestor of *S. cerevisiae*.

Trichothecene 3-O-Acetyltransferase HGT has been suggested for the gene encoding trichothecene 3-O-acetyltransferase (*Tri101*) identified from *Fusarium graminearum* and *Fusarium sporotrichioides* (103). *Tri101* is different from the other known trichothecene biosynthetic genes in that it is not part of a gene cluster (102). *Tri101* (like the trichothecene biosynthetic gene cluster, see below) is postulated to have arisen by HGT since it is not present in other related species (e.g. *F. oxysporum* and *Fusarium equiseti*). Likewise, since flanking housekeeping genes for UTP-ammonia ligase and phosphate permease are similarly linked in nonproducing strains, the position of *Tri101* likely did not arise by reciprocal translocation, such as has been postulated for the position of genes for T-toxin biosynthesis in *Cochliobolus heterostrophus* (108).

Experimental Nuclear Gene Transfer In addition to processes implied from DNA sequence comparisons, HGT of single nuclear genes can be demonstrated in experimental settings. *Aspergillus niger* was co-cultured in sterile soil with transgenic hygromycin B-resistant plants of *Brassica napus* and *B. nigra*, *Datura innoxia*, and *Vicia narbonensis* (76). Re-isolated fungal cultures from all four hosts displayed an increased number of hygromycin resistant colonies in comparison with re-isolated cultures from nontransgenic plants (ratios varied between 4:1 and 36:1). However, statistically higher values were observed only with *B. napus* and *B. nigra*. Also, the *hph* gene was detected only in 10 of approximately 200 resistant colonies. It was speculated that the majority of resistant colonies might have lost the *hph* gene during culturing. In addition to the *hph* gene, additional DNA pieces, similar to plant vector-like pUC sequences, were sometimes identified. Based on Southern hybridization, one fungal culture contained DNA that was similar to a repetitive element found in *B. nigra*, but the sequence of the repeated element and thus its ultimate origin were never determined. Stable retention of the *hph* gene was demonstrated for only one fungal colony that was originally isolated from *D. innoxia*. Horizontal transfer was also observed when the fungus was grown in soil in the presence of plasmid and/or plant material. The authors suggested that fungal lytic enzymes may release DNA from foreign sources, thereby providing the initial substrate for HGT.

Biotrophic fusion parasites are a rarity among fungi (175). Among the few known species, substantial research has been conducted only on the facultative parasite *Parasitella parasitica*. Research, mainly conducted by Wöstemeyer and co-workers, has concentrated on the interaction of *P. parasitica* with one of its hosts, *Absidia glauca*. *A. glauca* and *P. parasitica* both belong to the Mucoraceae (Zygomycetes). *P. parasitica* is a fusion biotroph, whereby infection is

characterized by the formation of a plasma bridge that allows for a limited cytoplasmic continuum between both fungal species (89, 175). A number of nuclei of *P. parasitica* invade the mycelium of *A. glauca*, and after several days, these nuclei lyse in the host mycelium (89). Successful infection requires the presence of complementary mating types in the different species. Action of both mating types produces trisporic acid, which has been hypothesized to be involved in host/parasite interaction (175).

The prolonged presence of foreign nuclei in the host and the fact that *A. glauca* develops vegetative sporangia with viable spores close to the infection structures stimulated experiments to assess the potential for HGT (89). Auxotrophic *A. glauca* (Met^-, His^-) were infected with a prototrophic strain of *P. parasitella*. Asexual progeny of *A. glauca* were then tested for prototrophy. Prototrophic mutants were identified at high frequency (mean: 0.42%), 10^4 times higher than the natural reversion frequency. The prototrophic progeny were considered para-recombinants. Para-recombinants were often unstable and lost their prototrophic phenotype at high rates during the developmental cycle from a single uninucleate spore to the subsequent formation of sporangia. Plasmid-encoded neomycin resistance was also horizontally transferred successfully from the parasite to the host under selective conditions. The transferred DNA was propagated extrachromosomally (89). Additional hybridization of 30 different DNA fragments of *P. parasitica* to digested DNA of para-recombinants of *A. glauca* did not identify additional horizontally tranferred DNA (89). This indicated that horizontal transfer is limited, rare, and may not persist in many cases.

Though intriguing, this research has left many questions unanswered. Mechanisms are unknown for the processes of DNA transfer, the selectivity of transfer, the basis for the replication, and instability of the transferred genes in the recipient (174). Working hypotheses favor a view in which foreign DNA may enter the nuclei in *Absidia* via a pathway analogous to transformation, after the nuclei in *Parasitella* disintegrate, and that the foreign DNA may be maintained unstably as extrachromosomal elements (89, 175).

Gene Clusters

The tendency of genes for enzymes of certain metabolic pathways to be clustered in filamentous fungi has been noted previously (88). Generally these gene clusters encode optional pathways for nutrient utilization (e.g. the optional carbon source, quinate) (58) or for synthesis of secondary metabolites (e.g. the mycotoxin, sterigmatocystin) (16). Unlike the clustering of genes as operons in prokaryotes, clusters of similar genes in fungi are not cotranscribed, nor has any vital regulatory function for clustering been established (88). Thus the reason for the existence of gene clusters in filamentous fungi has not been resolved (172).

Horizontal transfer of DNA between organisms has been proposed to be the driving force in the formation of gene clusters in bacteria, and similar reasoning could explain gene clusters in fungi as well. According to the "selfish operon"

hypothesis (116), horizontal transfer accelerates gene clustering because genetic rearrangements that bring genes with cooperating products (say, the genes for enzymes of a single metabolic pathway) closer together, increase the likelihood that the genes will be co-mobilized. If the cooperating genes are conditionally dispensable but have adaptive value for colonizing certain ecological niches, the incipient cluster can be maintained by positive selection in those environments (114). The model further proposes that following horizontal transfer, introgressed DNA containing the loosely clustered genes will be foreign to the host and thus will not be essential for the growth of the recipient cell. This DNA, between the cooperating genes with adaptive significance, can be subject to spontaneous deletion, ultimately bringing the loosely clustered genes into closer proximity. The authors suggest not only that HGT may result in formation of gene clusters but that the presence of gene clusters indeed may demonstrate the existence of HGT.

More tangible evidence for horizontal transfer of gene clusters in fungi also has been presented. The genes involved in the biosynthesis of the β-lactam antibiotic penicillin are clustered in the filamentous fungi *A. nidulans*, *Penicillium chrysogenum*, *Penicillium notatum*, and *Penicillium nalgiovense*, as are the genes for the related β-lactam cephalosporin in *Acremonium chrysogenum* (66). Similar genes for penicillin and cepahalosporin biosynthesis are found in both *Streptomyces* spp. and gram-negative eubacteria, and these genes are much more closely related than would be anticipated based on sequence divergence at other loci. *pcbC*, a gene common to both the penicillin and cephalosporin biosynthetic pathways, encodes isopenicillin-N-synthase, the enzyme responsible for creating the β-lactam ring of both antibiotics. Amino acid and DNA sequences between *pcbC* genes from fungi and bacteria are similar (Figure 2). For example, the gene from the fungus *Penicillium chrysogenum* shares 57.7% amino acid identity with *pcbC* from the gram-negative *Flavobacterium* and 57.1% amino acid identity with *pcbC* from the gram-positive *Streptomyces griseus* (2). While these high levels of sequence similarity are impressive, Smith et al (155) argue that HGT need not be invoked to explain the similarity. By rooting sequences using a presumptive parologous gene in the cephalosporin pathway, phylogenetic trees based on the *pcbC* sequence data are not inconsistent with vertical transmission.

Nevertheless, vertical transmission does not explain several other features more compatible with horizontal transfer of penicillin biosynthetic genes, likely from gram-positive bacteria to fungi. A strong case for horizontal transfer has been made based on inconsistencies in branch lengths, rather than branching order, of phylogenetic trees (17). The authors point out that although the gene tree for *pcbC* is consistent in topology to the presumed species tree (i.e. consistent with normal vertical transmission), that consistency in itself does not rule out HGT. They then use a maximum-likelihood approach to resolve two major inconsistencies among the gene sequences: (*a*) the high relative similarity between gram-positive bacteria and fungal *pcbC* sequences and (*b*) conversely, the high relative distance between the gene sequences from *Aspergillus* and *Penicillium* and those from other fungi. The best model to resolve these inconsistencies involves HGT of

pcbC from gram-positive bacteria to fungi and then rapid DNA sequence change of the genes in the lineage leading to *Aspergillus* and *Penicillium*. While this model favors an explanation involving HGT, several other observations are also consistent with this interpretation. First, there are a very limited number and variety of penicillin-producing fungal species. If penicillin biosynthetic genes were derived by vertical transmission from a common ancestor of bacteria and fungi, the distribution of extant penicillin producers would be unlikely to be so limited and clustered among similar fungi. Similarly, the range of biosynthetically related β-lactams produced by bacteria is much greater than that produced by fungi (2). Nearly the entire range of β-lactams produced by fungi could be explained by the horizontal transfer of one known gene cluster for the shared steps of the penicillin and cephalosporin pathways and a second known cluster for steps specific for cephalosporin biosynthesis.

The mechanisms by which DNA could potentially be mobilized for HGT can be hypothesized based on the study of the penicillin biosynthetic cluster in *P. chrysogenum*. The DNA sequence for the cluster has been determined for several naturally occurring and mutant strains of the fungus that differ in their relative ability to produce penicillin. While strains of the fungus producing normal levels of penicillin contain a single copy of the biosynthetic gene cluster, strains that overproduce penicillin may contain multiple copies of the genes as an amplified 58- or 107-kb unit (53). Remarkably, the borders of the different-sized, tandemly repeated units are flanked by the same hexanucleotide repeat (TTTACA). The penicillin biosynthetic unit is deleted from mutants of the fungus that do not produce penicillin (54) with either TTTACA, TGTAAA (its reverse complement), or TGTAAT sequences at the deletion borders (66). Genetic instability by way of amplification or excision of the cluster may be explained by recombination specific for these sequences (53). Looping out of the biosynthetic unit conceivably could be the beginning of a process leading to transfer of the genes in their extrachromosomal, and plausibly more mobile, state much as the circular extrachromosomal copies of transposons may lead to their ultimate transfer (94).

A number of genes, functionally related to pathogenicity, also are clustered in phytopathogenic fungi. Among *Fusarium* species, genes for biosynthesis of plant growth regulators, gibberellins (165), mycotoxin and protein synthesis inhibitory trichothecenes (77), and genes involved in pathogenicity to pea (69, 169) are all clustered, as are genes for host-selective toxin biosynthesis in *Alternaria alternata* (159), *Cochliobolus carbonum* (3, 145), and *C. heterostrophus* (176). These toxin biosynthetic clusters are distinctive because, like the pea pathogenicity genes of *N. haematococca*, the chromosomal region containing the clusters appears to be entirely absent in nonpathogenic strains within these fungal species. This discontinuous distribution of the clusters and other features such as distinctive codon usage and GC content along with the presence of transposable elements have all been cited as indications of horizontal transfer (140, 169, 176)

Further evidence of HGT may be implied for pathogenicity genes based on knowledge of population characteristics of the pathogen. While the molecular

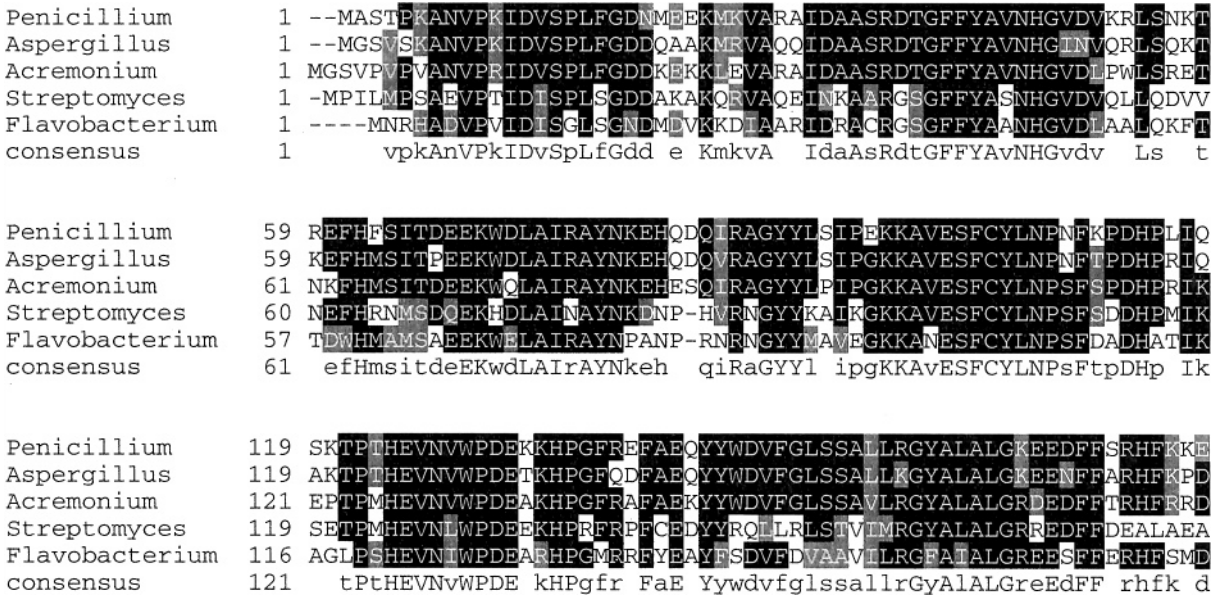

Figure 2 Clustal W alignment of the *pcbC* genes from *Penicillium* (accession number X17436), *Aspergillus* (A27355), *Acremonium* (M33522), *Streptomyces* (M36687), and *Flavobacterium* (P16020) and a consensus sequence illustrating the remarkable amino acid sequence conservation among these genes from distantly related microbes. Amino acid identities are highlighted in black and similarities in gray.

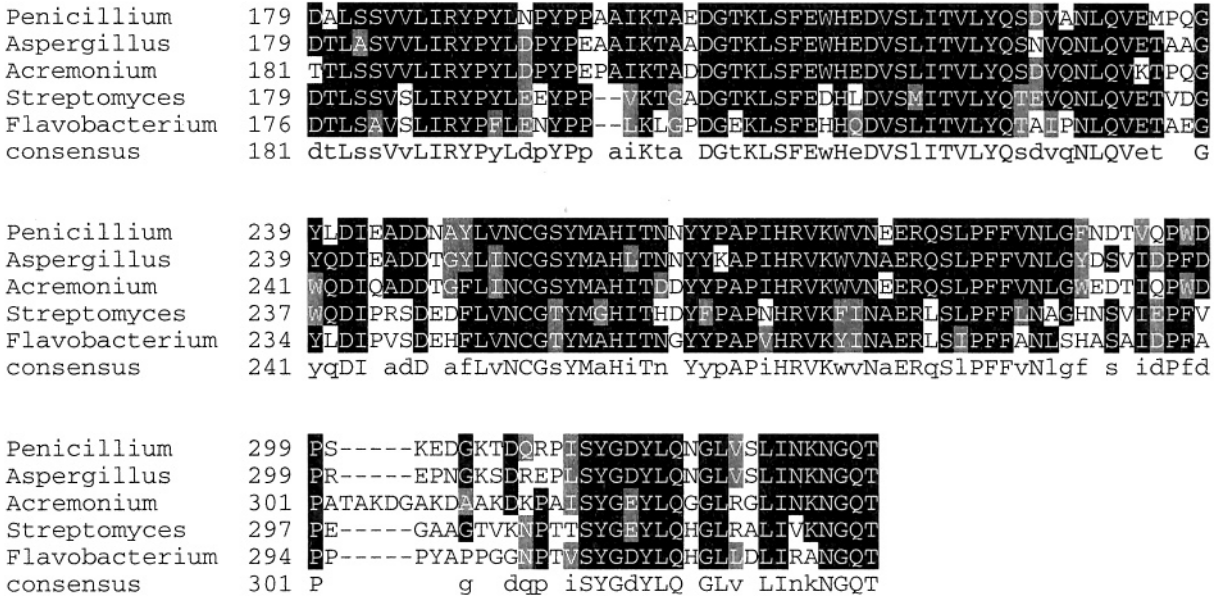

Figure 2 (*Continued*)

genetic basis for toxin production is unknown for the oat Victoria blight pathogen, *Cochliobolus victoriae*, study of mating-type genes suggests HGT has been involved in its evolution (27). The pathogenic species *C. victoriae* (though it is interfertile with the northern corn leaf spot pathogen, *C. carbonum*, in the laboratory) has been characterized based on the ability to produce a host-selective toxin known as victorin and to cause disease on oat with the dominant *Vb* allele (153). The toxic property of victorin is also selectively active toward oat carrying the *Vb* allele, making the acquisition of toxin production sufficient to allow the fungus to cause disease on *Vb* genotypes of the new host species. The sudden onset of the Victoria blight epidemic of the 1940s could be explained by the horizontal acquisition of the genes for biosynthesis of the victorin by a strain of *C. carbonum* weakly virulent on corn and the epidemic expansion of this strain on oats. Consistent with this proposal is the fact that all known strains of *C. victoriae* have a single mating-type gene, even though they have the potential to cross with *C. carbonum* (27). Thus, HGT could explain the epidemic emergence of new diseases and new host specificities in some instances, although the widespread planting of genetically uniform and susceptible plant material also plays a significant role.

The clustering and horizontal transfer of pathogenicity genes in bacteria is well documented. Pathogenicity islands are clusters of genes related to pathogenicity that are likely horizontally transmitted (67), and further defined by their genetic instability, discontinuous distribution within a bacterial species, dissimilarity to other regions of the chromosome based on GC content, and correlation with mobile genetic elements and affiliated repeated sequences. The structural and functional analogy between gene clusters in phytopathogenic fungi and pathogenicity islands, however, may be strained (172). For pathogenicity islands, horizontal transfer has been well documented and known mechanisms for HGT (e.g. bacteriophage transfer) have been described. For fungal gene clusters, HGT is still conjectural, and the mechanisms by which it could occur are only now being explored. Additionally, some gene clusters appear more complex than originally thought and may not be easily explained by a single HGT event (108, 144). DNA sequence comparison of clustered pathogenicity genes in different fungal species will be needed to address directly whether they have been acquired by HGT. One gene cluster where this may be addressed is the pea pathogenicity (PEP) locus, initially described for the pea root rot pathogen *N. haematococca*. Several genes of the gene cluster are also found in the pea wilt fungus *F. oxysporum* f. sp. *pisi*, but are absent in strains of *N. haematococca* and *F. oxysporum* that are nonpathogenic to pea (106, 131).

While gene clusters may facilitate HGT and may have evolved for the "selfish" reason of being more likely transferred than nonclustered genes, such clusters would require characteristics to maintain their integrity or the cluster would dissipate subsequent to transfer. Some clusters do show evidence for secondary dispersal within the resident genome [for example (3, 108)]. Other clusters, while showing evidence for mobility in the shuffling of gene order and direction of transcription, still remain as clusters, suggesting that clustering itself may have selective value, perhaps by shared regulatory elements (29). Some gene clusters

are developmentally regulated (139, 164) and the regulatory properties may be dependent, at least in part, upon their position within the cluster (134). Selection for appropriate gene expression resulting from position in the cluster may thus assure cluster integrity subsequent to a hypothesized HGT.

Whole Chromosomes

One of the most extensive and best characterized HGTs in filamentous fungi involves the selective transfer of an entire chromosome between two otherwise genetically isolated lineages of *Colletotrichum gloeosporioides*. Manners and coworkers first noted the possibility of selective chromosome transfer among naturally occurring A and B biotypes of this fungal plant pathogen (128). A and B biotypes are vegetatively incompatible, distinct in morphology and pathogenic phenotype, and contain numerous strain-specific dsRNAs, repetitive DNA elements, DNA polymorphisms, and distinctive electrophoretic karyotypes, indicating little or no recent history of genome-wide recombination (125, 127, 129). However, while a subset of B-biotype strains contains an optional 1.2-Mb chromosome that is clearly related to a supernumerary 2.0-Mb chromosome from the A biotype (129), an even more limited number of B strains contains both a 1.2-Mb and 2.0-Mb supernumerary chromosome. The 2.0-Mb chromosome in the A biotype and the corresponding chromosome in the small number of B-biotype strains are indistinguishable based on the presence of numerous chromosome-specific sequences and lack of repetitive elements found on all other B-biotype chromosomes. These observations have led to the deduction that the 2.0-Mb chromosome may be a recent transfer from the A biotype to a limited number of B-biotype strains (128).

Bolstering this conclusion is experimental evidence showing that the 2.0-Mb chromosome can be selectively transferred from the A biotype to a vegetatively incompatible B strain (73). The 2.0-Mb chromosome tagged with a hygromycin resistance gene was transferred to a B-biotype genetic background at a frequency of 10^{-7} in mixed cultures. No evidence was found for transfer of genetic material other than that of the 2.0-Mb chromosome. Similarly tagged A-biotype chromosomes >2.0 Mb in size were not detectably transferred by this same method. These experimental findings indicate that the 2.0-Mb chromosome is capable of horizontal transfer in the laboratory and that this HGT likely also has occurred in nature. The selective nature of chromosome transfer is still unresolved. The 2.0-Mb chromosome may encode genes that allow for the transfer and stable maintenance of itself in a different genetic background. However, a small (10 kb) autonomously replicating linear plasmid of foreign origin with no known genetic transfer capability was also shown to be transferred between the A and B biotypes at a similar frequency (147). So while the genetic basis and mechanism by which transfer takes place are unknown, selective horizontal chromosome transfer does occur and represents a hitherto unrecognized mode of genetic introgression in filamentous fungi. HGT of whole chromosomes may explain in part the abundant existence of supernumerary chromosomes in fungi.

Supernumerary chromosomes are present in some but not all members of a fungal species and often contain DNA not found in other portions of the genome (33). Certain features of supernumerary chromosomes suggest they may have originated by way of HGT. For example, they may contain middle repetitive sequences (often transposable elements) that are restricted to or especially prevalent on supernumerary chromosomes (47, 100) and conversely, they may lack repetitive elements found on all other chromosomes (2, 128, 129). The implication is that these sets of chromosomes may have different evolutionary sources; the supernumerary chromosome arriving with transposons from the source genome that have not yet been displaced to the newly resident genome and repetitive sequences on the resident genome that have not yet made their way to the more recently acquired supernumerary chromosome.

CONCLUSIONS AND PERSPECTIVES

Although horizontal gene transfer in fungi has been widely proposed, it remains difficult to prove beyond reasonable doubt. First, equally satisfactory alternative explanations often exist to resolve discrepancies between the phylogeny of a genetic element in question and a host genome (Figure 1). Second, since little is known about potential mechanisms by which HGT may occur for fungi, prudence has weighed against its acceptance to explain many potential instances of its occurrence. Also weighing against general acceptance of HGT is a lack of knowledge of the ecology of fungi that could explain potential environmental interactions that could lead to HGT.

HGT: Means, Motive and Opportunity

When considering suggestions of HGT, it may be useful to consider more than a single line of evidence to argue for or against its role. What, if any, are the "means, motive and opportunity" for HGT to occur?

The "means" or mechanisms by which HGT may occur, as previously stated, have not been elucidated. For example, fungi have no known infectious viruses analogous to transducing bacteriophage that transport foreign DNA from one individual to another in prokaryotes. However, several experimental systems point to potential ways in which DNA could move between fungi or between fungi and other organisms. Gene transfer through mechanisms similar to DNA transformation appears to take place in culture or in natural settings, as evidenced by transfer of genes and plasmids (76, 90). While experimentally contrived by use of genetically engineered drug-resistance genes, *Agrobacterium*-mediated transfer of DNA to fungi (40) indicates that a type IV secretion system is capable of efficient delivery of foreign DNA to fungal cells. Novel mechanisms for DNA transfer also may exist for fungi. Currently, no known mechanism can explain the selective chromosome transfer noted between biotypes of *C.*

gloeosporioides (73). This question, however, now can be addressed in an experimental system. In fact, fungi have great promise as model systems for studying mechanisms of HGT in eukaryotes since they are easily grown in culture in numbers large enough to detect processes that function at a very low frequency.

The "motive" for HGT in fungi also may be difficult to ascertain. Transferred elements presumably could be transmitted based solely on their proficiency for transfer. Even elements that might be imagined to have a selective potential, such as the penicillin biosynthetic cluster, may have arisen largely by neutral processes such as those described for the selfish operon hypothesis. Although it may not always be useful to assign, post hoc, a "motive" for HGT, certain biological explanations can reinforce reasoning otherwise based solely on comparison of gene genealogies or other indirect methods. For example, the hydrolytic enzymes of anaerobic rumen fungi give a dramatic picture of HGT; BLAST searches of these genes retrieve the most closely related sequences from other rumen organisms, regardless of phylogenetic affinity, including genes from bacteria and ciliates. Weaker similarity is exhibited toward enzymes from aerobic fungi. Since the anaerobic fungi lack mitochondria, they must depend entirely upon glycolysis for energy generation. Enzymes involved in glycolysis and those that feed into glycolysis such as the glucan hydrolases thus are key to living in this peculiar milieu, and enzymes finely adapted to function there may have strong selective value in such a bioreactor-type environment. This may be a strong "motive" for stable HGT.

The shared habitat of the rumen also provides the "opportunity" for intimate and continuous association of microorganisms that might be a prerequisite for HGT. If continuous, prolonged physical contact of organisms is indeed conducive for HGT, then the rumen, microbial symbioses, and host-parasite interactions could all provide this environment quite well. Likewise, the fungal life history, its absorptive nutrition, and its ability to generate abundant surface area in contact with other organisms, may provide a greater opportunity for HGT to occur than for other eukaryotes.

The future of research of HGT will rely on continued sequence analysis to discover additional aberrant DNA-based phylogenies. Undoubtedly, as more large-scale genomic sequence data become available, genome-wide comparisons will help to make judgments concerning the extent to which HGT has shaped the genetic profile of extant fungi. But the most persuasive arguments for HGT will begin to incorporate data from other aspects of the overall biology, ecology, and natural history of fungi to make a case.

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