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Adaptive Horizontal Gene Transfers between Multiple Cheese-Associated Fungi

Highlights
- New HTRs are found in cheese fungi
- HTRs are flanked by specific transposable elements
- HTRs have spread in cheese-associated fungi through recent selective sweeps
- Experiments link two HTRs to growth and competitive advantages on cheese

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In Brief
Ropars et al. report newly discovered horizontally transferred regions, flanked by specific transposable elements that allow cheese-making fungi and food spoilers to grow faster and be better competitors on cheese. These findings have industrial and food safety implications and also improve our understanding of adaptation processes.

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Adaptive Horizontal Gene Transfers between Multiple Cheese-Associated Fungi

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SUMMARY

Domestication is an excellent model for studies of adaptation because it involves recent and strong selection on a few, identified traits [1–5]. Few studies have focused on the domestication of fungi, with notable exceptions [6–11], despite their importance to bioindustry [12] and to a general understanding of adaptation in eukaryotes [5]. *Penicillium* fungi are ubiquitous molds among which two distantly related species have been independently selected for cheese making—*P. roqueforti* for blue cheeses like Roquefort and *P. camemberti* for soft cheeses like Camembert. The selected traits include morphology, aromatic profile, lipolytic and proteolytic activities, and ability to grow at low temperatures, in a matrix containing bacterial and fungal competitors [13–15]. By comparing the genomes of ten *Penicillium* species, we show that adaptation to cheese was associated with multiple recent horizontal transfers of large genomic regions carrying crucial metabolic genes. We identified seven horizontally transferred regions (HTRs) spanning more than 10 kb each, flanked by specific transposable elements, and displaying nearly 100% identity between distant *Penicillium* species. Two HTRs carried genes with functions involved in the utilization of cheese nutrients or competition and were found nearly identical in multiple strains and species of cheese-associated *Penicillium* fungi, indicating recent selective sweeps; they were experimentally associated with faster growth and greater competitiveness on cheese and contained genes highly expressed in the early stage of cheese maturation. These findings have industrial and food safety implications and improve our understanding of the processes of adaptation to rapid environmental changes.

RESULTS AND DISCUSSION

Multiple Recent Horizontal Gene Transfers between Distant *Penicillium* Species, Flanked by Specific Retrotransposons

We report here five newly sequenced and assembled *Penicillium* genomes, which we compared with previously available data [16–19]. The full dataset included the genome sequences of ten *Penicillium* species, six of which are either used as industrial inocula for cheese making (*Penicillium roqueforti* and *Penicillium camemberti*) or occur as contaminants in cheeses (Figure 1; Table S1). *P. camemberti* is only found in cheese and is thought to include a single clonal lineage originating from selection programs at the end of the 19th century from the blue-gray cheese molds used at that time, i.e., *Penicillium biforme* and *Penicillium tuscanoglaucum* [20, 21]. By contrast, *P. roqueforti* also occurs in habitats other than cheese, such as silage or wood, and displays substantial genetic diversity [22, 23]. For reconstructions of a rooted phylogeny of these ten *Penicillium* species, we used four *Aspergillus* species as an outgroup. We concatenated alignments of 3,089 single-copy genes shared by at least ten species and reconstructed a fully resolved and well-supported maximum-likelihood phylogeny (Figures 1A and 1B).

We used this rooted phylogeny to investigate the occurrence of horizontal gene transfers (HGTs) between *Penicillium* species. As HGTs (also known as xenology [24]) result in incongruences between gene genealogies and species trees, we compared all individual gene genealogies with the species tree. For this goal, we used the Notung software [25–27] to infer the number of duplication, loss, and HGT events that reconciled the gene genealogies with the species tree. Notung is conservative regarding the inference of HGTs because it tests their temporal feasibility, assumes that HGTs occur with a low probability, and forces the poorly supported nodes to follow the species
tree. Only orthologous groups with at least one homolog in at least eight genomes were analyzed, further rendering our estimates of HGTs a lower bound. We found overall 104 HGTs between Penicillium species, distributed among 77 orthologous groups. Notung inferred the highest number of HGTs relative to branch length in the clade encompassing P. camemberti, P. biforme, and their common ancestor (Figure 1B). P. roqueforti also acquired many xenologs relative to its branch length. 8 of the 21 horizontally acquired genes detected in P. roqueforti were inferred to come from P. camemberti, P. biforme, or their common ancestor, indicating recent transfers from species sharing the same ecological niche (Figure S1). Only five of these eight genes could be assigned putative functions, i.e., a protein kinase, two transcription factors, a cation transporter, and an integrase-like protein. Cation transport seems particularly relevant for growth in cheeses as several ions are limiting in this medium (e.g., iron ions), and pH drastically drops during cheese maturation [28].

Another line of evidence for the abundance of HGTs in Penicillium fungi came from the finding of multiple large genomic islands that were almost 100% identical at the nucleotide level between distant species, while being absent from closely related species (Figure 1A). The only substitutions detected in these genomic islands corresponded to repeat-induced point mutations, i.e., C-to-T transitions induced by a specific fungal defense mechanism against transposable elements (TEs) that can substitute multiple base pairs in a single meiosis [29]. In P. roqueforti, for example, seven genomic islands larger than 10 kb and displaying above 97% nucleotide identity with multiple other species were found. Only the largest region had previously been identified and was called Wallaby [16]. Such a high level of identity suggests that these genomic islands correspond to recent horizontally transferred regions (HTRs), although they could alternatively be recent introgressions. Two lines of evidence, however, support the HTR hypothesis rather than introgression: (1) the presence of several of these regions at non-homologous locations in the different Penicillium genomes (Figure S2; [16]) and (2) the low mean genome sequence identity between the Penicillium species sharing these regions, being less than 90%, an identity level at which no successful interspecific transfers, i.e., C-to-T transitions induced by a specific fungal defense mechanism against transposable elements (TEs) that can substitute multiple base pairs in a single meiosis [29]. In P. roqueforti, for example, seven genomic islands larger than 10 kb and displaying above 97% nucleotide identity with multiple other species were found. Only the largest region had previously been identified and was called Wallaby [16]. Such a high level of identity suggests that these genomic islands correspond to recent horizontally transferred regions (HTRs), although they could alternatively be recent introgressions. Two lines of evidence, however, support the HTR hypothesis rather than introgression: (1) the presence of several of these regions at non-homologous locations in the different Penicillium genomes (Figure S2; [16]) and (2) the low mean genome sequence identity between the Penicillium species sharing these regions, being less than 90%, an identity level at which no successful interspecific transfers can pass across species boundaries [31] and that they promote genomic rearrangements and recombination [32–34]. The capacity for mycelia fusions may also facilitate the exchange of genetic material in fungi [35].

Two Horizontally Transferred Genomic Regions Are Likely Involved in Cheese Adaptation and Have Spread in Cheese-Associated Penicillium through Recent Selective Sweeps

Five of the seven large HTRs detected in P. roqueforti were shared between Penicillium strains isolated from cheese, P. camemberti carrying four of them (Figure 1A). Two HTRs appeared of special relevance for cheese adaptation. Wallaby [16] carries a gene encoding an antifungal protein, known for inhibiting the growth of competitors. The second largest HTR was found at terminal edges of scaffolds and was therefore named CheesyTer. This 80-kb region, found as a single block in all the genomes studied, carried 37 putative genes, among which two had relevant putative functions for adaptation to cheese, i.e., lactose permease and beta-galactosidase (Figure 2A). Lactose
is present during the first few days of cheese maturation, and it acts as a primary carbon source, being rapidly consumed by lactic acid bacteria [37]. The two lactose metabolism genes present in CheesyTer were among the most strongly expressed in *P. camemberti* during the first step of cheese rind maturation, during which lactose is available (Figure 2B; Table S2; Supplemental Experimental Procedures), indicating a role in the use of the cheese substrate.

We investigated the presence of CheesyTer and Wallaby by PCR in 416 strains from 65 fungal species from various environments (Figure 1C; Table S3; Supplemental Experimental Procedures). The presence of the transfers was found highly significantly associated with dairy environment both among species ($\chi^2 = 55.7$; degrees of freedom [df] = 1; p value = 8.571e$^-14$) and among strains within species ($\chi^2 = 45.2$; df = 1, p value = 1.774e$^-11$). Amplicons were actually obtained only for *Penicillium* species that are frequently isolated in the dairy environment, with the only exception of *P. rubens*, the penicillin-producer fungus, in which 16 strains out of 20 carried either one of the two HTRs. The CheesyTer and Wallaby fragments obtained by PCR showed zero substitution among all strains from all species, including synonymous sites and non-coding regions, as previously found for Wallaby [16]. This result confirms that the presence of CheesyTer and Wallaby is not ancestral in *Penicillium* species and that these genomic islands have instead been acquired very recently. This also indicates that the two HTRs have spread in several species through recent selective sweeps. *P. roqueforti* was found polymorphic for the presence of Wallaby and CheesyTer, with all tested strains carrying either both of these regions or neither of them (Table S3). Within *P. roqueforti*, these regions were present only in strains isolated from the cheese environment, suggesting a role in adaptation to the cheese environment.

The Wallaby and CheesyTer Horizontally Transferred Regions Are Experimentally Associated with Faster Growth and Greater Competitiveness on Cheese

We therefore investigated whether strains carrying Wallaby and CheesyTer showed higher fitness in terms of growth on cheese substrate or for competitiveness. We set up three experiments, focusing on *P. roqueforti*, because a large collection of strains was available, isolated from various environments, and including strains carrying both Wallaby and CheesyTer (hereafter named *W+C*+) and strains lacking them (hereafter named *W−C−*).

We first compared the growth of 50 *P. roqueforti* strains on a cheese medium and on a minimal medium (26 *W+C*+ and 24 *W−C−*; Table S4, tab a). Neither the presence of Wallaby and CheesyTer, as a main effect independent of the medium, nor the origin of the strain (i.e., cheese versus other environments) significantly influenced the growth of *P. roqueforti* (Table S1). By contrast, the effect on growth of the medium and its interaction with the presence of the two genomic islands were significant (Figure 3; Table S1): *W+C*+ strains had a growth advantage on cheese medium but a slower growth on minimal medium.

Second, we investigated whether *P. roqueforti* strains carrying Wallaby and CheesyTer had a higher ability to exclude competitors. We measured the growth of three fungal strains belonging to species commonly found in cheese but lacking Wallaby and CheesyTer (*P. nalgiovense* FM193, *P. biforme* LCP05529, and *Geotrichum candidum* FM074) on plates covered with lawns of *P. roqueforti* either *W+C*+ (n = 11) or *W−C−* (n = 12) strains (Table S4, tab b). These experiments were carried out on minimal, cheese, and malt agar media. No difference in growth was detected for the yeast *G. candidum* between lawns of *W+C*+ or *W−C−* *P. roqueforti* strains (Table S1). By contrast, *W+C*+ *P. roqueforti* strains significantly impaired the growth of the two *Penicillium* challengers on the cheese and malt media (Figure 3B). This was not the case on the minimal medium: the interaction between the presence of the transfers and the medium was significant (Table S1). Using the same experimental design, we then investigated the effect of the two genomic islands when present in the challengers. For this goal, we inoculated on *P. roqueforti* lawns (*W+C*, n = 2, or *W−C−*, n = 2), on cheese medium, different strains of species displaying a polymorphism in the Wallaby and/or CheesyTer presence (Table S4, tab c). We used as challengers different strains of *P. camemberti* (*W+C−*, n = 1, or *W+C+*, n = 3), *P. biforme* (*W−C−*, n = 2, or *W+C+*, n = 2), and *P. rubens* (*W−C−*, n = 1, or *W+C−*, n = 3). For all three species, we found that the *P. roqueforti* lawns significantly inhibited the growth of the challengers and significantly more so when the *P. roqueforti* lawn carried Wallaby and CheesyTer. Interestingly, the presence of either CheesyTer or Wallaby in the challengers allowed better growth on *W−C−* *P. roqueforti* lawns while neither had significant effect on the growth on *W+C*+ *P. roqueforti* lawns (Table S1).

Third, we investigated competition among *P. roqueforti* strains carrying (*W+C*, n = 8) or lacking (*W−C−*, n = 11) Wallaby and CheesyTer. We grew *P. roqueforti* strains on cheese medium as pairwise face-to-face confrontations, and we measured the deviations from symmetrical growth (Table S4, tab d; Figure 3C; Supplemental Experimental Procedures). For the *W+C*+ versus *W+C* confrontations, the mean growth deviation from a boundary in the exact middle of the Petri dish was not significantly different from zero (t test, t = 1.5; df = 36; p value = 0.14). Similar results were obtained for the *W−C−* versus *W−C−* confrontations (t test, t = 0.25; df = 70; p value = 0.80). For the *W+C*+ versus *W−C−* confrontations, deviations were measured by...
taking the W+C+ strain as the focal strain; the mean growth deviation was significantly different from zero and positive, the W+C+ strains thus growing farther than the W+C− strain (t test, t = 12.32; df = 90; p value < 0.0001). The mean deviations were significantly higher in the W+C− versus W+C+ confrontations than in the W+C− versus W+C+ or W−C− versus W−C− confrontations (Tukey-Kramer test, p value < 0.0001), while the means between these two latter were not significantly different.

This experiment shows that the competitive advantage of W+C+ strains against W+C− strains also holds within the species P. roqueforti. Altogether, these experimental results strongly support the existence of fitness advantages for the Penicillium strains carrying the horizontally transferred genomic islands, both in the use of cheese substrate and in competition with fungal competitors.

Conclusions
Our present study on domesticated fungi shows how adaptation can occur rapidly in eukaryotes. The two cheese species studied here underwent parallel adaptation to the cheese medium, and this involved the transfers of identical regions across species boundaries. HGT events have been reported in fungi [35, 38, 39], particularly in environments created by humans, in domesticated yeasts and fungal pathogens of crops [10, 40, 41]. The extent and timing of gene transfers and the number of species having received the same HTRs are here particularly striking. Furthermore, we provide experimental evidence of fitness advantages for strains carrying these HTRs on a human-made medium. These findings altogether are potentially useful for guiding modern strain improvement programs. Indeed, together with the protocol for inducing sex in P. roqueforti [22, 42], the identification here of several key candidate genes important for cheese metabolism and competition may allow further selecting interesting traits for cheese industry using the great genetic variability present in P. roqueforti strains without Wallaby or CheesyTer [22]. In addition, our results suggest that caution is required concerning the introduction of genes into microorganisms, as these genes could readily be transferred to other species in the food environment. Indeed, the rapid spread of Wallaby and CheesyTer into many species of the dairy environment, even when occurring only as contaminants, indicates that transgenes may readily cross species boundaries in the food chain. Finally, the findings here of rapid adaptation through frequent horizontal gene transfers among distant species under selection in novel, human-made media contribute to our understanding of the evolutionary genomic mechanisms allowing rapid adaptation to environmental changes in eukaryotes.

Figure 3. Fitness Advantages of P. roqueforti Carrying Wallaby and CheesyTer, for the Use of the Cheese Substrate and for Outgrowing Competitors
(A) Left: pictures of two P. roqueforti strains, with (LCP06166, top) and without (LCP06040, bottom) Wallaby and CheesyTer on minimal medium and cheese medium. Right: mean growth ± SE (in mm) of P. roqueforti strains with and without Wallaby and CheesyTer on the two media.
(B) Left: pictures of a P. biforme challenger (LCP05529, without Wallaby and CheesyTer) on two different P. roqueforti lawns, on cheese, malt agar, and minimal media (bottom: strain LCP06149 with Wallaby and CheesyTer; top: strain LCP05885 without the genomic islands; the first line is a control, i.e., with no P. roqueforti lawn). Right: mean growth ± SE (in mm) of a P. biforme (top) or a P. camemberti (bottom) challenger on P. roqueforti strain lawns with or without Wallaby and CheesyTer.
(C) Growth asymmetry (mean ± SE in mm of deviations from the middle of the Petri dish) in pairwise confrontations of P. roqueforti strains with (W+C+) or without (W+C−) Wallaby and CheesyTer, on cheese medium, for the three types of possible pairs. The A and B letters correspond to significantly different means according to a Tukey-Kramer test. The picture shows examples of confrontations, at left LCP06271 (W+C+) against LCP06157 (W+C−) and at right LCP00148 (W+C+) against LCP06157 (W+C−).
Accession Numbers

The accession numbers for the Penicillium genome sequences reported in this paper are GenBank: HG813601–HG814182 for P. biforme; HG816029–HG818118 for P. carneum; HG814183–HG815135 for P. tuscoflaglum FM041; HG815136–HG815288 and HG815290–HG816004 for P. nalgiovense FM193; and HG813308–HG813531 for P. paneum FM227.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.08.025.

Author Contributions


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