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Diversity and mechanisms of genomic adaptation in *Penicillium*

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Abstract

Penicillium **is a diverse fungal genus with hundreds of species occurring worldwide in various substrates, from soil to food, and with various lifestyles, from necrotrophic pathogenicity to endophytic mutualism. Several species are important for human affairs, being widely used in industry, such as the penicillin-producer *P. rubens*, the two cheese starters *P. camemberti* and *P. roqueforti*, and the mold used for fermenting sausages, *P. nalgiovense*. Other species are food spoilers that produce harmful mycotoxins or cause damages in fruit crops. Currently, 30 genomes of *Penicillium* belonging to 18 species are available. In this chapter, we reconstruct a phylogenetic tree based on available *Penicillium* genomes and outline the main features of the genomes, such as gene and transposable element content. We then review the recent advances that the available genomic and transcriptomic resources in the *Penicillium* genus have allowed regarding our understanding of the genomic processes of adaptation, including changes in gene content, expression and strikingly frequent and recent horizontal gene transfers. In addition, we summarize recent studies using genetic markers on the level of genetic diversity, mode of reproduction and population structure within *Penicillium* species. Overall, the *Penicillium* genus appears highly suitable models for studying the mechanisms of adaptation.**

Introduction

Penicillium is a diverse fungal genus with 354 accepted species today (Visagie et al., 2014b), occurring worldwide in various substrates, from soil to food. Their lifestyles also cover a broad range, from necrotrophic pathogenicity to endophytic mutualism, although most are saprotrophs. As a consequence of these ecological niches, many *Penicillium* species have important economic and social relevance for human populations. Several species are widely used in industry, such as the penicillin-producer *P. rubens*, the two cheese starters *P. camemberti* and *P. roqueforti*, and the mold used for fermenting sausages, *P. nalgiovense* (Bernáldez et al., 2013). Other species cause damages and yield loss in fruit crops, *e.g.*, *P. digitatum* and *P. italicum*, while others are a concern for food safety because of their production of mycotoxins, such as patulin (Eckert and Eaks, 1989; McCallum et al., 2002).

In addition to their economic significance, *Penicillium* molds also provide a tractable model for understanding the genetic and genomic processes underlying adaptation, due to the diversity of their ecological niches, their small genomes, their long haploid phase, their short generation time, and their easy manipulations in laboratory. Therefore, they can help addressing the current key challenges in evolutionary biology, including the identification of the genes involved in ecologically relevant traits as well as the understanding of the nature, time course, and architecture of the genomic changes involved in the origin and processes of population adaptation and divergence (Gladieux et al., 2014).

The *Penicillium* species used in industry (*e.g.*, for cheese maturation, sausage fermentation or for penicillin production) represent particularly well-suited organisms for studying domestication, a selection-based process studied since the dawn of evolutionary thinking as a model of rapid adaptation and diversification (Darwin, 1868). Yet, the process of domestication has been much less studied in eukaryote microorganisms than in plants or animals. Several traits are typically modified in domesticated fungi, such as color of colonies, growth rate, thallus density, length of conidiophores, rate and rapidity of spore germination (Eichler, 1968; Moreau, 1979). In the baker's yeast *Saccharomyces cerevisiae*, the genomic processes associated with domestication include large-scale duplications leading to genome size expansions (Liti and Louis, 2005; Machida et al., 2005), the acquisition of new traits by horizontal gene transfers (HGT) (Hall et al., 2005; Khaldi and Wolfe, 2008; Khaldi et al., 2008; Novo et al., 2009) and hybridization (Liti et al., 2006). These mechanisms have been suggested

as ways for fungi to increase their biochemical repertoire and their ability to adapt to new ecological niches, but its generality remains to be assessed (Friesen et al., 2006; Khaldi and Wolfe, 2008; Khaldi et al., 2008; Rosewich and Kistler, 2000; Wisecaver et al., 2014).

In this chapter, we aimed at summarizing the insights that the available genomic resources in the *Penicillium* genus have contributed to our understanding of the genomic processes of adaptation. In addition, we review recent studies using genetic markers on the level of genetic diversity, mode of reproduction and population structure within *Penicillium* species. We start with a section describing the *Penicillium* species for which genomes are available, with special emphasis on their ecological niches and life history traits. Second, we reconstruct a phylogenetic tree based on available *Penicillium* genomes and outline the main features of the genomes, such as gene and transposable element content. Third, we summarize recent findings on strikingly frequent and recent horizontal gene transfers. Fourth, we review some of the recent transcriptomic studies performed in *Penicillium* fungi. We finally review recent investigations on genetic diversity and population structure within *Penicillium* species.

Ecological niches and life history traits

Currently, 30 genomes of *Penicillium* belonging to 18 species are available in public databases (Table 1); these species are necrotrophic plant pathogens (*P. digitatum, P. expansum, P. italicum*), common food spoilers (*P. biforme*, *P. fuscoglaucum, P. carneum, P. paneum*), or key industrial species for food production (*P. camemberti, P. roqueforti, P. nalgiovense*), pharmaceutic industry (*P. rubens*) or biorefinery *(P. decumbens*). The genome described as *P. aurantiogriseum* in the databases (Yang et al., 2014) most likely belongs to *P. expansum* (Ballester et al., 2015). We detail below some of the specific traits and lifestyles of the *Penicillium* species with a sequenced genome.

The two species used for cheese production, *P. camemberti* and *P. roqueforti*, though sharing the same nutrient-rich ecological niche, are not closely related and have different domestication histories, thus providing ideal models to study parallel adaptation (Elmer and Meyer, 2011). The fungus *P. camemberti*, used for the maturation of soft cheeses like Brie and Camembert, is the result of selection programs aiming at improving the texture of the colony, the color of the conidia and physiological characteristics of mycelia. This human-created white species is thought to be derived from a single clone of *P. commune* (Pitt et al., 1986), a species complex split into *P. biforme* and *P. fuscoglaucum* (Giraud et al., 2010). *Penicillium camemberti* has never been isolated from other substrates than dairy products, in contrast to *P. fuscoglaucum* and *P. biforme*, considered as contaminants by stakeholders because of their

blue-grey color (Frisvad and Samson, 2004). *Penicillium roqueforti,* used as a starter culture in the production of blue veined-cheeses, is in contrast widespread in food environments and has also been isolated from plant environments such as silage or wood (Frisvad and Samson, 2004).

Penicillium carneum and *P. paneum*, two sister species of *P. roqueforti*, are common food spoilers responsible for the production of harmful mycotoxins (O'Brien et al., 2006; Petersson and Schnürer, 1999). *Penicillium nalgiovense* is used in the food industry as an inoculum on fermented dry sausages in Italy, Spain and France (Bernáldez et al., 2013). It contributes to the taste of sausages and it helps preventing desiccation and protecting them from undesirable microorganisms through antibacterial and antifungal activities (Lücke, 1997; Lücke and Hechelmann, 1987).

The discovery by Alexander Fleming and subsequent mass production of β-lactam antibiotics (including penicillin) have revolutionized medicine and greatly contributed to reduce the mortality due to infectious bacterial diseases in the world (Fleming, 1929; Hersbach et al., 1984). The production of antibiotics in large amounts has been made possible through strain improvement in *P. rubens*. Industrial strains are all derived from a single strain, NRRL1951, isolated from a spoiled cantaloupe during World War II (Raper et al., 1944). Until recently, *P. rubens* was considered as a synonym of *P. chrysogenum,* but it is now accepted as a closely related but separated species (Houbraken et al., 2011). *Penicillium solitum* is a common food spoiler (in cheeses and dry meats such as sausages and salami), a pomaceous fruit pathogen (Frisvad, 1981; Pitt and Leistner, 1991; Sanderson and Spotts, 1995) and it has also been isolated from apple orchards' soil and house dust (Frisvad and Samson, 2004; Papagianni et al., 2007; Visagie et al., 2014a). It is also used for production of compactin,a cholesterol-lowering agent with also an antifungal effect (Frisvad and Samson, 2004).

Penicillium digitatum is a necrotrophic pathogen responsible for up to 90% post-harvest losses in citrus storage, particularly in arid and sub-tropical climates (Eckert and Eaks, 1989). In contrast to most other necrotrophic fungi, *P. digitatum* seems highly specialized as it has never been collected in any other substrate than citrus (Barkai-Golan, 2001). It is therefore a model for the study of specialization in the necrotrophic lifestyle. *Penicillium italicum* presents a very similar lifestyle on the same substrate (Palou, 2014).

Penicillium expansum is famous for being the first described *Penicillium* species; it is an important post-harvest spoilage agent that can cause great losses in apple storage facilities (Jurick et al., 2011). In contrast to *P. digitatum*, *P. expansum* can be found in a wide array of other substrates and is a concern for health care as it can produce patulin, a highly toxic

mycotoxin (McCallum et al., 2002). The role of patulin in the capacity to colonize fruits and diverse ecological niches remains debated (Ballester et al., 2015).

Penicillium capsulatum and *P. decumbens* both produce secondary metabolites useful for the industry, in particular highly efficient enzymes for degrading cellulose; *P. decumbens* is also used in biorefinery as a renewable source for oil production (Li et al., 2010). *Penicillium verrucosum* and *P. nordicum* are both known for producing ochratoxin A, one of the most common mycotoxins in spoiled food (Castella et al., 2002). *Penicillium paxilli* is the species which the potent tremor-inducing blocker of calcium-activated potassium channels paxillin was originally isolated from (Berry et al., 2015).

Phylogenetic relationships, genome size and content, and changes in gene content

Despite the economically and ecologically important role of *Penicillium* fungi, their phylogenetic relationships still remain unclear, with only a few phylogenetic trees published, based on single genes (Houbraken and Samson, 2011; Samson et al., 2004; Seifert et al., 2007). We therefore constructed a phylogenetic tree, including all *Penicillium* strains with an available sequenced genome and four *Aspergillus* species as outgroups, based on 3,986 shared single-copy orthologs, corresponding to a concatenated alignment of 1,198,500 bp (Figure 1). In the *Penicillium* clade, some internal nodes remained poorly supported; this may reflect a rapid radiation in this clade, leading to incomplete lineage sorting, and/or hybridizations or recurrent horizontal gene transfers (Cheeseman et al., 2014; Ropars et al., 2015).

Genomes in the *Penicillium* genus appear highly dynamic, with estimated genome sizes ranging from 25 Mb to 36 Mb (Table 1). A relationship between host range and genome size has been proposed for fruit pathogens (Ballester et al., 2015; Marcet-Houben et al., 2012). For example, *P. digitatum*, which presents the smallest genome (25.7Mb in average), is only able to infect citrus fruits, whereas the generalist *P. expansum* (pathogen of pome and stone fruits) has the largest genome among fruit pathogens (ca 31 Mb); *P. italicum* presents intermediate host range (mainly citrus fruits) and genome size (ca 29 Mb) (Table 1).

We analysed the repeat content of all available genome using RepeatMasker (Smit et al., 2013). Overall, *Penicillium* genomes have a low proportion of interspersed repeats, ranging from 0.32 to 1.71% of total genome assembly lengths, and yet all classes of transposable elements (TEs) are represented (Figure 2). The 10 most abundant types of TEs include four non-LTR *i* retroelements (I-1_AO, I-4_AO, I-5_AO and I-6_AO), three *mariner* DNA transposons (Mariner-6_AN, MarinerL-1_AO and Mariner-1_AF), one *hAT* DNA transposon

(hAT-1_AN), one *gypsy* LTR retrotransposon (Gypsy1-I_AO) and one *R1* non-LTR retroelement (RTAg4). *Gypsy* elements are by far the most abundant TE class found in *Penicillium* genomes as they account for 20% of all TEs; *mariner* elements constitute the second most abundant class (13%).

Penicillium biforme, P. camemberti and *P. fuscoglaucum,* three closely related species occurring in cheese, show the largest genome sizes among available *Penicillium* genomes (ca. 35-36 Mb, Table 1), which is not associated with a particularly high TE content (Figure 2). Interestingly, a large expansion of the proteome seems to have occurred in the ancestor of this clade, indicating that the increase in genome size might be correlated with the acquisition of new genes (Rodríguez de la Vega et al., 2015). In contrast, *P. digitatum* is characterized by a much lower number of genes than other *Penicillium* lineages (Marcet-Houben et al., 2012; Ropars et al., 2015), which probably relates to its highly specialized necrotrophic lifestyle.

Comparative genomic studies have revealed interesting patterns of gains or losses in genes involved in the production of secondary metabolites, such as penicillin, patulin or small secreted proteins acting as effectors in pathogens. *Penicillium rubens* for instance has acquired its capacity to produce high quantities of penicillin through the duplication of its penicillin biosynthetic gene cluster (Fierro et al., 1995) A comparison of two industrial *P. rubens* genomes, one of a high-penicillin producing strain and one of a low-penicillin producing strain, revealed in the high-penicillin producing strain an even higher number of copies of the penicillin biosynthetic gene cluster, as well as many genomic structural variations, such as translocations and gene gains/losses, likely related to an enhanced nitrogen and energy metabolism (Wang et al., 2014). In the necrotrophic species *P. expansum,* a large number of secondary metabolism gene clusters were identified that were absent in other sequenced *Penicillium* genomes and may be involved in pathogenicity (Ballester et al., 2015). Actually, despite a major genome contraction compared with other *Penicillium* species, *P. expansum* had the largest repertoire of secondary metabolites genes, indicating high numbers of gene gains and losses in this species. The patulin gene cluster was inferred to be present in the ancestor of *P. expansum* and *P. roqueforti,* its absence in other lineages of this clade implying gene losses (Ballester et al., 2015). Overall, *Penicillium* genomes thus appear highly dynamic, with changes in gene content relating to genomic adaptations.

Horizontal gene transfers

The multiple available *Penicillium* genomes have further allowed detecting dozens of horizontal gene transfers (HGTs) that are transmissions of genetic material between species by other means than sexual reproduction. HGT events are most often detected by the existence of incongruences between gene genealogies and the species tree. Indeed, the finding of orthologs from distantly related species placed close together in a gene tree most likely indicates that this gene has recently been horizontally transferred between the two species instead of having followed vertical inheritance and divergence along the species tree. Despite long thought to be rare in eukaryotes, recent studies have shown that HGTs may play a major role in adaptation in this lineage, in particular in fungi (Gladieux et al., 2014; Keeling, 2009; Keeling and Palmer, 2008; Wisecaver et al., 2014).

Fungi are the eukaryotic group for which the largest number of HGT events has been described so far. Most of described HGTs have a prokaryotic origin, likely reflecting the abundance of prokaryotes in all environments and the relative ease of detecting such HGT events compared to those from a eukaryotic origin (Gladieux et al., 2014). However, many HGTs between fungi have also been described, such as transfers of genes involved in secondary metabolite pathways in *Aspergillus* and *Penicillium* clades (Wisecaver et al., 2014).

Among *Penicillium* species, the penicillin-producer *P. rubens* may have acquired several important genes from bacteria by horizontal gene transfers, including some of the penicillin biosynthetic genes, *pcbAB* and *pcbC*, and the arsenate-resistance cluster (van den Berg et al., 2008). Genome analysis of the necrotrophic fungus *P. digitatum* revealed four putative genes that have been horizontally acquired from prokaryotes, including DEC1, likely playing a role in pathogenicity (Marcet-Houben et al., 2012); indeed, it belongs to a gene family associated with virulence in maize infections, with homologs in plant pathogenic fungi and in bacteria, but without any homolog in non-pathogenic *Penicillium* species.

Several other horizontal gene transfers in *Penicillium* have occurred in the cheese environment, being striking by 1) the size of the transferred regions (*i.e.*, several kilobases), 2) the eukaryotic origin of these transfers (likely among cheese-associated *Penicillium* species), 3) the number of species in which the same regions have been horizontally transferred, and 4) the very recent date of the transfers, likely associated with the human history of cheese production (Cheeseman et al., 2014; Ropars et al., 2015). These horizontally transferred regions (HTRs) indeed occurred between *Penicillium* species from the cheese environment, and were completely identical at the nucleotide level between distant species (otherwise having a pairwise sequence identity of 85-90%) while lacking in other closely related species. One of

these HTRs, *Wallaby*, is a 575 kb region that accounts for 2% of the *P. roqueforti* genome, and it can be in a single block or in a few fragments depending on the species (Cheeseman et al., 2014). Another HTR, *CheesyTer,* is 80 kb long, and is always found in a single block (Ropars et al., 2015).

These two HTRs are flanked by copies of transposable elements (TEs) belonging to a specific family, the *i* non-LTR retrotransposons, that are rare elsewhere in the genomes (Ropars et al., 2015). This suggests that these TEs may be involved in the horizontal gene transfers. In fungi, the transfer of genetic material is thought to occur through conjugation, natural and agrobacterial transformation, viral transduction, or anastomosis (Coelho et al., 2013; Wisecaver and Rokas, 2015). In *Fusarium*, for example, the transfer of an entire chromosome can occur by simple co-incubation of mycelial of two strains (Ma et al., 2010).

The gene content in *Wallaby* and *CheesyTer* suggests that these transfers may play an important role in the adaptation of these fungi to the cheese environment. Among the 248 genes that *Wallaby* was predicted to contain, two genes, *paf* and Hce2, encoded proteins that may be involved in antagonistic interactions with other microorganisms (Cheeseman et al., 2014). *CheesyTer* carries 37 putative genes, including genes coding for lactose permease and beta-galactosidase, which likely provide advantages in terms of use of the cheese substrate (Ropars et al., 2015). Actually, these two genes were found to be overexpressed in the first days of cheese maturation (Lessard et al., 2014; Ropars et al., 2015).

In *P. roqueforti*, all strains were found to carry either both or none of these two HTRs. The two HTRs were only present in strains found in the dairy environment, while lacking in some strains from cheese and in all the strains isolated from other environments, such as silage or wood (Cheeseman et al., 2014; Ropars et al., 2015). This further indicates an advantage conferred by these two HTRs in cheese.

Experiments of growth and competition on different media have further supported a role of the two HTRs in adaptation to cheese. Indeed, *P. roqueforti* strains carrying the two HTRs showed a significantly higher growth rate on cheese medium and a significantly lower growth rate on minimal medium (Ropars et al., 2015). Furthermore, *P. roqueforti* strains carrying the two HTRs showed a significant competitive advantage, both against *P. roqueforti* strains lacking the HTRs and against other *Penicillium* species also lacking the HTRs. Interestingly, this effect was only significant when strains were grown on cheese medium and not on minimal medium (Ropars et al., 2015).

Transcriptomics

The availability of *Penicillium* genomes have also facilitated transcriptomic studies, *i.e.*, investigations of mRNA expression in different conditions, which allows studying the regulation of genes and therefore detecting important genes involved in the adaptation to particular environments. Transcriptomics in *Penicillium* has focused so far on penicillin production in *P. chrysogenum,* on secondary metabolites production in plant pathogens, such as *P. expansum, P. digitatum* and *P. italicum,* and on cheese-making fungi.

The transcriptome analyses of the pathogenic *P. expansum* on apple revealed the induction of several metabolic pathways during infection and thus identified putative pathogenicity factors, such as proteases, cell-wall degrading enzymes and oxidoreductases (Ballester et al., 2015). Putative effectors that are able to modulate host physiology were also identified (Ballester et al., 2015).

A metatranscriptome analysis of *P. camemberti* and *Geotrichum candidum* was performed in a camembert-type cheese matrix (Lessard et al., 2014). The functional annotation allowed the identification of the biological processes involved in cheese ripening. Globally, similar functions appeared involved in the use of the cheese substrate in both the yeast *G. candidum* and the mold *P. camemberti* (Lessard et al., 2014).

A system biology approach, including transcriptomic but also metabolome and metabolic flux analyses, was used to understand the loss of penicillin production capacity by the high-producing *P. chrysogenum* strain during long-term ethanol-limited cultivation, a phenomenon called degeneration. The findings indicated that degeneration was due to the production of a lower quantity of the first two enzymes acting during penicillin biosynthesis, which may be due to a decrease of translation efficiency (Douma et al., 2011).

Population genetic diversity within *Penicillium* species and mode of reproduction

The genetic diversity has also been investigated within *Penicillium* species in some cases, although only with neutrally-evolving markers so far. The population genetic variability has been found to differ drastically among *Penicillium* species. In *P. camemberti,* no genetic polymorphism could be detected using either DNA fragments or microsatellites. This is consistent with this species being a clonal lineage originating from a white mutant in the cheese-making mold formerly used for making Brie, *P. commune* (Giraud et al., 2010). In contrast, the genetic diversity in *P. roqueforti* was revealed to be substantial using microsatellites (Ropars et al., 2014) and DNA fragment sequences (Gillot et al., 2015). A strong population structure

was found, with one population containing only cheese strains, most of which carried the HTRs described above, *Wallaby* and *CheesyTer*, and a second population containing cheese and non-cheese strains, all lacking the HTRs. These two populations showed further, although weaker, subdivisions that corresponded to different morphologies and different cheese types (Gillot et al., 2015).

To date, genetic diversity has not been investigated within other *Penicillium* species, with the exception of *P. chrysogenum*, which has actually led to the identification of cryptic species and in the subsequent renaming of the penicillin-producing strain in *P. rubens* (Houbraken et al., 2011).

Although sexual stages have been described in many *Penicillium* species (Visagie et al 2014b), others has been historically considered to be exclusively asexual. This actually holds true for one fifth of fungal species (Taylor et al., 1999), but was mainly due to the difficulty of observing sex in this phylum in nature. Indeed, direct or indirect evidence of sex have been observed in most cases when thoroughly investigated. Indirect evidence include 1) the presence of the complete meiotic toolbox (*i.e.*, all the genes known to be necessary for meiosis and for mating-type determinism, with sequences apparently functional, *i.e.*, under purifying selection), 2) footprints of recombination in populations, and 3) footprints of RIP (repeat-induced point-mutation), a defense mechanism of fungal genome inducing C/T transition mutations in repeated sequences during sexual reproduction in ascomycetes (Galagan and Selker, 2004). Recent studies have improved our knowledge of the reproduction mode and breeding system in *Penicillium.* All species studied so far were shown to be heterothallic, as haploid genomes carried a single mating-type allele, either MAT1-1 or MAT1-2 (Hoff et al., 2008; Ropars et al., 2012).

After discoveries of indirect evidence of sex in populations of the penicillin-producer *P. rubens*, with occurrence of both mating-type alleles (Hoff et al., 2008) and of RIP footprints (Braumann et al., 2008), a sexual cycle could be induced in this species (Böhm et al., 2013). Similarly, in the cheese species *P. roqueforti*, mating types were shown to occur in balanced ratios in populations, RIP footprints were observed and purifying selection was inferred on genes involved in mating (Ropars et al., 2012). Later, population analyses showed no linkage disequilibria among markers, suggesting recurrent recombination events and fruiting bodies and recombinant sexual ascospores could be successfully produced *in vitro* (Ropars et al., 2014).

Conclusions and future prospects

Genomic and transcriptomic analyses have revealed several interesting genes and mechanisms likely involved in the adaptation of *Penicillium* species to various environments. In particular, the domesticated penicillin-producing and cheese-making *Penicillium* appear ideal model eukaryotes for studying the genomic processes of adaptation, given the recent and strong selection by humans. These genomic inferences now need to be validated using functional genetics, which will be allowed by the recent development of transformation and gene silencing methods (Durand et al., 1991; Gil-Durán et al., 2015; Goarin et al., 2014; Kosalková et al., 2015; Ullán et al., 2008). It will also be very interesting to explore the population genomics of adaptation within species, in particular in the *Penicillium* fungi with high genetic diversity and a variety of ecological niches, such as *P. roqueforti.* Footprints of selective sweeps for instance may reveal selection having acting recently on other genes that the horizontally-transferred regions.

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Figure Legends

Figure 1: Maximum likelihood tree of genome sequenced *Penicillium* based on concatenated alignment of 3,986 single copy orthologs using RaxML (Stamatakis, 2014). Node labels correspond to the proportion of gene trees supporting the node.

Figure 2: (A) Number and classification of transposable elements found in *Penicillium* genomes; (B) Percentage of the *Penicillium* genomes composed of interspersed repeats.

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