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# Sex in *Penicillium*: Combined phylogenetic and experimental approaches

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## ABSTRACT

We studied the mode of reproduction and its evolution in the fungal subgenus *Penicillium Biverticillium* using phylogenetic and experimental approaches. We sequenced mating type (MAT) genes and nuclear DNA fragments in sexual and putatively asexual species. Examination of the concordance between individual trees supported the recognition of the morphological species. MAT genes were detected in two putatively asexual species and were found to evolve mostly under purifying selection, although high substitution rates were detected at some sites in some clades. The first steps of sexual reproduction could be induced under controlled conditions in one of the two species, although no mature cleistothecia were produced. Altogether, these findings suggest that the asexual *Penicillium* species may have lost sex only very recently and/or that the MAT genes are involved in other functions. An ancestral state reconstruction analysis indicated several events of putative sex loss in the genus. Alternatively, it is possible that the supposedly asexual *Penicillium* species may have retained a cryptic sexual stage.

## 1. Introduction

Despite the costs of sex (Otto and Lenormand, 2002), most eukaryotes engage in sexual recombination at least at some point in their life cycle. The predominance of sexual reproduction suggests that sex must provide some advantages. Although asexual reproduction is common in nature, exclusively asexual taxa are rare and they are considered to be short-lived (Judson and Normark, 1996). Many models have been built to explore short- and long-term advantages of sex to explain its maintenance, but evidence from natural cases is still scarce (but see De Visser and Elena, 2007, for recent experimental evidence for direct benefits of sex). More information is needed on suitable biological models that could be used to tackle these issues.

The empirical study of sex and recombination has been based on vertebrates, insects and plants models, while other groups of eukaryotes, including fungi, have been neglected (Birky, 1999). Biological groups that exhibit a diversity of reproductive strategies provide unique opportunities to study the evolution of sex. Groups such as fungi are thus excellent models as they present a great range of reproductive strategies, including obligatory sexual species, those that alternate sexual and asexual reproduction, and others that appear to be strictly asexual (Taylor et al., 1999). Fungi also appear to have had multiple transitions from sexuality to asexuality (Lobuglio et al., 1993). In Fungi, asexual reproduction by production of asexual propagules (e.g. conidia) has been considered to be particularly common, with a quarter of fungal species thought to reproduce only by asexual means (Taylor et al., 1999). However, recent studies have shown that a great number of fungal "asexual" species are in fact capable of sexual reproduction. Sex in these species is only difficult to observe in nature and challenging to induce in the laboratory. Some species for which sex has not been observed, like *Coccidioides immitis*, present population structures consistent with recombination, suggesting the existence of cryptic sex in nature (Burt et al., 1996). Sex has been successfully induced under controlled conditions in species such as *Candida albicans* (Hull et al., 2000), *Aspergillus fumigatus* (O'Gorman et al., 2009), *A. flavus* and *A. parasiticus*, which were long thought to be asexual (Horn et al., 2009a,b). Finally, apparently functional mating type genes (i.e. genes that define mating compatibility in fungi) have recently been identified and characterized in several species with no sexual cycle described, such as *A. oryzae* (Galagan et al., 2005), and recently in *Penicillium chrysogenum* and *Acremonium chrysogenum* (Hoff et al., 2008; Pöggeler et al., 2008). Other fungal species shown to be truly clonal from a population genetic standpoint, such as *Penicillium marneffei*, also present mating type genes, suggesting that sex has been lost recently (Woo et al., 2006; Fisher, 2007). Alternatively, sexual reproduction that would always occur between identical clones, as allowed under homothallism, would not be distinguishable from strictly asexual reproduction using population genetics.

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Besides the fact that Fungi represent suitable biological models for studying the maintenance of sex and recombination, the study of reproductive strategies in this group has important direct applications. Until very recently, many fungal plant pathogens and some important animal pathogens and species with biotechnological importance were assumed to be clonal with widespread distributions (Taylor et al., 1999). However, recent studies have revealed the existence of recombination in some of these important species (Burt et al., 1996; Couch et al., 2005). Deciphering the mode of reproduction of pathogens may have profound implications for our understanding of the biology and for the management of the species. Recombination should maintain genetic variation within populations and generate new genotypes, which can present new or increased virulence, pathogenicity or drug resistance (Dyer and Paoletti, 2005; Taylor et al., 1999). Furthermore, many of the fungi used in industry are thought to have only asexual reproduction, and the discovery of a sexual stage could help improving strain quality by crosses (Pöggeler, 2001).

Here, we studied the evolution of reproduction in the fungal subgenus *Penicillium Biverticillium* using phylogenetic and experimental approaches. This group includes important species, such as the opportunistic pathogen *P. marneffei*, food and feed spoilers as well as species of importance in the food and biotechnology industries, such as *P. pinophilum* and *P. funiculosum* (Domsch et al., 1980). In the fungal genus *Penicillium* counting ca. 250 species (Pitt, 1979), only few species have a complete life cycle described, the teleomorphs being then *Talaromyces* or *Eupenicillium*. The remaining species are considered as strictly asexual fungi, corresponding to several independent losses of sex (Lobuglio et al., 1993). Asexual species have been classified into four subgenera, *Aspergilloides, Biverticillium, Penicillium* and *Furcatum* on the base of the morphology of their penicilli (Pitt, 1979). The subgenus *Biverticillium* is phylogenetically related to *Talaromyces* (Lobuglio et al., 1993), while the remaining three subgenera are related to *Eupenicillium* (Peterson, 2000) and are close to *Aspergillus* and related teleomorphs (Berbee et al., 1995). The teleomorphs *Talaromyces* and *Eupenicillium* constitute two distinct phylogenetic lineages within the family Trichocomaceae.

We first constructed a robust phylogeny of the fungal subgenus *Penicillium Biverticillium* to (1) improve our knowledge of their relationships, (2) test if the criterion of concordance between multiple gene genealogies supported the extant described morphological species (Dettman et al., 2003; Le Gac et al., 2007), and (3) evaluate the relationships between sexual and asexual species, in order to estimate the number of transitions towards asexuality. We used more strains per species, more genes, and partly different species, than those analyzed previously by Lobuglio et al. (1993). For building phylogenies, we used several genes recently proposed by Aguileta et al. (2008) as having a high phylogenetic performance in fungi, in addition to some genes commonly used for fungal phylogenies. Most of the published fungal phylogenies are indeed based on the same DNA markers, for instance the ribosomal genes or spacers, and genes coding for elongation factor proteins, RNA polymerase and beta tubulin (James et al., 2006; Lobuglio et al., 1993). However, a recent study has shown that these genes may be suboptimal phylogenetic markers, inferring different relationships among species than those supported by most genes in the genomes (Aguileta et al., 2008). We therefore wanted to compare the utility of the genes typically used in fungal phylogenies vs. that of the genes found to have a high phylogenetic performance by Aguileta et al. (2008).

We were also interested in assessing whether the species considered as asexual were in fact so. For this goal, we first tried to detect mating type genes, and sequenced them in sexual and asexual species. Mating-type loci are called "idiomorphs" rather than alleles in ascomycetes due to the uncertainty of the origin by common descent. Most ascomycetes present two idiomorphs MAT 1-1 and MAT 1-2. These genes code for transcription factors that induce the production of pheromones and pheromone receptors. The MAT 1-1 idiomorph includes a gene encoding a protein with a motif called the $\alpha$1 domain, while the MAT 1-2 idiomorph presents a gene encoding a protein with a DNA-binding domain similar to that of the high mobility group (HMG) (Coppin et al., 1997). Homothallic fungi can undergo intra-haploid mating (Giraud et al., 2008), the proximal cause being in most of the filamentous homothallic ascomycetes, each haploid possesses two alternate forms of the MAT locus in its genome (Coppin et al., 1997). In contrast, heterothallic fungi carry a single MAT idiomorph and two strains carrying complementary MAT idiomorphs are required for sex to occur.

Selective pressures acting on mating type genes are expected to be different in sexual vs. asexual species, and these pressures can be detected based on sequences (*i.e.* O'Donnell et al., 2004). In sexually reproducing species, mating type genes must remain functional in order for sex to take place, implying that purifying selection should act (Devier et al., 2009; O'Donnell et al., 2004). On the other hand, in asexual species still carrying mating type genes, mutations driving loss of function should be selectively neutral (Fisher, 2007), leading to relaxed selection. Positive selection may act on MAT genes in sexual species, involving rapid and recurrent changes that proved advantageous (Wik et al., 2008). We therefore looked for footprints of acceleration in substitution rates (indicating relaxed selection or positive selection) on MAT genes. Finally, when species were found to have both MAT 1-1 and MAT 1-2 alleles, we attempted to induce a sexual cycle experimentally.

## 2. Material and methods

### 2.1. Group of study and fungal isolates

Most of the isolates of *Talaromyces* and *Penicillium* used in this study were obtained from the LCP culture collection (Laboratoire Cryptogamie Paris) at the French Natural History Museum (Muséum National d'Histoire Naturelle), Paris. Additional isolates used were donated by the MUCL collection (Mycothèque de l'Université Catholique de Louvain, Louvain La Neuve, Belgium) and by CMPG (Collection Mycologie Pharmacie Grenoble) and are now included in the LCP collection (Supplementary material Table S1).

### 2.2. Phylogenetic marker selection and primer design

We used the FUNYBASE (Marthey et al., 2008) in order to search for single-copy orthologs and estimate their phylogenetic performance at different taxonomic scales within the *Penicillium* genus. We chose genes having an ortholog, and no paralog, in all complete fungal genomes analyzed by Aguileta et al. (2008), having a high phylogenetic performance (*i.e.* topological score higher than 91, see Aguileta et al., 2008), and having different levels of divergence among species. The exact functions of these genes are uncertain and their putative functions were inferred by BLAST analyses. We therefore only used their abbreviated names as in Aguileta et al. (2008) (for further details on the genes and their annotations see Marthey et al., 2008). Three genes were chosen: MS277 (putative ribosome biogenesis protein), MS456 (putative DNA replication licensing factor), and FG610 (putative chaperonin complex component TCP-1). MS277 and MS456 were the two sole genes found to yield the same topology as whole-genome sequences by Aguileta et al. (2008). Using FUNYBASE, the protein sequences of the chosen genes were downloaded from *A. fumigatus*, as this species was one of the closest to the group *Penicillium*/*Talaromyces* available in the database and could therefore be used as an outgroup. To obtain the