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Fertility depression among cheese-making *Penicillium roqueforti* strains suggests degeneration during domestication

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Genetic differentiation occurs when gene flow is prevented, due to reproductive barriers or asexuality. Investigating the early barriers to gene flow is important for understanding the process of speciation. Here, we therefore investigated reproductive isolation between different genetic clusters of the fungus *Penicillium roqueforti*, used for maturing blue cheeses, and also occurring as food spoiler or in silage. We investigated premating and postmating fertility between and within three genetic clusters (two from cheese and one from other substrates), and we observed sexual structures under scanning electron microscopy. All intercluster types of crosses showed some fertility, suggesting that no intersterility has evolved between domesticated and wild populations despite adaptation to different environments and lack of gene flow. However, much lower fertility was found in crosses within the cheese clusters than within the noncheese cluster, suggesting reduced fertility of cheese strains, which may constitute a barrier to gene flow. Such degeneration may be due to bottlenecks during domestication and/or to the exclusive clonal replication of the strains in industry. This study shows that degeneration has occurred rapidly and independently in two lineages of a domesticated species. Altogether, these results inform on the processes and tempo of degeneration and speciation.

KEY WORDS: **Purifying selection, prezygotic, postzygotic, reproductive isolation, sterility, species criteria, speciation, sex evolution.**

Speciation, the process by which new species arise, is of fundamental interest in evolutionary biology. In sexually reproducing organisms, speciation occurs when gene flow is sufficiently reduced, due to premating or postmating reproductive barriers (Coyne and Orr 2004; Kohn 2005; Giraud et al. 2008). Premating barriers prevent syngamy between individuals whereas postmating barriers act after syngamy, decreasing viability, or fertility. Elucidating what barriers to gene flow evolve in the early stages of divergence and how rapidly, is thus essential for understanding the process of speciation. The type, strength, and tempo of establishment of the early barriers to gene flow are considered to depend on the geographical distribution of incipient species. In sympatry, models predict the evolution of strong and rapid premating intersterility (Coyne and Orr 2004). Intersterility is one of the many possible components of reproductive isolation, representing failure of crosses, either due to inability of initiating crosses or to produce hybrids. In allopatry, extrinsic barriers restrict mating between individuals, so that intersterility barriers are expected to

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arise gradually and slowly as by-products of divergence and to be mostly postmating (Coyne and Orr 2004). Theoretical expectations of strong premating intersterility in sympatry but not in allopatry have been widely validated experimentally in different groups of plants and animals (Coyne and Orr 2004).

Fungi have long been neglected in the study of speciation, despite being tractable models and showing interesting patterns. For example, homobasidiomycetes (mushrooms) show enhanced premating intersterility in sympatry compared with allopatry, whereas ascomycetes (moulds and yeasts) display no or little premating intersterility, both among sympatric and allopatric sibling species (Le Gac and Giraud 2008). This is because ascomycetes mate within their habitat after mycelial growth, so that only individuals adapted to the same substrate can mate. Ecological premating barriers are therefore often very strong in ascomycetes, even in sympatry, allowing divergence without intersterility, that is without the evolution of either mate choice or hybrid inviability or sterility (Le Gac and Giraud 2008; Giraud et al. 2010). On the other hand, intersterility barriers have been found to be highly congruent with species delimited by multiple genes (Dettman et al. 2003; Le Gac et al. 2007a,b; Giraud et al. 2008; Cai et al. 2011), thus providing good species criteria. While intersterility can be less discriminating than other species criteria for ascomycetes isolated by ecological barriers, mate choice remains the barrier to gene flow that is expected to evolve between sympatric species co-occurring in the same environment (Giraud et al. 2008).

Domesticated species represent excellent models for the study of evolutionary processes, because domestication corresponds to recent and strong selective events, thus allowing studying evolution in action. In particular, the barriers to gene flow between domesticated populations and their wild, and often sympatric, relatives are interesting to study for understanding the evolution of reproductive isolation and its tempo. In domesticated plants for example, polyploidy and selfing have been prominent reproductive barriers (Dempewolf et al. 2012). In addition, degeneration may affect domesticated species (Lu et al. 2006; Larson and Fuller 2014; Schubert et al. 2014; Marsden et al. 2015), degeneration being a decrease in fitness due to the accumulation of deleterious mutations because of relaxed selection and bottlenecks. Such degeneration may play a role in reproductive isolation if it targets the ability to undergo sex in partially clonal organisms, such as fungi. There have indeed been reports of loss of sex after several generations of asexual propagation in fungi, although this was not in domesticated species but in pathogenic fungi replicated in laboratories (Xu 2002; Saleh et al. 2012).

Despite the assets of fungi as suitable models for studying reproductive isolation and sex, that is simple morphologies, well-identified ecological niches, short-generation times, the possibility to clonally replicate genotypes, to store them alive for decades and to induce sex in vitro (Kohn 2005; Giraud et al. 2008), studies dealing with reproductive isolation in domesticated fungi remain scarce, with a single well-studied case, the yeast *Saccharomyces cerevisiae*. Strong genetic differentiation has been found between *S. cerevisiae* lineages from different food processes, that is bread, beer, wine, and sake, as well as from natural ecological niches (Ben-Ari et al. 2005; Fay and Benavides 2005; Legras et al. 2007). The differences in ecological niches seem to play a role in reproductive isolation between these different domesticated yeasts lineages, directly by reducing crossing opportunities, and indirectly by inducing postmating barriers (Clowers et al. 2015; Hou et al. 2015). Chromosomal rearrangements also contribute to postmating isolation between yeast lineages (Albertin et al. 2009; Hou et al. 2014). In other yeast species, hybridization and mismatch repair system have been shown to be efficient barriers to gene flow (Greig et al. 2002, 2003). While reproductive isolation has been extensively studied between populations of domesticated yeasts (Ben-Ari et al. 2005; Fay and Benavides 2005; Legras et al. 2007), no case of degeneration in fertility has been reported to our knowledge in domesticated yeasts or in any other domesticated fungi.

Here, we investigated reproductive isolation between lineages of another domesticated ascomycete fungus, *Penicillium roqueforti*, used in the production of blue cheeses such as French Roquefort, Spanish Cabrales, or Italian Gorgonzola. *Penicillium roqueforti* does not exclusively occur in cheese, it can also be found as a spoilage agent in refrigerated stored foods or other habitats, such as silage, wood, and forest soil (Samson 2000; Pitt and Hocking 2009; Ropars et al. 2012a). Previous population studies using microsatellites have shown the existence of differentiated genetic clusters within *P. roqueforti*, that are sympatric (Ropars et al. 2014b; Gillot et al. 2015). Ropars et al. (2014) have revealed the existence of two main genetic clusters: the A cluster showed little diversity and contained only strains isolated from the cheese environment, whereas the B genetic cluster displayed higher genetic diversity and included strains from cheese and other environments. Within the B cluster, further genetic subdivision separated strains collected in cheese from those collected in other environments. The A cluster, containing only industrial cheese strains, possesses genomic islands acquired by horizontal gene transfers, that are shared between several *Penicillium* species isolated from cheese environment, such as *P. camemberti*, used for the production of soft cheeses such as Camembert (Ropars et al. 2014a, 2015). These genomic islands seem to carry crucial metabolic genes providing a competitive advantage as well as better use of the cheese substrate. The strains from cheeses carrying these genomic islands indeed show better fitness on cheese medium while growing less well on poor medium (Ropars et al. 2015). Ecological barriers to gene flow may therefore play a role in the genetic differentiation within *P. roqueforti*. However, as cheese strains occur in the same environment (Ropars et al. 2014b),

ecological isolation cannot be a barrier to gene flow between the two cheese clusters and there may therefore also be intersterility. Furthermore, inability of sexual reproduction, possibly due to genomic degeneration during domestication, could also be a cause for the lack of gene flow. Sexual reproduction has been successfully induced in lab conditions using two noncheese *P. roqueforti* strains (Ropars et al. 2014b) and population genetics analyses have revealed footprints of recombination in populations, suggesting that sex is occurring or has occurred until recently in *P. roqueforti* (Ropars et al. 2012b). However, neither the capacity of sexual reproduction of individuals belonging to the cheese clusters nor interfertility between clusters could be tested so far, as only a few strains were used in the previous crosses reported, preventing any statistical analyses (Ropars et al. 2014b). Examining the fate of crosses between clusters may clarify the taxonomic status of the different *P. roqueforti* genetic clusters. The use of the phylogenetic species recognition criterion of genealogical concordance between multiple gene genealogies (GC-PSR) has provided no evidence for the existence of cryptic species (Gillot et al. 2015), but intersterility may be a more discriminant species criterion, especially between cheese clusters that co-occur in the same environment.

We therefore aimed at testing whether intrinsic barriers to gene flow can be detected between *P. roqueforti* genetic clusters, focusing on premating and postmating fertility, that is crosses initiation and ascospore production, and loss of sex ability in domesticated strains. We addressed more specifically the following questions in *P. roqueforti*: (1) Can we induce sexual reproduction in all the genetic clusters, even in the domesticated strains used for cheese production, or has genomic degeneration impacted sex ability? (2) Are there premating and/or postmating intersterility, that is failure of crosses or of ascospore production, between the different genetic clusters of *P. roqueforti*, and in particular those occurring in the cheese environment? For these goals, we needed publicly available strains from the different genetic clusters of *P. roqueforti*. We therefore built a new collection of worldwide *P. roqueforti* strains isolated from cheeses and from other environments and we genotyped the strains using microsatellite markers to assign them to the previously reported genetic clusters. We then attempted to cross strains within and between genetic clusters to explore interfertility and sex ability. The crosses were also analyzed using optic and electronic microscopy (SEM).

Material and Methods

STRAIN ISOLATION

Because most of the cheese strains used for the previous studies was not publicly available, we built a new collection. We collected 97 blue cheeses from 16 countries around the world (e.g., Roquefort, Gorgonzola, Stilton, Cabrales, Blue Gouda, Danish blue, Cheddar blue). Spores were sampled from the cheeses, spread on Petri dishes containing malt-agar medium and were incubated for three days at 25°C. For each plate, single monospores cultures were grown using a dilution method to guarantee that only a single haploid genotype was obtained. All the strains were included in the public LCP (Laboratoire de Cryptogamie, Paris) collection belonging to the National Museum of Natural History and are available upon request (Table S1). We used here overall 240 *P. roqueforti* strains including 67 strains previously analyzed (Ropars et al. 2014b, 2015) and 173 new strains. Of the 240 strains analyzed here, 207 strains were collected from the cheese environment, 31 from noncheese environments, such as silage, fruits, and bread, and two strains were of unknown origin. Some noncheese strains were obtained from other public databases (i.e., from the CBS-knaw fungal biodiversity centre in Utrecht, Netherlands and from the Belgian co-ordinated collections of micro-organisms BCCM/MUCL in Louvain-la-Neuve, Belgium).

DNA EXTRACTION, STRAIN GENOTYPING, AND POPULATION GENETICS ANALYSES

Genomic DNA was extracted from fresh mycelium of the single-genotype strains grown for five days on malt agar. The Qiagen DNeasy Plant Mini Kit (Qiagen, Ltd. Crawley, UK) was used for DNA extraction and purification. To ensure that the strains belonged to *P. roqueforti* we sequenced for each of them the 5′ end of the β-tubulin gene using the oligonucleotide primer set Bt2a/Bt2b (Glass and Donaldson 1995), as the β-tubulin sequence is a good marker to discriminate species in the *Penicillium* Section *Roqueforti* (Samson et al. 2004). We considered that strains belonged to *P. roqueforti* when their β-tubulin sequence showed 100% identity with the sequences of either one of the two clades previously described in this species (Samson et al. 2004), as assessed by blast search in public databases.

We used for the genetic analyses only the strains available in public collections. All the 240 strains were genotyped using the eight polymorphic microsatellite markers giving the clearest patterns among those described previously (Ropars et al. 2014b): Proq12, Proq13, Proq73, Proq74, Proq78, Proq80, Proq81, and Proq88. These microsatellite loci are not linked to *Wallaby* or *CheesyTer*. Furthermore, the *P. roqueforti* collection was screened for the presence/absence (noted hereafter ±) of the two horizontally transferred genomic islands that have been suggested to be involved in adaptation to cheese environment for the industrial strains (Ropars et al. 2014a, 2015), *Wallaby* (noted W), and *CheesyTer* (noted C), using the primers developed previously (Ropars et al. 2015).

Individual-based Bayesian clustering method implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000) was used to assign strains to the different genetic clusters. Ten independent analyses were carried out for each number of clusters, from $K = 1$ to

$K = 10$, using admixture models and 500,000 MCMC iterations, after a burn in of 50,000 steps. The output was processed using CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007), to identify clustering solutions in replicated runs for each value of K. Population structure was then displayed graphically with DISTRUCT v1.1 (Rosenberg 2003). We computed the ΔK statistics (Evanno et al. 2005) via the Structure Harvester website (Earl and vonHoldt 2012) (http://taylor0.biology.ucla.edu/structureHarvester/), to identify the *K* value corresponding to the strongest structure.

Discriminant analyses of principal components (DAPC) were computed using the *adegenet* package (Jombart 2008) implemented in the R software (R Development Core Team 2008).

The diversity indices (numbers of alleles and expected heterozygosity), F_{ST} and linkage disequilibrium were computed using Genepop on the web (Raymond and Rousset 1995; Rousset 2008). Genetic distances between strains used for the crosses were computed using the suboption 5 from Genepop on the web (Raymond and Rousset 1995; Rousset 2008), which computes estimates $\hat{a}$ of genetic distances between pairs of individuals as described in (Rousset 2000), which are somewhat analogous to $F_{ST}/(1\text{-}F_{ST})$ estimates.

CROSSES

Penicillium roqueforti is a heterothallic species, meaning that haploid individuals can mate only if they carry different mating types. Its breeding system is bipolar with two alleles, that is there are only two mating types in the species, MAT1-1 and MAT1-2. We identified the mating type of each strain using the primers previously described (Ropars et al. 2012b). We launched crosses between strains of opposite mating types, belonging either to the same genetic cluster or to different clusters (hereafter named intracluster and intercluster crosses, respectively). The 31 strains used for the crosses are described in Table S1, in which they are highlighted in bold and in gray rows.

Crosses were performed in duplicate, on Petri dishes with biotin-supplemented oatmeal medium, following the protocol previously described (Ropars et al. 2014b). Briefly, for each isolate, spore suspensions containing 1×10^5 conidia per mL were prepared from five days old cultures. On each Petri dish, two strains of opposite mating types were inoculated onto the agar surface, each being inoculated on two points, at opposite edges of a diameter (5 μL of spore suspension were deposited at each inoculation point); the diameters along which the two strains were inoculated on a given Petri dish were perpendicular (Fig. 1A). Petri dishes were then left at 15°C in the dark (Ropars et al. 2014b). After four weeks, Petri dishes were observed under a binocular microscope to look for fruiting bodies (cleistothecia). Cleistothecia produced were then observed under an optic microscope to search for ascii and ascospores.

SEM OBSERVATIONS

Cleistothecia were isolated after 4–5 weeks. They were manually opened, mounted on aluminum or brass blocks with a mixture of Tissue Tek O.C.M.™ compound and colloidal graphite, and directly fixed using slushy nitrogen freezing and a cryo-transfer system (Quorum PT3000T) to prevent artifacts of chemical fixation and freeze drying. Once in the cryo-transfer system, water was sublimed at –90°C for 8 min, the specimens were sputtered with platinum (90 s) and analyzed at –140°C at high vacuum with the SE2 detector using a Sigma VP at 8kV and an aperture of 20 μm.

STATISTICAL ANALYSES

Differences in genetic diversity between clusters were tested using ANOVAs. Deviations from balanced mating-type ratios were tested using χ^2 tests. Logistic regressions were used to test for differences in fertility between different types of crosses. Student's *t* was used to test for differences in genetic distances between groups of strains that were more or less interfertile. All statistical analyses were performed using JMP (SAS Institute).

Results

GENETIC DIVERSITY AND POPULATION STRUCTURE IN *P. roqueforti*

Our set of *P. roqueforti* isolates includes 210 isolates from cheeses, 28 strains from noncheese environments and two strains from unknown origin. Out of these 240 strains used in the present study, 173 were isolated and genotyped de novo. The 100% identity in the β-tubulin sequences of all these strains to published *P. roqueforti* sequences in databases confirmed their species identity. The ΔK value pointed to $K = 2$ as the strongest structure level in the data set (Fig. S1), separating strains carrying both horizontally transferred regions *Wallaby* (W) and *CheesyTer* (C) (the previously identified A cluster in (Ropars et al. 2014b), hereafter called the W+C+ strains), all isolated from dairy environments, from strains lacking both (the previously identified B cluster, hereafter called the W–C– strains). At $K = 3$, the B cluster was split into two well-delimited clusters, one with only cheese strains and a second with strains from various environments, and mostly other environments than cheese (Fig. S2A). We therefore hereafter call these three clusters "W–C– cheese," "W+C+ cheese," and "various environments." The F_{ST} values between the three clusters were high (Table S2). The DAPC also supported a genetic structure clearly separating three populations, with the axis 1 separating well W–C– cheese strains from those isolated from various environments (Fig. S2B). Although the ΔK value pointed to $K = 2$ as the strongest structure in the dataset, the barplots, the F_{ST}, and the DAPC all showed that the population subdivision at $K = 3$ was also strong. Furthermore, we expected different

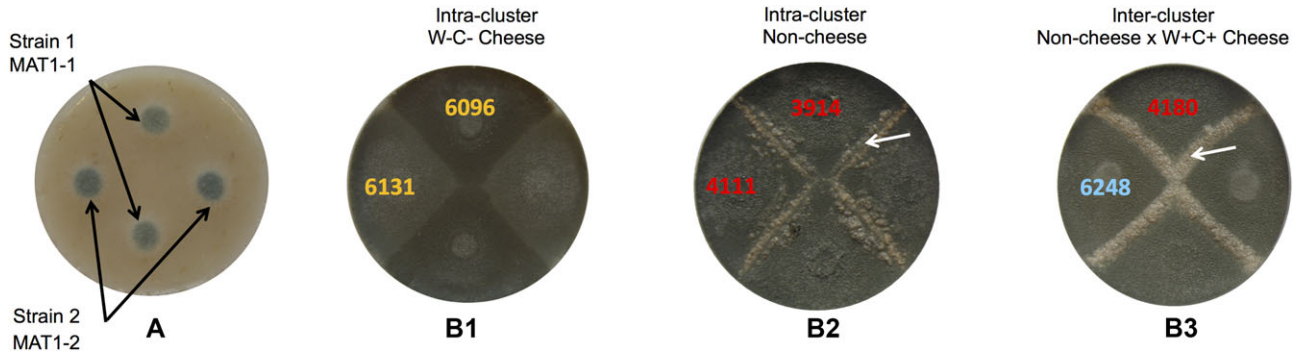

Figure 1. Crosses between strains of *Pencillium roqueforti* of opposite mating types. (A) inoculation scheme for setting up crosses between two strains. (B) Pictures of more or less fertile crosses. Cleistothecia were absent from infertile crosses (B1) while successful crosses (B2, B3) showed cleistothecia along junctions of intersecting colonies (white arrows). The strains used in the B2 and B3 crosses display contrasted mycelium morphologies but these two crosses were fully fertile, with no obvious difference in their cleistothecia or ascospore morphologies. The strain IDs are indicated in colors corresponding to those of the clusters in Figures 2 and S2: yellow indicates W–C– cheese strains, blue W+C+ cheese strains, and red noncheese strains.

fertility levels between strains from cheese, clonally replicated for long, and strains from other environments. Therefore, for investigating fertility in crosses, we focused on the genetic subdivision observed with three genetic clusters.

Virtually all strains in the W+C+ cheese cluster carried the MAT1-2 mating type (134 strains out of the 141 strains genotyped at the MAT locus, i.e., 95.0% of the strains) while almost all strains in the W-C- cheese cluster harbored the MAT1-1 mating type (40 strains out of the 49 strains genotyped at the MAT locus, i.e., 81.6% of the strains; Figs. S2; S1). In contrast, the "various environments" cluster showed a balanced mating type ratio (eight strains out of the 19 strains genotyped at the MAT locus, i.e., 42.1% of MAT1-1; Fig. S2). A χ^2 test indicated that this proportion was not significantly different from a 50:50 ratio ($\chi^2 = 0.80$, df $= 1$, $P = 0.3711$). ANOVAs indicated significant differences in genetic diversities at the microsatellite loci between the three clusters, in terms of both number of alleles ($F = 5.95$, df $= 2$, $P = 0.0089$), and expected heterozygosities ($F = 10.33$, df $= 2$, $P = 0.0008$). The genetic diversity was much lower in the two cheese clusters than in the "various environments" one (Fig. 2), the lowest diversity being found in the W-C- cheese cluster. Levels of linkage disequilibrium were higher in the two cheese clusters than in the "various environments" one (Fig. 2), suggesting less recombination in cheese strains.

CROSSES

We set up 189 crosses in total (Table S3), either within or between the three main genetic clusters. We hereafter call the three clusters "W–C– cheese," "W+C+ cheese," and "noncheese," as for the third cluster with strains from various environments we only used strains from other environments than cheeses in the crosses. We were limited in crossing possibilities by the imbalanced mating-type ratios in the cheese genetic clusters. We launched crosses using 12, 12, 7 strains in total from the noncheese, W–C– cheese and W+C+ cheese clusters, respectively. They were combined as a full design taking into account their mating types, yielding 24 crosses within the noncheese clusters, 20 crosses within the W–C– cheese cluster, 10 crosses within the W+C+ cheese cluster, and 140 intercluster crosses (Table S3).

After four weeks, we searched for cleistothecia and ascospores using binocular and optic microscopes. We considered as fertile the crosses where cleistothecia, asci, and ascopores were produced (Fig. 1). When no cleistothecia were produced, we inferred premating intersterility, as crosses were then not initiated at all. The presence of cleistothecia but lack of asci and ascospores was considered as postmating intersterility, because syngamy then occurred but did not yield progeny. We did not quantify precisely the number of cleistothecia per cross, but in crosses in which cleistothecia were produced we did not notice any striking differences in their quantity between different crosses.

Results are presented in Table S3 and summarized in Figure 3, giving the degree of fertility for the six types of crosses (three intracluster and three intercluster types). We considered as three nominal classes the degree of fertility observed in the crosses, that is full fertility, premating intersterility and postmating intersterility. A multinomial logistic regression showed that the different types of crosses displayed significantly different fertility levels ($\chi^2 = 138.87$, df $= 8$, $P < 0.0001$).

Regarding intracluster crosses, only the noncheese ones were all fully fertile. Only half of the crosses within the W–C– cheese cluster were fertile, while the other half showed post- or premating intersterility. All the crosses within the W+C+ cheese cluster but one showed postmating intersterility.

Regarding intercluster crosses, we did not find evidence of strong intrinsic reproductive isolation between clusters of *P. roqueforti*. Indeed, all intercluster types of crosses included

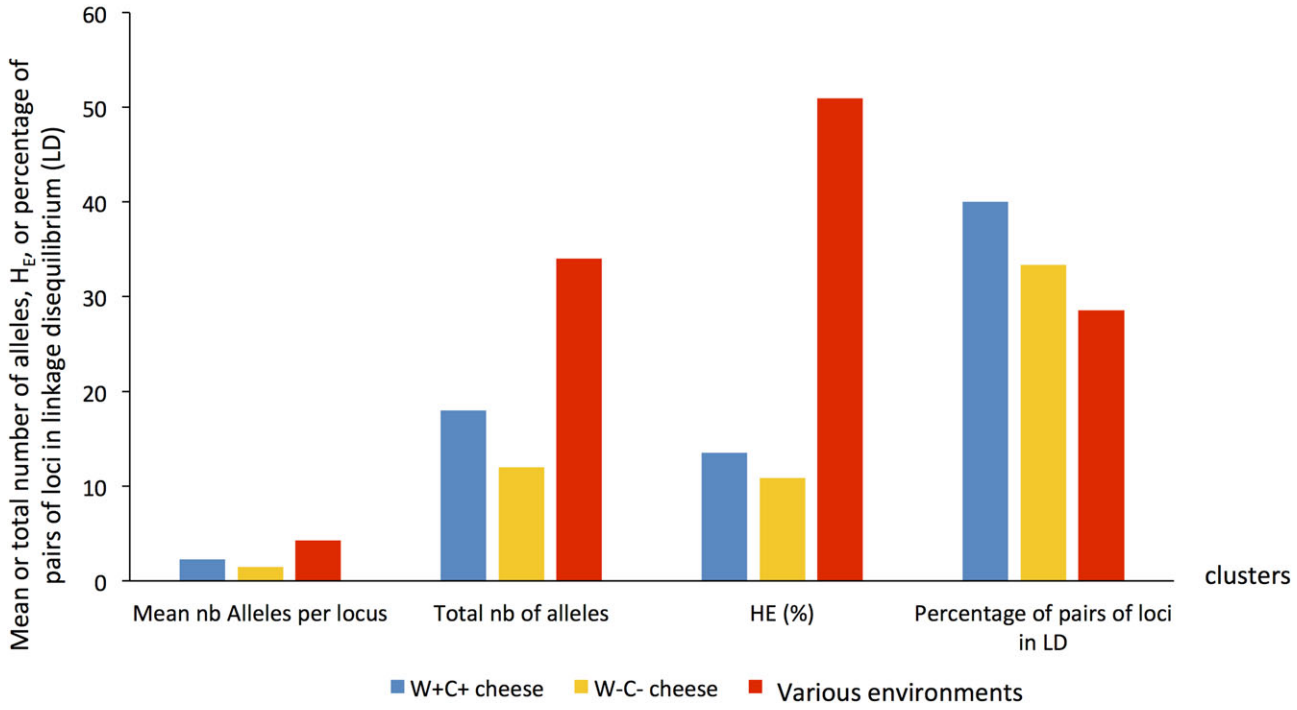

Figure 2. Histograms showing the genetic diversity (mean of the expected heterozygosity H_E, mean number of alleles per marker, total number of alleles across all markers) and linkage disequilibrium levels in the three genetic clusters of *Penicillium roqueforti*, corresponding to W–C– cheese strains, W+C+ cheese strains and noncheese strains, respectively. Colors correspond to those in Figures 1 and S2.

some fertile combinations, and some even showed higher levels of fertility than the crosses within cheese clusters (Fig. 3). The intercluster crosses involving noncheese strains showed more postmating intersterility when crossed with the W+C+ than the W–C– cheese strains, but this may be due the lower intrinsic fertility of cheese strains, the W+C+ cheese strains displaying high levels of postmating sterility even in intracluster crosses.

We then performed tests separately for pre- and postmating fertility. When considering only premating fertility (i.e., the production of cleistothecia), a logistic regression showed significant differences between the types of crosses ($\chi^2 = 56.95$, df $= 5$, $P < 0.0001$). However, this was only due to the W–C– cheese intracluster crosses that mostly gave no cleistothecia, while other types of crosses all produced cleistothecia. When considering only postmating fertility (i.e., the production of ascospores given that cleistothecia were produced), a logistic regression showed significant differences between the types of crosses ($\chi^2 = 81.92$, df $= 5$, $P < 0.0001$). However, this was mainly due to crosses involving W+C+ cheese strains, that all showed high levels of postmating sterility. In contrast, the other types of crosses, once having produced cleistothecia, most often produced ascospores (Fig. 3).

We then investigated more finely whether genetic distance affected the fertility of the crosses. The mean pairwise genetic distances between strains whose crosses yielded cleistothecia with ascospores, without ascospores or no cleistothecia, were all significantly different ($t = 1.97$; d.f. $= 2$, $P < 0.001$). The mean pairwise genetic distance was the lowest in the class of crosses giving no cleistothecia (mean $\pm$ SE $\hat{a} = 0.34 \pm 0.06$), was intermediate in the class yielding cleistothecia and ascospores (mean $\pm$ SE $\hat{a} = 0.56 \pm 0.03$), and the highest in the class yielding cleistothecia without ascospores (mean $\pm$ SE $\hat{a} = 0.73 \pm 0.04$). This further supports the inference that the fertility of crosses is less related to the genetic distances between strains than to intrinsic fertility levels.

SEM/TEM showed the ultrastructure of cleistothecia and ascospores that had not been described in this species so far (Fig. 4) and did not reveal any striking difference in these structures between the different clusters (Fig. S3).

Discussion

Our goal was here to test what barriers to gene flow may explain the genetic differentiation observed between the three main *P. roqueforti* clusters, that is the noncheese strains, the W+C+ cheese strains and the W–C– cheese strains, respectively. This could not be tested in previous studies due to the small number of strains used in crosses (Ropars et al. 2014b). We investigated here in particular (i) intrinsic premating barriers (inability to form cleistothecia), postmating barriers (inability to form ascospores), and

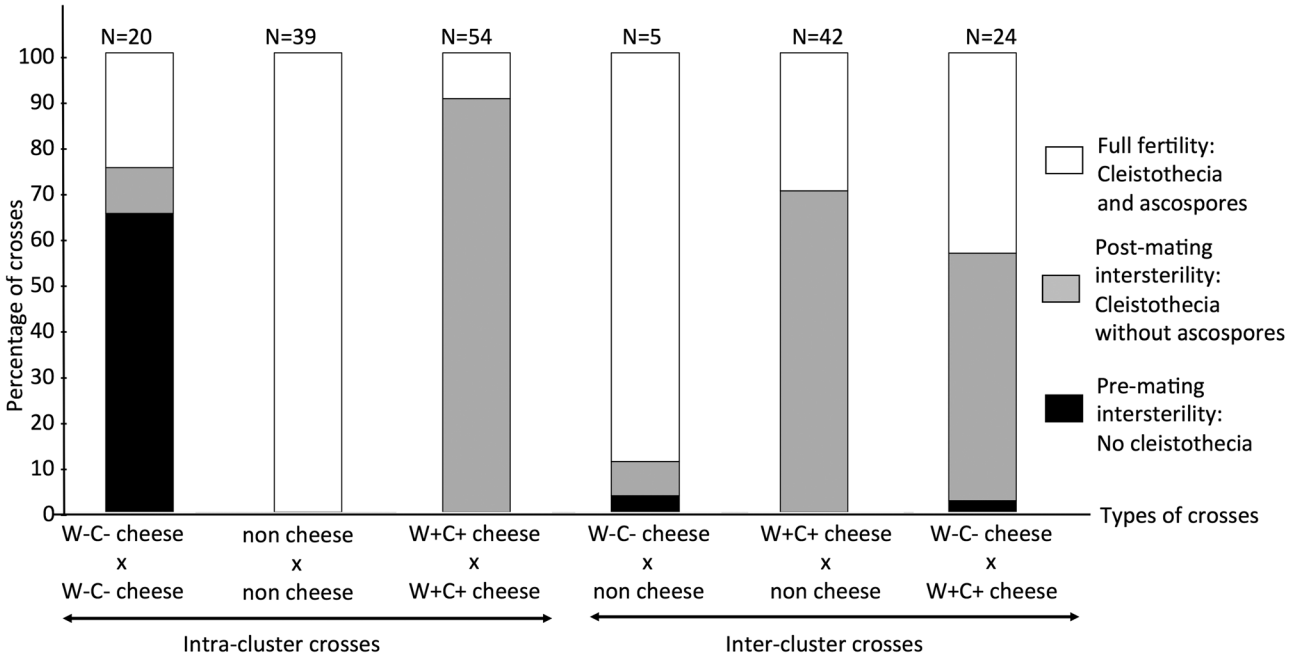

Figure 3. **Results of the different types of crosses (within and between the three genetic clusters of *Penicillium roqueforti*, including W–C– cheese strains, W+C+ cheese strains and noncheese strains, respectively). In black is shown the proportion of crosses with no cleistothecia (premating intersterility), in gray the proportion of crosses with cleistothecia but without ascospores (postmating intersterility), and in white the proportion of crosses with ascospores in the cleistothecia (full fertility).**

(ii) decrease in fertility in domesticated cheese strains, by looking at the ability to undergo sex within clusters. For this, we built a new strain collection of *P. roqueforti* from worldwide cheeses, which we made publicly available. We assigned our strains to the main genetic clusters found in previous studies (Ropars et al. 2014b; Gillot et al. 2015). Only 31 strains from other environments than cheese were however available while they represented the most genetically diverse cluster. This is because the "natural" environment of *P. roqueforti* is unknown and it is only rarely isolated from silage, and even less frequently from wood or soil. Finding strains in other environments than food is therefore difficult, preventing a comprehensive picture of the diversity of the noncheese cluster so far.

We focused here on the three main genetic clusters found within *P. roqueforti*. A finer genetic subdivision has previously been reported (Ropars et al. 2014b), with the W+C+ cheese strains split into three subclusters and the wild strains into two subclusters. Because these subdivisions corresponded to low levels of genetic differentiation, we considered here only the three main genetic clusters, that is W+C cheese, W–C– cheese and noncheese strains. The results of the experimental crosses revealed significant differences in fertility between the different types of crosses but this did not result from a lower fertility in the intercluster crosses. All intercluster types of crosses indeed showed some fertility, sometimes even at higher levels than intracluster crosses, with asci and ascospores looking similar in inter- and intracluster crosses. In addition, the less successful crosses on average were not the ones among the genetically most distant strains. Therefore, we did not find any evidence of mate choice barriers to gene flow (i.e., premating intersterility) between the different clusters of *P. roqueforti,* in particular between domesticated and wild strains. This reinforces the view that mate choice is not required for adaptation to different environments in ascomycetes (Le Gac and Giraud 2008; Giraud et al. 2010). Furthermore, the crosses between the two cheese clusters displayed even higher fertility than within each of the cluster, which stands against the prediction that premating intersterility would be an important barrier to gene flow between genetic clusters co-occurring in the same environment. This lack of intrinsic barriers to gene flow reinforces the previous inference based on multiple gene genealogies (Gillot et al. 2015) that the *P. roqueforti* genetic clusters likely do not represent cryptic species, but instead differentiated populations from a single species. The lack of differences in the ultrastructure of asci and ascospores further supports this view.

Intracluster crosses showed a decrease in fertility in the cheese clusters. Indeed, only the crosses among noncheese strains were all fertile. In contrast, all crosses among W+C+ cheese strains but one showed postmating sterility. Some crosses among W–C– cheese strains showed fertility but more than half were sterile, and mostly without even any cleistothecia. This suggests a reduced fertility of industrial cheese strains, being stronger and occurring earlier in the mating process in the W–C– than in the

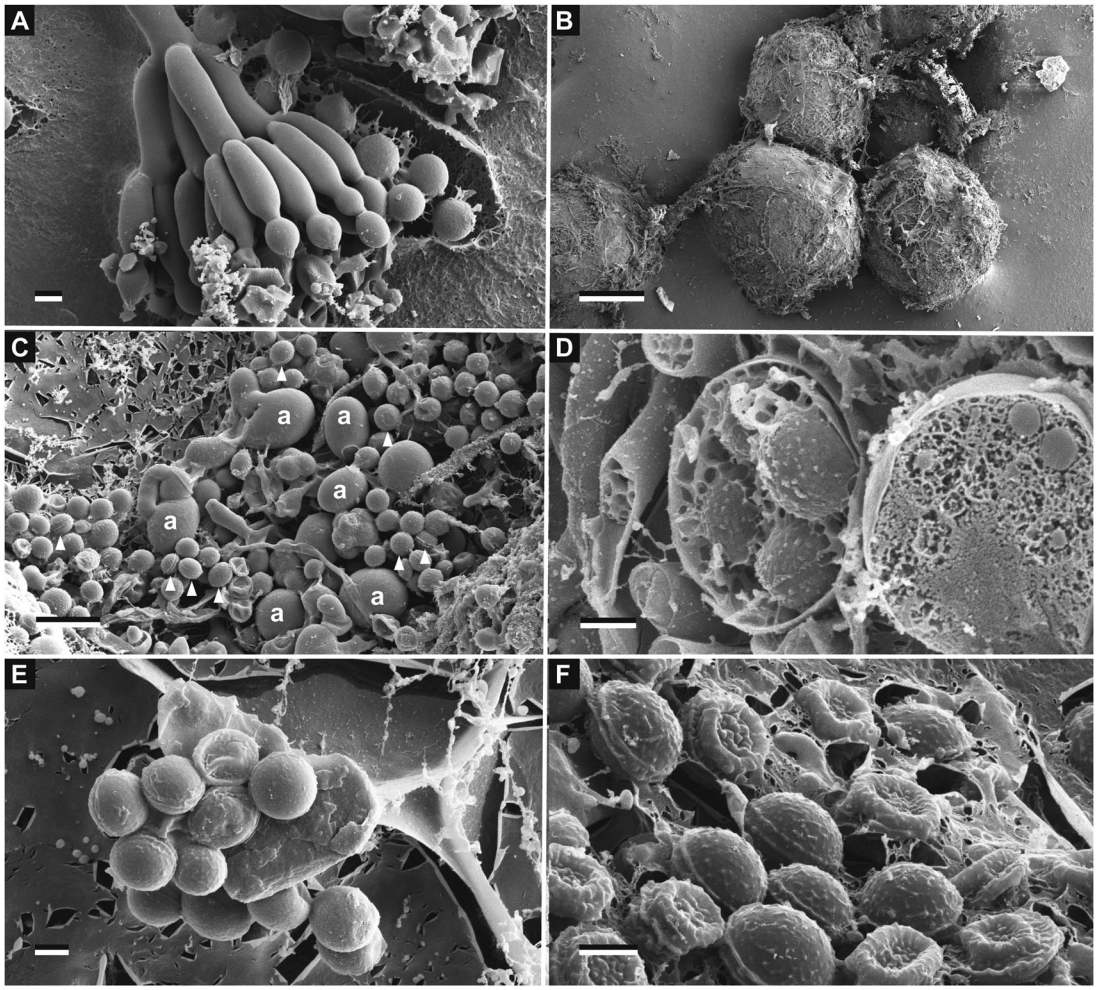

Figure 4. Sexual and asexual structures of *Penicillium roqueforti* taken using scanning electron microscopy. (A) Conidiophore, bearing asexual spores (conidia) on the plate of the cross 6248 × 3969 (W+C+ cheese × noncheese); the white bar corresponds to 2 μm. (B) Cleistothecia, from the cross 6248 × 3969 (W+C+ cheese × noncheese); the white bar corresponds to 200 μm. (C) Ascospores and asci from the cross 6201 × 6145 (W–C– cheese × W–C– cheese); the white bar corresponds to 10 μm; asci are indicated by the letter "a" and ascospores by white arrows. (D) Ascospores in an ascus in the cross 6133 × 6145 (W+C+ cheese × W–C– cheese); the white bar corresponds to 2 μm. (E) Ascospores in the cross 6201 × 6145 (W–C– cheese × W–C– cheese); the white bar corresponds to 2 μm. (F) Ascospores in the cross 6037 × 6136 (noncheese × W–C– cheese); the white bar corresponds to 2 μm.

W+C+ cheese cluster. The two cheese clusters are thus not only genetically differentiated, but they also show contrasted fertility levels. This may be because they represent two distinct domestication events. A previous studies have in fact shown that the two cheese clusters corresponded to different cheese protected designations of origin and showed different morphologies, colors, and growth rates (Gillot et al. 2015). The two clusters may therefore correspond to independent selection events, of different strains, with contrasted phenotypic and physiologic traits for making different types of cheeses.

The reduced fertility in the cheese strains likely represents degeneration. Degeneration has been previously reported in other domesticated species, for example dogs, horse, and rice (Lu et al. 2006; Larson and Fuller 2014; Schubert et al. 2014; Marsden et al. 2015), but not in domesticated fungi so far. In animals and plants, the decrease in fertility during domestication is likely mainly due to strong genetic drift in small populations while in fungi it may be also due to fitness being disconnected from fertility because of clonal multiplication. The degeneration in *P. roqueforti* indeed likely results both from bottlenecks reducing the efficacy of purifying selection during domestication and clonal replication for a long time without sex. The hypothesis of bottlenecks in cheese strains of *P. roqueforti* is supported here by the lower diversity at microsatellite markers and at the mating-type genes in the two cheese clusters, and even more in the W–C– cheese cluster. The hypothesis of lack of sex in recent times is supported by the higher levels of linkage disequilibrium among markers in the cheese clusters than in the noncheese one and by the uneven

mating-type proportions, because sex restores balanced mating types immediately within populations. The imbalanced mating-type ratios cannot be the only cause of lack of sex because the two cheese clusters are of opposite mating types. In the noncheese cluster in contrast, our findings of a balanced mating-type ratio, of low linkage disequilibrium levels, and of high fertility altogether suggest recurrent and relatively recent sex events. We cannot exclude that the cheese strains have evolved different requirements for sex induction, but this appears unlikely given that *P. roqueforti* strains used for cheese-making have been grown clonally for long by humans and that sexual structures are never observed in cultures, even in the gluten-rich media used for hundreds of years to grow the cheese strains.

The lack of sex in cheese clusters is likely due to recent industrial processes of cheese-making. Originally, *P. roqueforti* was not inoculated during the blue cheese production; it came from the environment, from a yet unknown source. For more than a century in the blue cheese industry, *P. roqueforti* asexual spores (conidia) have been inoculated into the cheese curd (Labbe and Serres 2004, 2009; Vabre 2015). Since ca. 1790–1830, the spores were initially collected from rotten bread, the bread being initially let to be rotten spontaneously; contaminating spores thus came from the environment, likely from wild recombining populations of the fungus in caves or farms (Labbe and Serres 2004, 2009; Vabre 2015). Later, the breads were inoculated with spore powder kept from previous inocula, selecting those that had yielded good cheeses (Labbe and Serres 2004, 2009; Vabre 2015). For the last 30–40 years, the inoculated strains have been much more carefully controlled, consisting mostly in monospore isolations cultivated in vitro, in order to avoid contamination issues, possibly with toxic micro-organisms, and to render the cheese maturation process more replicable and reliable (Labbe and Serres 2004, 2009; Vabre 2015). This represents recent strong selection of a few clonal lineages and subsequent exclusive asexual culturing, which has likely contributed to reduce the sexual ability of the cheese-making strains. In other fungal species with mixed reproductive systems, that is with facultative sexual and asexual cycles, the loss of the ability to reproduce sexually has been observed in experimental conditions after only a few cycles of exclusive mitotic divisions. Loss of sexual reproduction ability has been observed repeatedly for example in the human pathogen *Cryptococcus neoformans* after only ca. 600 mitotic divisions (Xu 2002), and in the rice pathogen *Magnaporthe oryzae* after only 10–20 rounds of weekly transfers and exclusive asexual growth (Saleh et al. 2012). The ease and rapidity of sex loss in these previous examples and in *P. roqueforti* during domestication and strain improvement is likely due to the numerous essential genes involved in this finely tuned process (Hornok et al. 2007). The genetic differentiation between the genetic clusters is therefore most likely due to both strong bottlenecks changing allelic frequencies and the lack of sex in cheese populations impairing genetic homogenization with other clusters. The finding of well-defined cheese clusters, despite lack of sex ability that could unite each of these clusters, is likely due to the recent origin of a single lineage per cluster, with still some footprints of more ancient recombination events. Some gene flow within cheese clusters may also be possible via parasexuality, and in fact, horizontal gene transfers have been reported among cheese strains even between distant species (Cheeseman et al. 2014; Ropars et al. 2015).

In conclusion, this study provides evidence for an important evolutionary process, that is degeneration in the form of reduced fertility after bottlenecks and strong selection. This degeneration occurred independently in two different lineages, representing convergent evolution at very short-time scale. This shows that the loss of an important biological phenomenon can occur very rapidly and repeatedly, at human time scale, if not used regularly and thus not under purifying selection any more. This is important for understanding general processes in evolution. In addition, this study informs on the tempo and nature of early barriers to gene flow, showing that genetic isolation, and adaptation to different substrates have evolved rapidly, but without being associated with intrinsic intersterility. In addition, our results are consistent with the theory of speciation, that predicts the evolution of mostly premating isolation at small genetic distances between sympatric fungal incipient species adapted to the same ecological niche, and of mostly postmating isolation at larger distances between fungal incipient species occurring in distinct ecological niches. Altogether our study thus yields important findings for understanding the tempo and mechanisms of speciation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Implementation of the Evanno's method for detecting the number of K showing the strongest genetic subdivision.

Figure S2. Population structure of *Penicillium roqueforti*.

Figure S3. Pictures of ascospores and asci of different crosses of *Penicillium roqueforti*, showing no striking differences between different types of crosses.

Table S1. *Penicillium roqueforti* strains used in this study: LCP (Laboratoire de Cryptogamie, Paris) collection number, genotype at the eight microsatellite markers, mating-type allele (1 for MAT1-1, 2 for MAT1-2, and "NA" when unknown), presence/absence of the *Wallaby* and *CheesyTer* genomic islands, indication of whether the strain have been used in previous studies, origin (substrate and geography), date of collection, cluster assignation at $K = 2$ (1: W+C+ strains; 2: W–C– strains) and $K = 3$ (1: W+C+ Cheese cluster; 2: Various environments cluster; 3: W–C– Cheese cluster) by STRUCTURE, and details on the cheese brand and location when available and relevant.

Table S2. Mean F_{ST} values across microsatellite markers between genetic clusters in *Penicillium roqueforti*.

Table S3. Results of the different types of crosses (within and between the three genetic clusters of *Penicillium roqueforti*, including W–C– cheese strains, W+C+ cheese strains and noncheese strains, respectively).