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1 **Generation of diversity in the blue cheese mold *Penicillium roqueforti* and**
2 **identification of pleiotropic QTL for key cheese-making phenotypes**

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23 **Abstract**

24 Elucidating the genomic architecture of quantitative traits is essential for our understanding of
25 adaptation and for breeding in domesticated organisms. *Penicillium roqueforti* is the mold used
26 worldwide for the blue cheese maturation, contributing to flavors through proteolytic and lipolytic
27 activities. The two domesticated cheese populations display very little genetic diversity, but are
28 differentiated and carry opposite mating types. We produced haploid F1 progenies from five crosses,
29 using parents belonging to cheese and non-cheese populations. Analyses of high-quality genome
30 assemblies of the parental strains revealed five large translocations, two having occurred via a circular
31 intermediate. Offspring genotyping with genotype-by-sequencing (GBS) revealed several genomic
32 regions with segregation distortion, possibly linked to degeneration in cheese lineages. We found
33 transgressions for several traits relevant for cheese making, with offspring having more extreme trait
34 values than parental strains. We identified quantitative trait loci (QTLs) for colony color, lipolysis,
35 proteolysis, extrolite production, including mycotoxins, but not for growth rates. Some genomic
36 regions appeared rich in QTLs for both lipid and protein metabolism, and other regions for the
37 production of multiple extrolites, indicating that QTLs have pleiotropic impacts. Some QTLs
38 corresponded to known biosynthetic gene clusters, e.g., for the production of melanin or extrolites. F1
39 hybrids constitute valuable strains for cheese producers, with new traits and genetic diversity, and
40 allowed identifying target genomic regions for traits important in cheese making, paving the way for
41 strain improvement. The findings further contribute to our understanding of the genetic mechanisms
42 underlying rapid adaptation, revealing convergent adaptation targeting major regulators.

43

44 **Introduction**

45 Most traits of agricultural or evolutionary significance are quantitative and genetically complex,
46 involving multiple genes that interact with one another and the environment (Falconer and Mackay
47 1995). Genomic loci showing diversity associated with quantitative trait variation are called QTLs, for
48 “quantitative trait loci”. Domesticated species are good models for understanding the genomic
49 architecture of quantitative traits involved in adaptation. Indeed, they have been subjected to recent
50 and strong selection on known traits, and display highly contrasting phenotypes compared to wild
51 populations, as well as between different domesticated varieties. In addition to the fundamental
52 interest in understanding the genomic basis of adaptive changes, the agronomically important traits
53 targeted for genetic improvement generally follow quantitative inheritance.

54 In the QTL mapping approach, genotyping and phenotyping of progenies allow assessing statistical
55 associations between genotypes and phenotypes, thereby allowing identifying genomic regions
56 affecting traits of interest (Falconer and Mackay 1995). The QTL approach has largely advanced our
57 understanding of the genomic architecture of important traits, for example in crops and cattle
58 (Adamczyk et al. 2013; Silva et al. 2014; Kumar et al. 2017). Studies of the genomic architecture of
59 adaptation in crops have revealed that traits involved in domestication were often controlled by only a
60 few QTLs with pleiotropic effects, often being major regulators (Sweeney and McCouch 2007; Baach
61 et al. 2008; Bachlava et al. 2010; Wang et al. 2010; Telias et al. 2011; Wirén and Jensen 2011;
62 Andargie et al. 2014; Johnsson et al. 2014; Wright 2015; Wright et al. 2015; Kongjaimun et al. 2012;
63 Kantar et al. 2017; Somta et al. 2020; Bomblies and Doebley 2006; Martínez-Ainsworth and Tenaillon
64 2016). The most emblematic example is the *teosinte branched 1 (tb1)* locus in maize, that determines
65 apical dominance as well as other domestication traits, which is due to a transposable element
66 insertion in a regulatory region (Doebley et al. 1997; Martínez-Ainsworth and Tenaillon, 2016).

67 Domesticated fungi used for food production, such as *Saccharomyces cerevisiae* for fermentation,
68 filamentous fungi for cheese maturation or the button mushroom *Agaricus bisporus* for direct human
69 consumption, also represent good models for understanding the genomics of adaptation, in particular

70 as they have small and compact genomes (Gladieux et al. 2014). QTL mapping proved to be an
71 efficient tool for identifying the genes affecting phenotypes impacting technological performances of
72 industrial yeast strains, such as thermotolerance, chemical resistance, dehydration stress tolerance, and
73 phenotypes associated with the fermentation process, such as volatile compounds production, ethanol
74 production, in nitrogen-limited fermentations and in nitrogen consumption and utilization (Nguyen *et*
75 *al.* 2022; Kessi-Pérez et al. 2020; Eder *et al.* 2018; Wilkening *et al.* 2014; Swinnen, Thevelein and
76 Nevoigt 2012; Liti and Louis 2012; Steinmetz *et al.* 2002). In addition to classic QTLs, traits have also
77 been reported to be impacted in yeasts by aneuploidy (Todd, et al. 2017) and genomic rearrangements
78 (Zimmer *et al.* 2014). QTL mapping has also been successfully used in *A. bisporus*, for identifying the
79 genomic regions controlling color and spore number (Foulongne-Oriol et al. 2012; Imbernon et al.
80 1996), in the causal agent of the cereal disease *Fusarium* head blight, *Fusarium graminearum*, for
81 identifying the genetic determinants of aggressiveness (Laurent et al. 2021), and in the *Lachancea*
82 *waltii* yeast, for identifying genomic regions controlling the growth rate in the presence of various
83 drugs (Peltier et al. 2021).

84 *Penicillium roqueforti* is the mold used for the maturation of all types of blue cheeses worldwide,
85 responsible for the typical blue-veined aspect, and contributing to their specific flavor and aroma, in
86 particular through high levels of proteolytic and lipolytic activities (Moreau 1980; Cerning et al. 1987;
87 Collins et al. 2003). When this study was performed, four populations had been identified in *P.*
88 *roqueforti*, two populations being used to produce cheeses and two populations being found in molded
89 silage and lumber or in spoiled food (Dumas et al. 2020). The two populations used for cheese
90 maturation each show footprints of bottlenecks and a domestication syndrome, with phenotypes
91 contrasting with those of the non-cheese populations and beneficial for several important aspects of
92 cheese safety, appearance and flavor (Dumas et al. 2020; Caron et al. 2020; Crequer et al. 2023). The
93 two cheese populations each correspond to a clonal lineage, with contrasted phenotypes and diversity
94 levels, and inoculated in different types of cheeses, *i.e.* Roquefort PDO (protected designation of
95 origin) versus other blue cheeses (Gillot et al. 2015; Dumas et al. 2020). The *P. roqueforti* cheese

96 populations produce cheeses with higher percentages of blue area and with higher quantities of desired
97 volatile compounds than non-cheese populations (Caron et al. 2020).

98 The Roquefort population, found in Roquefort PDO cheeses, displays some level of genetic diversity
99 and exhibits traits beneficial for pre-industrial cheese production, *e.g.* slower growth in cheese models
100 and greater spore production on bread, the traditional multiplication medium (Gillot et al. 2017;
101 Dumas et al. 2020). The Roquefort population also produces higher quantities and more diverse
102 positive aromatic compounds in cheeses, which is due to its faster and different proteolysis and
103 lipolysis activities (Caron et al. 2020; Dumas et al. 2020). In addition, the Roquefort population
104 produced cheese with lower water activity, which could restrict spoiling microorganisms (Caron et al.
105 2020). Lipolysis and proteolysis are important traits in *P. roqueforti*, involved in cheese maturation,
106 yielding the specific volatile and metabolic compounds responsible for the desired strong and spicy
107 blue cheese flavors (Cerning et al. 1987; Collins et al. 2003; Gillot et al. 2017).

108 The other cheese population, named non-Roquefort, is constituted by a single clonal lineage, due to a
109 recent strong selection for a single “performant” strain, and is found in all types of blue cheese
110 worldwide except Roquefort PDO cheeses. The non-Roquefort population display phenotypes more
111 suited for industrial cheese production, such as a more efficient cheese cavity colonization ability,
112 higher tolerance to salt, to acidic pH and to lactic acid compared to other populations (Dumas et al.
113 2020; Ropars et al. 2020; Crequer et al. 2023), and the production of volatiles important for aroma and
114 flavor, *e.g.* methyl ketones (Caron et al. 2020). The non-Roquefort clonal lineage acquired large
115 genomic regions by horizontal transfers (Dumas et al. 2020; Ropars et al. 2015), mediated by giant
116 Starships mobile elements (Gluck-Thaler et al. 2022). These horizontally transferred regions include in
117 particular two very large regions called *Wallaby* and *CheesyTer*, which encompass genes with
118 functions in lactose metabolism or competition against other microorganisms. The non-Roquefort
119 lineage further displays footprints of positive selection in genes involved in volatile compound
120 production (Dumas et al. 2020) and has lost the ability to secrete the mycophenolic acid (MPA)
121 mycotoxin, an immunosuppressant used for preventing transplant organ rejection (Matas et al. 2013),
122 because of a deletion in a key gene (*mpaC*) of its biosynthesis pathway (Gillot et al. 2017; Crequer et

123 al. 2024). *Penicillium roqueforti* can secrete other metabolites, *i.e.* extrolites, including some
124 considered as toxins (*e.g.* Roquefortine C and PR toxin), but not found in blue cheeses, or in such low
125 concentration that they do not cause acute health hazards (Scott, 1981; Fontaine et al. 2015; Hymery et
126 al. 2017). Other *P. roqueforti* extrolites can be of interest, such as the potential antitumor compounds
127 andrastin A and (iso)-fumigaclavine A (Ge et al. 2009; Matsuda et al. 2013). These active compounds
128 may be used by the fungus in the competition against other microorganisms (Jakubczyk et al. 2020;
129 Conrado et al. 2022), and thus avoid cheese spoilage, although little is known on these aspects.

130 Identifying the genomic regions controlling the phenotypes contrasting between the four *P. roqueforti*
131 populations thriving in different niches would have important fundamental implications for our
132 understanding of the genomic architecture of adaptation and also applied consequences for strain
133 improvement by marker-assisted selection in progenies. However, because of the clonal population
134 structure of the two domesticated cheese lineages, it is not possible to identify the genetic determinants
135 of important traits for cheese making through phenotype/genotype association without producing a
136 recombinant population, which would also allow generating genetic and phenotypic diversity from the
137 two single clonal lineages used in most blue cheeses worldwide (Dumas et al. 2020). Fortunately,
138 sexual reproduction can be induced in *P. roqueforti* (Ropars et al. 2014), the two cheese lineages carry
139 opposite mating types, and some strains have retained some level of sexual fertility, despite the general
140 degeneration in sexual reproduction ability in the cheese populations (Ropars et al. 2016). As the life
141 cycle of *P. roqueforti* has a dominant haploid phase, direct access to this phase allows association
142 genetics to be carried out on first-generation offspring.

143 Here, we therefore aimed at generating diversity in cheese *P. roqueforti* populations and at identifying
144 the genomic regions controlling the phenotypic differences between the non-Roquefort and Roquefort
145 lineages, and more generally between *P. roqueforti* populations. For this goal, we produced five sexual
146 F1 haploid progenies by crossing six fertile haploid *P. roqueforti* strains from the four originally
147 identified populations at the beginning of the study, focusing particularly on the cross between the two
148 cheese populations, Roquefort and non-Roquefort. We studied in the progenies several phenotypes
149 important for cheese making, *i.e.* lipolysis, proteolysis, growth, color and the production of the main

150 known extrolites: roquefortine C and PR toxin, with its intermediates eremofortins A and B, as well as
151 metabolites with lower toxicity risk, *i.e.* andrastin A, (iso)-fumigaclavine A and MPA (Coton et al.
152 2020; Chàvez et al. 2023). Depending on the desired cheese product, different values may be wanted
153 for the various traits analyzed. For example, slower growth, lipolysis and proteolysis may allow to
154 store cheeses longer without product degradation, while faster lipolysis may contribute to stronger
155 aromas and faster growth to bluer cheeses (Caron et al. 2020). We analyzed 1,073 offspring with
156 single nucleotide polymorphisms (SNPs) obtained using genotype-by-sequencing (GBS). Because
157 genotyping revealed the presence of the two parental alleles in some genomic regions in the otherwise
158 haploid offspring, we also generated high-quality genome assemblies for the parental strains, to
159 compare the parental genomes and identify genomic translocations. We built genetic maps and ran
160 QTL analyses to identify the genetic architecture of the phenotypes important for cheese making.

161

162 **Results**

163 **Five fertile crosses involving at least one cheese strain**

164 We chose the five most fertile crosses among 17 trials involving in each case at least one cheese strain.
165 We identified the isolated spores that were actually recombinant ascospores and not asexual conidia by
166 using 11 microsatellite markers. We focused on the cross between the two cheese populations, *i.e.*
167 Roquefort (R) and non-Roquefort (N), as it may be the most interesting for strain improvement in the
168 cheese making context, and we isolated 387 recombinant offspring (Table 1). The other crosses each
169 involved a cheese strain (either R or N) and a strain from a non-cheese population, either silage (S) or
170 lumber/food spoiler populations (L), and we isolated between 157 and 185 recombinant offspring for
171 each of these four crosses (Table 1).

172 **Transgression and heterosis in phenotype distributions**

173 We measured several phenotypes important for blue cheese production, *i.e.*, mycelium growth, colony
174 color, lipolysis, proteolysis and extrolite production. We analyzed nine variables summarizing these
175 traits in the five progenies, plus seven traits related to extrolite production in three crosses, resulting in

176 66 trait distributions (9x5+7x3). Among these 66 distributions, 28 did not significantly deviate from
177 normality, 10 best fitted a unimodal distribution and 30 a bimodal distribution (Supplementary Figures
178 1 and 2; Supplementary Table 1). None of the phenotype distributions were trimodal or amodal
179 (Supplementary Table 1).

180 The distribution of the five progenies on the principal component analysis based on trait values
181 (Supplementary Figure 3) shows that they represent overall a higher phenotypic diversity than each
182 cross considered separately. A few traits appeared positively or negatively correlated (Figure 1 D),
183 some associations being expected, such as between color traits (*e.g.* blue and red) or between the
184 production of different targeted extrolites (Marcano et al. 2023; Torrent et al. 2017; Rojas-Aedo et al.
185 2018; Gillot et al. 2017). Other associations were more surprising, such as the negative correlation
186 between proteolysis rate on the one hand, and green and hue level on the other hand (Figure 1 D).

187 We found heterosis in the progenies, computed as the difference in mean trait values between the
188 parents and their progenies (Supplementary Table 2). Out of the 66 phenotype distributions, 53
189 displayed significant positive or negative heterosis. We found negative heterosis in all crosses for the
190 production of PR toxin (the most toxic *P. roqueforti* mycotoxin) and andrastin A, indicating that
191 progenies produced on average less of these two extrolites than their parent mean, which can be
192 beneficial for cheese making. In contrast, we found positive heterosis in all crosses for MPA
193 production, a less toxic extrolite. All tested phenotypes exhibited both positive and negative
194 transgressions across all crosses, *i.e.* with some offspring displaying higher values and others lower
195 values than either parent, which holds promise for the generation of phenotype diversity and strain
196 improvement. We found a large proportion of offspring with low or no PR toxin production in the
197 RxN and SxN crosses. We detected in all crosses negative transgression in roquefortine C production
198 level (*i.e.* the offspring mean was lower than the parental mean) and many offspring with no MPA
199 production, which is promising for the selection of hypotoxinogenic strains.

200 **High values of phenotype heritability**

201 Narrow-sense heritability (h^2) represents the part of phenotypic variance explained by additive genetic
202 variance, *i.e.* the part on which selection can act. The narrow-sense heritability of growth rate,
203 lipolysis, proteolysis and color across the five progenies, estimated by regressions of the progeny trait
204 means on the parent trait means, were relatively high, with for example two-thirds of traits with h^2
205 above 30% (Table 3). Some estimates were below zero or higher than 100% (Figure 1), but narrow-
206 sense heritability estimates based on regressions between offspring and parent trait means may not be
207 highly reliable with only five crosses, and even only three crosses in the case of the targeted extrolites.
208 For extrolite production, we also estimated broad-sense heritability (H^2) as we measured two replicates
209 for each strain (Supplementary Table 3). The broad sense heritability estimates for extrolite production
210 were high, ranging between 91.3 % for roquefortine C and 99.6% for isofumigaclavine A, which is
211 promising for selection (Supplementary Table 3).

212 **Translocations between parental genomes**

213 The comparison of high-quality genome assemblies revealed five large genomic chromosomal
214 rearrangements between the parents in four crosses (LxN, RxN, RxS, RxL; Figure 2), which had
215 impacts on genetic maps. These translocations ranged from 60 to 530 kb in size. The two silage
216 parents and the non-Roquefort parent showed syntenic genomes between each other, suggesting that
217 they kept the ancestral arrangement order. We could therefore infer that four of the translocations (L1,
218 L2, L3 and L4) occurred in the lumber population while the fifth one, R1, occurred in the Roquefort
219 population (Figure 2). We detected no particular transposable element family or clusters at the margin
220 of the translocations or DUF domains specific to Starship elements.

221 The L1 and L4 regions displayed internal rearrangements between their copies in different genomic
222 locations: the last 3' portion of each of the L1 and L4 regions in the parents with the ancestral
223 arrangement was located in 5' of the L1 and L4 regions in the translocated parent (Supplementary
224 Figure 4). This internal rearrangement suggests that the translocation occurred via a circular
225 intermediate mechanism, with an insertion cutting site in each of the L1 and L4 regions different from
226 their excision site (Supplementary Figure 4).

227 Both parental alleles for the R1 region insertion were found together in 38, 31 and 24 % of the RxN,
228 RxL and RxS offspring in GBS data, respectively (Figure 2). The Oxford Nanopore long-read
229 mapping depths confirmed the presence of two R1 region copies in the six analyzed offspring from the
230 RxN cross with both R1 region parental alleles detected in GBS data. Similarly, the long-read mapping
231 confirmed that the five offspring with a single parental allele in GBS data carried a single copy of the
232 translocated region in their genomes. The absence of this translocated region is likely lethal as we did
233 not detect any offspring with no R1 copy, while some recombination events should have generated
234 offspring with none of the two copies. We detected no offspring with both parental alleles nor an
235 absence of the region for the translocations L1 to L4, indicating that both the duplication or absence of
236 these regions lead to inviability.

237 We checked that the presence of the two R1 copies in haploid genomes was stable after culture and
238 replication of 24 offspring of the RxN cross carrying the two parental alleles. After cultivating these
239 24 offspring from the RxN cross for one week on plates and transferring conidia from the edge of the
240 colony to new plates 19 times, PCRs showed that the cultivated lineages still carried the two R1
241 parental alleles. We did not detect any RIP footprints (C-to-T repeat-induced point mutations) in the
242 R1 regions of the six offspring with a duplicated R1 region and with high-quality genome assemblies.
243 However, RIP footprints would only be expected in the following generation (F2), after a sex event
244 involving genomes with the duplicated region, as RIP is known to occur during the short dikaryotic
245 phase of sexual reproduction in ascomycetes (Galagan and Selker 2004).

246 **Genetic maps, recombination pattern and segregation biases**

247 We identified between 2,462 and 2,943 reliable markers in the progenies across the five crosses,
248 evenly distributed along the non-Roquefort reference genome. For constructing genetic maps, we
249 excluded redundant markers, *i.e.* those physically close and with identical segregation patterns in a
250 given cross. We thus constructed the genetic maps with 1,448 markers for the RxN cross, and 676 to
251 785 markers for the other crosses with fewer analyzed offspring (Table 2). The genetic maps for the
252 five crosses displayed lengths between 775 and 913 cM, with a mean of 857 cM (Table 2). We

253 detected four linkage groups in each genetic map, corresponding to the four chromosomes in the
254 parental genome assemblies (Figure 3). The mean number of crossing-overs per chromosome was
255 2.15, with a maximum of 2.58 for chromosome 1, the longest chromosome, and a minimum of 1.25 for
256 chromosome 4, the smallest chromosome. The mean recombination rate was estimated between 27.0
257 cM/Mb and 31.8 cM/Mb, with a mean of 29.8 ± 1.8 cM/Mb (Table 2). The LxN cross had a
258 particularly low mean recombination rate, mainly due to the presence of two cold spots of
259 recombination on chromosome 1 (indicated by a plateau in the plot of genetic map positions against
260 genomic positions; empty rectangles in Figure 3), with local recombination rates of 6.7 cM/Mb and
261 3.1 cM/Mb, respectively (Table 2). The edges of the two cold spots corresponded to the translocated
262 regions in the chromosome 1 of the LCP06039 strain (regions L1, L2, L3, Figure 2 and 3). This is
263 likely due to the inviability of offspring carrying either zero or two copies of the translocated region
264 because of recombination events between their insertion sites (Figure 2). Other regions with low or no
265 recombination rates corresponded to horizontally transferred regions only present in the reference
266 genome, putative centromeres, other translocated regions and TE-rich regions (Figure 2 and 3).

267 In the five crosses, large regions presented segregation biases, with under-representation of alleles
268 from the cheese populations (*i.e.* either from the Roquefort or non-Roquefort parents; Figure 4). Such
269 segregation biases could allow the purge of deleterious mutations in the cheese clonal lineages, but
270 could also render the selection of valuable alleles that are in linkage with deleterious alleles in these
271 regions more challenging. The segregation bias against the non-Roquefort parent allele in the second
272 half of chromosome 4 (between 3.5Mb and 4.2Mb) was present in all crosses involving the non-
273 Roquefort parent (Figure 4) and was very strong (>90% of the alternative allele in all three crosses).
274 Such strong under-representation of alleles from the cheese population in all of these three crosses can
275 be due to the lower fertility of domesticated populations (Ropars et al. 2016), with likely deleterious
276 alleles accumulated through clonal replication that lead to low viability of the ascospores carrying
277 them. We observed another segregation bias with strong over-representation (> 90%) of silage parental
278 alleles, in the RxS and SxN crosses, in chromosome 1 from 4.2 to 4.5Mb (Figure 4). The under-
279 represented allele, that of the cheese parents, displayed no segregation bias in the other crosses

280 involving the cheese parents (Figure 4), suggesting the presence of either a selfish element (*e.g.* a
281 spore killer) or a highly beneficial allele for early growth in the corresponding region in the
282 silage/spoiled food parents rather than a deleterious one in the cheese parent. This interpretation is
283 supported by the over-representation of the silage allele in two different crosses and the lack of bias in
284 the RxL and LxN crosses in this region, while these observations would not be expected if the cheese
285 parents had deleterious alleles in this region. The segregation bias with over-representation of
286 Roquefort alleles at the end of chromosome 1 is likely due to the lack of the R1 translocated region
287 that leads to offspring inviability. Symmetrically, the alleles from the other parent tended to be over-
288 represented at the R1 locus on chromosome 2, but not significantly so. This suggests that the location
289 of R1 may be more advantageous on chromosome 1 than on chromosome 2.

290 **Detection of QTL with pleiotropic effects**

291 We investigated QTLs for the nine traits associated with lipolysis, proteolysis, growth and color in all
292 descendants, as well as for the seven traits associated with extrolite production specifically in the RxN,
293 RxL and SxN crosses (Supplementary Table 4). We found QTLs for all tested phenotypes except for
294 growth rate (Figure 4; Supplementary Table 4), indicating that marker-assisted selection for these
295 traits may be performed for strain improvement and diversification. We detected 123 QTLs across all
296 phenotypes and crosses, 109 explaining more than 5% of the variance (hereafter called “major
297 QTLs”). We identified 58 major QTLs for the eight parameters related to lipolysis, proteolysis and
298 color, across the five crosses, with 8 to 17 QTLs per cross, with a mean of 1.3 major QTLs and a
299 maximum of 3 major QTLs per trait and cross. For extrolite production, we identified 13 to 23 major
300 QTLs across the three progenies analyzed for these traits, with a mean of 2.4 and a maximum of 5
301 major QTLs per extrolite and cross (Supplementary Table 4, Figure 4).

302 The identified QTLs and the direction of their effects were in agreement with the past occurrence of
303 selection for color, proteolysis, lipolysis and extrolite production in cheese populations. We indeed
304 identified QTLs impacting colony color, bluer colonies being associated with cheese parent alleles,
305 and in particular in the genomic region containing the dihydroxynaphthalene-melanin biosynthesis

306 cluster, known to be involved in melanin production (Figure 4). The cheese parent alleles were also
307 both associated with slower proteolysis and faster lipolysis, in connection with firmer texture and
308 longer cheese storage and the production of typical blue cheese flavors, respectively. We identified
309 QTLs for the production of three extrolites, MPA, PR toxin and roquefortine C, the non-Roquefort
310 alleles being associated with lower production levels (Figure 4). This is consistent with a selection for
311 bluer color, more efficient lipolysis and less efficient proteolysis in domesticated lineages, as well as
312 lower extrolite production levels.

313 Most of the major QTL regions presented pleiotropic effects. The 109 major QTLs were indeed not
314 distributed homogeneously across the genome, with instead regions carrying clustered QTLs for
315 different phenotypes (Figure 4, Supplementary Figure 5). We considered as pleiotropic the regions
316 presenting overlaps in QTL regions of at least two different trait classes in a given cross (proteolysis,
317 lipolysis, color and extrolite production). We identified five such regions, among which three regions
318 displayed pleiotropic impact on the four trait classes, *i.e.* proteolysis, lipolysis, color and extrolite
319 production, across the different progenies, and also within some progenies for multiple traits (empty
320 vertical rectangles in Figure 4, Supplementary Figure 5). For the pleiotropic region on chromosome 2,
321 the Roquefort allele was associated with faster lipolysis and lower proteolysis. This QTL region
322 displayed significant interactions for lipolysis with two other QTLs, in chromosomes 3 and 4; such
323 interactions between loci in determining a trait value constitute positive epistasis. The same region
324 also impacted color. For the pleiotropic regions detected in chromosomes 1 and 3, the non-Roquefort
325 alleles were associated with slower proteolysis, faster lipolysis and bluer colony color (Supplementary
326 Table 4, Figure 4). We further found epistasis for other types of phenotypic traits, particularly between
327 different QTLs controlling proteolysis (Supplementary Table 4, Figure 4). QTL interactions had the
328 same sign (positive or negative) across the various crosses in some cases and different signs in other
329 cases, indicating that epistatic interactions are dependent on the genetic background. Such epistasis
330 could impair selection, as recombination breaks up allelic combinations, while specific allelic
331 combinations are beneficial under positive epistasis.

332 We also identified QTLs for extrolite level production, including some outside of their known
333 biosynthesis gene clusters, suggesting that these QTLs correspond to *trans*-acting regulators. For three
334 extrolites (MPA, PR toxin and roquefortine C), we identified QTLs with intervals included in their
335 biosynthesis cluster, with non-Roquefort alleles associated with lower production levels. The lower
336 MPA production level associated with the non-Roquefort allele is likely due to the deletion in the
337 *mpaC* gene that was identified in the non-Roquefort population (Gillot *et al.* 2017; Crequer *et al.*
338 2024). We also found a QTL in a region including the PR toxin biosynthesis gene cluster, with lower
339 production level of the PR toxin but accumulation of its production intermediates, eremofortins A and
340 B, associated with the non-Roquefort allele at the PR toxin biosynthesis cluster. This suggests a
341 disruption in the cluster driving low production levels of the toxin and accumulation of its
342 intermediates in offspring presenting the non-Roquefort allele likely due to a premature STOP codon
343 in the ORF11 gene of the cluster (Crequer *et al.* 2024). The offspring harboring a non-Roquefort allele
344 for the various biosynthesis gene clusters displayed lower production levels of the two most toxic *P.*
345 *roqueforti* extrolites, *i.e.* PR toxin and roquefortine C, and of the less toxic MPA extrolite, which is
346 consistent with a selection for an hypotoxinogenicity trait in the non-Roquefort population (Fontaine *et*
347 *al.* 2015; Hymery *et al.* 2017; Crequer *et al.* 2024). Most of the QTLs associated with extrolite
348 production were not located within their biosynthesis gene cluster, which suggests the implication of
349 *trans*-acting regulators. These QTLs were often shared between different extrolites, suggesting a co-
350 regulation of their pathways, which could facilitate the selection for low extrolite production in strains
351 bred for cheese production. In particular, isofumigaclavine A production shared five of its eight major
352 QTLs with andrastin A production, suggesting co-regulation of the production of the two extrolites.
353 We detected QTLs for all tested extrolites, except roquefortine C, in the R1 region for its two insertion
354 sites (Figure 4), with lower production associated with the presence of the R1 region. Such a QTL
355 clustering suggests the presence of a master regulator of extrolite production in the R1 region. We in
356 fact found an ortholog of a master regulator gene in the R1 region, namely the *srkl* gene, regulating
357 osmotic and oxidative responses in fungi (Marquina *et al.* 2022). The selection of offspring with two
358 R1 regions could thus allow further decreases in overall extrolite content, and in particular that of the
359 studied mycotoxins in cheese.

360 We detected an andrastin A QTL next to the *Wallaby* horizontally transferred region (HTR) in the
361 RxN cross and a proteolysis QTL in the region containing the *CheesyTer* HTR in each of the SxN and
362 LxN crosses (Figure 4). Multiple other phenotypes have previously been suggested to be controlled by
363 these HTRs, such as milk sugar metabolism and interactions with other microorganisms (Ropars *et al.*
364 2015), but the indel nature of these HTRs may render hard to detect their associations with
365 phenotypes, as markers are lacking in one of the parents.

366 We also checked whether QTL regions encompassed other regulators known to be involved in
367 extrolite production (*prlaeA*, Marcano *et al.* 2023, *pcz1*, Gil-Durán *et al.* 2015, and *sfk1*, Torrent *et al.*
368 2017) as well as other essential phenotypes in *P. roqueforti*, such as conidiation or growth rate. We
369 located *pcz1* in the pleiotropic region of chromosome 2, encompassing QTLs for andrastin A
370 production and color, melanin being involved in both color and conidiation (Gil-Durán *et al.* 2015);
371 *pcz1* could therefore be a candidate for the QTLs controlling andrastin A production and color via
372 expression regulation. While no QTL interval overlapped with *prlaeA*, a QTL region controlling MPA
373 production in one cross encompassed *sfk1*. However, this QTL region overlaps with a shorter MPA
374 QTL present in another cross without *sfk1* in the confidence interval, which suggests no implication of
375 *sfk1* in MPA production regulation.

376 The QTL intervals were too large to identify particular genes or functions beyond the horizontal gene
377 transfers, biosynthesis gene clusters of the targeted extrolites or other *a priori* candidate genes. Indeed,
378 with on average one gene every 3kb in the annotated Roquefort parental strain and a median QTL
379 length of 760 kb, half of the QTL regions are expected to encompass more than 250 genes. We
380 therefore only tried to identify candidate genes in the smallest QTL intervals explaining more than
381 20% of the phenotypic variance. We found an enrichment in genes involved in carbohydrate
382 catabolism for a QTL associated with color in the pleiotropic region of chromosome 2 (g3621 to
383 g3634), and a pepsin gene (endopeptidase, g5778) for a QTL associated with proteolysis in
384 chromosome 3. Carbohydrate catabolism and pepsin are of paramount importance for secondary
385 starters, to use the remaining lactose residues to establish themselves early in the cheese matrix

386 (Cerning *et al.* 1987), and to degrade caseins and therefore to participate in proteolysis (Grippon *et al.*
387 1977), respectively.

388

389 **Discussion**

390 In this study, we aimed at generating genetic and phenotypic diversity in the blue cheese fungus *P.*
391 *roqueforti* by analyzing haploid F1 progenies from five crosses between cheese or non-cheese strains,
392 and identifying QTLs for key traits in cheese making. This will be essential for both strain breeding
393 purposes and for studying the genomic architecture of traits selected during domestication. Generating
394 diversity and reshuffling alleles is crucial as deleterious mutations have accumulated in the two clonal
395 lineages currently used for cheesemaking. Moreover, their low diversity raises concern for the
396 sustainable use of *P. roqueforti* for cheese making. We successfully generated offspring by crossing
397 strains from different populations of *P. roqueforti*, despite the known low fertility of the cheese
398 populations (Ropars *et al.* 2016). The progenies displayed a broad diversity for the phenotypes
399 important for cheese making, in terms of growth, color, proteolysis and lipolysis rates, as well as
400 extrolite production, with more extreme trait values than the parents. We detected QTLs for all these
401 traits except growth, often displaying pleiotropic effects. These findings will be highly valuable for
402 strain breeding, and further suggests that humans have domesticated this cheese fungus by selecting
403 major regulators, as reported in crops (Telias *et al.* 2011; Wirén & Jensen, 2011; Wang *et al.* 2010;
404 Sweeney & McCouch, 2007; Wright, 2015; Wright *et al.* 2015; Baach *et al.* 2008; Johnsson *et al.*
405 2014; Bachlava *et al.* 2010; Andargie *et al.* 2014; Kongjaimun *et al.* 2012; Kantar *et al.* 2017; Somta
406 *et al.* 2020; Bomblies & Doebley, 2006; Martínez-Ainsworth & Tenaillon, 2016).

407 *Variability in phenotypes in progenies, with heterosis and positive transgression for most traits*

408 We found large phenotypic variation, transgression and heterosis in progenies for multiple relevant
409 phenotypes for blue cheese production, *i.e.* mycelium growth, lipolysis, proteolysis and extrolite
410 production. Progenies displayed transgression, with the trait values of some offspring being much
411 more extreme than those of the parents. All progenies also exhibited heterosis, indicating mean trait
412 values in offspring that may be better than the mean value of the parents. Both transgression and

413 heterosis are promising for strain improvement and diversification. Furthermore, we detected QTLs
414 for most traits, indicating that selection is possible, and even marker-assisted selection. The generation
415 of variation is particularly important in *P. roqueforti*, as the two main cheese populations, Roquefort
416 and non-Roquefort, have lost most of their genetic diversities due to recent bottlenecks followed by
417 strong selection in industrialization times (Dumas *et al.* 2020). These clonal lineages even degenerate
418 in terms of sexual fertility and probably for other traits of interest for cheese making (Ropars *et al.*
419 2016). Sexual reproduction is the only way to purge deleterious mutations that accumulate during
420 clonal propagation. The progeny between the Roquefort and non-Roquefort strains is particularly
421 promising, as a European regulation requires a food safety assessment (“Novel food” law; UE No
422 2015/2283) for the use of microorganisms for food production except if they have already been used
423 for food before May 1997. The cross of parental strains already used for cheese production for decades
424 should therefore meet the European Union requirements. The other crosses may also be of great
425 interest, even for the European market, as the regulation might be interpreted in terms of species rather
426 than strains, as already done for example in wine and beer yeasts.

427 *Translocations and segregation biases*

428 The obtained high-quality genome assemblies led to the identification of five translocations between
429 the parental strains, having occurred independently in three lineages. We also identified offspring
430 carrying twice the R1 rearranged region. For these translocations, we did not find any offspring
431 completely lacking them, indicating that their absence in genomes may lead to offspring unviability.
432 For the L1, L2, L3, and L4 translocations, we detected no offspring with twice the regions either,
433 implying that their duplications are also lethal. We detected lower production levels for the targeted
434 metabolites in offspring carrying two copies of the R1 region. These offspring may therefore be highly
435 valuable for cheese making, especially as the presence and locations of the translocations were stable
436 through multiple replications and culture events. For further improvement, it should however be
437 checked that future crosses with these offspring will not induce RIP in the duplicated region. RIP
438 footprints (*i.e.* mutations from CA to TA in repeated sequences) have indeed been already detected in
439 *P. roqueforti* (Cheeseman & Ropars *et al.* 2014).

440 We detected no particular transposable element types or abundance at the margin of the translocations.
441 In particular, we detected no DUF3435 domain, typical of Starship elements, that move within and
442 between fungal genomes via a circular intermediate (Bucknell & MDonald, 2023) and are involved in
443 horizontal gene transfers in *P. roqueforti* (Gluck-Thaler et al. 2022). Nevertheless, the L1 and L4
444 translocations appeared to have occurred via a circular form. These regions may therefore correspond
445 to a new type of large mobile element with a circular intermediate. These findings reinforce the view
446 that fungal genomes are highly dynamic, with frequent gene movements, within genomes, between
447 individuals or even between species, and that this contributes to adaptation (Bucknell & MDonald,
448 2023).

449 For several translocated regions, either their lack or duplication in genomes led to to unviability, which
450 induced the cosegregation of the two insertion sites, potentially generating linkage drag in future
451 breeding, *i.e.* cosegregation of desired and undesired alleles in progenies. For other translocated
452 regions, only their absence was lethal, and this led to segregation biases in progenies. We detected
453 additional segregation biases, in other genomic regions, mostly with under-representation of the
454 parental strain from the parental domesticated cheese populations. Such biases are likely due to
455 degeneration in the cheese clonal lineages, with alleles less fit than in the wild populations, especially
456 under sexual reproduction and ascospore germination (Ropars *et al.* 2016). We detected a strong
457 segregation distortion in chromosome 4, with an absence of the non-Roquefort alleles in the progeny.
458 This suggests an unviability of the ascospores carrying the non-Roquefort allele, in agreement with the
459 low fertility of the non-Roquefort population, showing high post-mating sterility, *i.e.* formation of
460 cleistothecia without ascospores (Ropars *et al.* 2016). The region with segregation distortion in
461 chromosome 4 contained two orthologs of genes involved in sporulation in the *Saccharomyces*
462 *cerevisiae* yeast, g9043 and g9061 coding for SPS22-like (Coluccio *et al.* 2004) and RMD1-like
463 proteins (inferred by electronic annotation, UniprotKB-KW; Enyenihi & Saunders, 2003), suggesting
464 that the region contains genes essential for sexual reproduction. The region with segregation distortion
465 in chromosome 4 also included genes with premature stop codons in the non-Roquefort parent,
466 although with either no predicted function or a function with *a priori* no relationship with sexual

467 reproduction (g8973 and g8998). In the case of the RxS and SxN segregation bias in chromosome 1,
468 the under-represented Roquefort and non-Roquefort alleles displayed no segregation bias in the other
469 crosses involving the same Roquefort and non-Roquefort parents. This indicates that the bias depends
470 on the genetic background and may therefore be due to epistatic interactions leading to a deleterious
471 effect or a selfish meiotic drive element (Grognet *et al.* 2014; Turner & Perkins, 1979).

472 *QTL identification and the genomic architecture of adaptation*

473 We identified QTLs for all phenotypes but growth, confirming that selection of these traits for strain
474 improvement can be considered. There were only a few major QTLs per trait and cross, which should
475 facilitate strain breeding. The QTLs for color, lipolysis and proteolysis on the one hand, and for
476 extrolite production on the other hand, clustered in the same genomic regions, suggesting pleiotropy,
477 or clustering of genes with effects on these different pathways. The three pleiotropic regions affecting
478 color, and lipid and protein degradation have the same effects, with faster lipolysis, slower proteolysis
479 and bluer colony color for the cheese strain allele. This is consistent with a selection for a bluer color,
480 stronger flavors and a longer conservation in domesticated lineages of *P. roqueforti* for cheese
481 making. Furthermore, such pleiotropy or gene clustering may render selection for these traits easier or
482 more challenging depending on whether alleles coding for desired and unwanted traits are associated.
483 Actually, pleiotropic regions controlling multiple important traits for cheese making may be the result
484 of human selection targeting major regulators, and their finding informs on the genomic architecture of
485 adaptation. The control of traits involved in domestication by only a few QTLs and their pleiotropy
486 have been reported in animals and plants, often due to major transcription regulators, human selection
487 having targeted “masterminds” (Martinez-Ainsworth & Tenailon, 2016), but also to the genetic
488 determinant of important crop traits being clustered in genomes (Wright, 2015; Kantar *et al.* 2017).
489 Here, we show that such pleiotropic regions are present in two independently domesticated lineages of
490 *P. roqueforti*, with the same type of effect on the phenotype, suggesting evolutionary convergence
491 (Ropars & Giraud, 2022) and selection of major regulators with pleiotropic effects.

492 Known shared regulators of various secondary metabolites include *prlaeA*, *sfk1* and *pcz1*, their
493 silencing or knock-out decreasing the production of roquefortine C, andrastin A et MPA (Marcano *et*
494 *al.* 2023; Torrent *et al.* 2017; Rojas-Aedo *et al.* 2018). Over-expression of *pcz1* decreases the
495 production of roquefortine C and andrastin A, and increases the production of MPA; *pcz1* also impacts
496 growth, conidiation and spore germination (Gil-Durán *et al.* 2015); *pcz1* was found in one of the
497 pleiotropic regions with an impact on andrastin A production and on color, which is mostly due to
498 conidia pigmentation.

499 In addition, we found QTL regions containing the biosynthesis gene clusters involved in PR toxin,
500 MPA and roquefortine C production. In the RxS and LxN crosses, we found a QTL affecting the
501 colony color in a region containing the DHN melanin biosynthesis gene cluster, known to code for the
502 production of *P. roqueforti* conidia pigment (Cleere *et al.* 2024; Seekles *et al.* 2021). Besides these *a*
503 *priori* candidates, the QTL analysis did not point to particular genes or regulatory regions, as the
504 regions with significant impacts were large, encompassing many genes, which impaired searching for
505 relevant gene functions. Furthermore, as shown in plant domestication, several important traits have
506 evolved due to transposable element insertions in regulatory regions (Martínez-Ainsworth &
507 Tenailon, 2016; Yao *et al.* 2015), in which case approaches based on gene functions may not be
508 powerful.

509 *QTLs for desired traits in Penicillium roqueforti*

510 We observed a large diversity of phenotypes within each of the generated progenies compared to
511 current commercial strains, and an even greater diversity across the five progenies. We detected
512 pleiotropic regions, acting on multiple metabolism traits, which could facilitate or impair the selection
513 for desired combinations of lipolysis, proteolysis and color. The presence of additional QTLs
514 elsewhere in the genome suggests that a finer selection could be possible for one of the traits without
515 affecting the others. Therefore, our study paves the way for strain improvement and diversification in
516 cheese-making strains.

517 Toxin production is a concern in *P. roqueforti*, in particular the production of PR toxin and
518 roquefortine C. The low toxin concentrations found in cheese and the long history of safe consumption
519 of blue cheeses, combined with the recent discovery that both Roquefort and non-Roquefort
520 populations are unable to produce PR toxin, indicate the innocuity of commercial strains in cheese
521 production (Crequer *et al.* 2024). Nevertheless, selecting strains with even lower toxin production
522 would be of major interest. We found several QTLs with effects on extrolite production. In particular,
523 QTLs in the biosynthesis gene clusters of PR toxin, roquefortine C and MPA indicated that the
524 corresponding non-Roquefort alleles decreased extrolite production. This suggests selection for lower
525 toxicity in the domesticated non-Roquefort lineage. In addition, lower production of several extrolites
526 was associated with the duplication of the R1 region. Selection of these alleles could thus further
527 improve the safety of *P. roqueforti* use in cheese production or for new products such as vegan cheese.
528 While toxin production is detrimental for cheese safety, other extrolites can be interesting to produce
529 for medical applications: for example, MPA is an immunosuppressant used for preventing transplant
530 organs rejection (Matas *et al.* 2013), and andrastin A, a potential antitumor compound (Ge *et al.* 2009;
531 Matsuda, Awakawa & Abe, 2013). Our study shows large positive transgression for the production of
532 these extrolites and multiple QTL regions. These results are highly promising to increase their
533 production level in the pharmaceutical context.

534 We detected QTLs for color as well as for protein and lipid degradation. In blue cheeses, lipolysis and
535 proteolysis by *P. roqueforti* influence texture, taste and the production of aromatic compounds, the
536 latter being mainly influenced by lipolysis (Cantor *et al.* 2004). Currently, the texture and the amount
537 of aromatic compounds in the final product is mostly controlled by the length of the ripening period. A
538 longer ripening time increases the duration of lipolysis and proteolysis in cheeses, leading to a softer
539 texture and the production of more pronounced aromatic compounds in the final product (Cantor *et al.*
540 2004). Although the general consumer demand is moving towards milder products (Michel Place,
541 L.I.P. S.A.S. pers. com.), producers aim to develop a range of products with varying degrees of flavor
542 to meet the diversity of consumer preferences (*i.e.* product segmentation). For example, the Carles
543 firm producing Roquefort PDO cheeses generated a new strain (called “Maxime”) producing milder

544 cheeses, with lighted blue color, called “Elegance” ([https://www.professionfromager.com/salon-](https://www.professionfromager.com/salon-virtuel/france/occitanie/article/carles)
545 [virtuel/france/occitanie/article/carles](https://www.professionfromager.com/salon-virtuel/france/occitanie/article/carles)). Another interest of producers is the visual aspect of the cheese,
546 which depends on the initial color of the spores and the ability of the strain to maintain this color in
547 conditioned atmospheres (Fairclough, Cliffe & Knapper, 2011). We identified QTLs for these traits,
548 indicating that selection for strain improvement and diversification should be possible, for example the
549 selection of strains with different colors, and proteolysis or lipolysis activities.

550 Breeding could thus be performed in *P. roqueforti* using crosses between different populations and
551 then back-crosses to retain only the desired new traits while purging deleterious mutations. It would
552 thus be possible to use the QTLs identified here for marker-assisted selection, as done in
553 *Saccharomyces cerevisiae* for malic consumption (Vion *et al.* 2021). As *Penicillium roqueforti* is
554 haploid, there is no issue of inbreeding depression and selection is particularly efficient, as there is no
555 dominance effect of masking alleles. Marker-assisted selection is all the more useful in *P. roqueforti*
556 as the assessment of cheese features requires a long and time-consuming process of cheese making
557 with multiple replicates in controlled environments (Caron *et al.* 2021).

558

559 **Conclusion**

560 In this study, we managed to generate F1 offspring from different crosses, including one between
561 parents from the two domesticated cheese populations, and obtained high variability in phenotypes in
562 progenies. This is crucial given the very low diversity currently available in each of the Roquefort and
563 non-Roquefort cheese populations. Despite genomic rearrangements and segregation biases due to
564 deleterious alleles, the QTL approach identified regions involved in important traits for cheese
565 making. The high heritabilities and the QTLs identified for all traits but growth indicate that selection
566 should be efficient for strain improvement. Segregation biases, linkage drag, pleiotropy and genomic
567 rearrangements may however reduce the efficacy of selection.

568 Further efforts could increase the number of offspring and use the Termignon cheese population
569 recently discovered in non-inoculated French cheeses produced at a small scale in the Alps, as this
570 population is genetically and phenotypically differentiated from the two other cheese populations

571 (Crequer *et al.* 2023). With a better understanding of the demands of consumers and cheese producers,
572 association genetics could become a very promising tool for the improvement of *P. roqueforti* strains,
573 increasing blue cheese quality and diversity. Few studies have addressed these questions in the
574 domestication of fungi so far, beyond yeasts and the button mushroom (Foulongne-Oriol *et al.* 2012;
575 Imbernon *et al.* 1996; Nguyen *et al.* 2022; Kessi-Pérez, Molinet & Martínez, 2020; Eder *et al.* 2018;
576 Wilkening *et al.* 2014; Swinnen, Thevelein & Nevoigt 2012; Liti & Louis, 2012; Steinmetz *et al.*
577 2002). From a fundamental point of view, identifying the genomic regions controlling traits of interest
578 to humans helps understand the genetic mechanisms behind the adaptation of organisms to their
579 environment. In particular, our findings of similar QTLs between independently domesticated *P.*
580 *roqueforti* lineages, with pleiotropic effects, indicate convergent adaptation targeting major
581 regulators.

582

583 **Materials and methods**

584 **Progeny production**

585 The production of progenies was obtained following the protocol in Ropars *et al.* (2014) with some
586 modifications. Six *P. roqueforti* strains (Table 1) were selected for their fertility as observed in a
587 previous study (Ropars *et al.* 2016) and based on their phenotypic differences estimated from
588 proteolysis and lipolysis measurements (Dumas *et al.* 2020). They were crossed in all possible pairs
589 given their mating types, either MAT1-1 (three strains) or MAT1-2 (three strains) (Table 1).

590 For each cross, three Petri dishes were inoculated on an oat medium supplemented with biotin after
591 sterilization (6.4 µg/L; Böhm *et al.* 2012). On each Petri dish, 5 µL of an uncalibrated spore
592 suspension was used to inoculate agar sections at opposite sites on the Petri dish, located perpendicular
593 to the sections inoculated with aliquots of conidia of the opposite sexual type (Figure 5 A; O'Gorman,
594 Fuller & Dyer, 2009). Petri dishes were sealed with food-grade plastic film and incubated upside down
595 at 19°C in the dark. The contact zone between strains (Figure 5 A) was examined with a binocular
596 loupe and an optical microscope regularly for four to six weeks to check for the presence of
597 cleistothecia (sexual structures). When cleistothecia were found, three to five of them were sampled,

598 crushed with tweezers, and observed under an optical microscope with lactophenol cotton blue dye
599 solution for ascospore detection (Figure 5 B and C). We took the pictures with a Nikon DS-Fi2
600 camera, on an inverted optical microscope with apodized phase contrast (Nikon France, Champigny-
601 sur-Marne, France). When ascospores were identified, all the cleistothecia of the corresponding Petri
602 dish were collected in a 0.05% Tween-20 water suspension (P1379-100mL Sigma Aldrich, Saint
603 Quentin Fallavier, France). Because cleistothecia are covered with conidia derived from asexual
604 reproduction, we developed a treatment to kill them before releasing ascospores from cleistothecia.
605 Approximately 500 μ L of the cleistothecia suspension was pooled with 500 μ L of a commercial 2.6%
606 sodium hypochlorite solution and gently agitated for 20 seconds; then 1 mL of water was added to stop
607 the sodium hypochlorite action. Cleistothecia were washed twice with one-minute centrifugation at
608 7,000 rpm, removal of the supernatant and resuspension in Tween 20. Then, we crushed cleistothecia
609 with a sterile piston (Piston Pellet Eppendorf, Montesson, France) and filtered residues with a 40 μ m
610 filter (cell screen, Greiner, Les Ulis, France). Single-ascospore isolation was carried out with a dilution
611 method on malt extract agar Petri dishes (MEA, Galloway and Burgess 1952).

612 **Recombinant offspring detection**

613 We chose five fertile crosses for further analyses (Table 1). Genomic DNA was extracted from fresh
614 mycelium and conidia suspension after single-ascospore isolation and growth for five days on MEA
615 media using the Nucleospin soil kit (Macherey-Nagel, Düren, Germany). For checking that isolated
616 spores were recombinant offspring and not asexual conidia, we used 11 microsatellite markers (Ropars
617 *et al.* 2014) labeled with fluorescence, out of which between five and nine were polymorphic
618 depending on the cross. These markers were amplified with the Multiplex PCR kit (Qiagen, Les Ulis,
619 France) using a touchdown program with an initial denaturation of 15 min at 95°C, 35 cycles of 30 s at
620 94°C, a decrease of 1°C every 90 s from 60 to 50°C, and 60 s at 72°C. The PCR program ended with a
621 final 30 min extension step at 60°C. Genotyping by capillary fractionation electrophoresis was
622 performed at INRAe Clermont-Ferrand (INRAe Platform GENTYANE, Clermont-Ferrand,
623 FRANCE). The profiles obtained were analyzed with the GENEMAPPER v4.0 software (Applied
624 Biosystem, Villebon-sur-Yvette, France) to detect recombination between parental genotypes. Only

625 strains with recombinant genotypes were retained, *i.e.* carrying alleles from one parent at some
626 markers and alleles for the other parent at other markers.

627 **Genome sequencing, assembly and analysis**

628 We generated long-read-based genome assemblies by sequencing the genomes of the LCP06136
629 parental strains, as well as 24 F1 offspring, with Oxford Nanopore MinION technology with an R9
630 flow cell, a high-quality genome assembly of the other parental strain (LCP06133) being available
631 (Crequer *et al.* 2023). We also generated genome assemblies for the other parents, based on Illumina
632 sequencing and assemblies guided by the Oxford Nanopore assemblies of the LCP06136 and
633 LCP06133 parents.

634 DNA was extracted from mycelium and conidia with the Nucleospin soil kit for the progeny and with
635 the NucleoBond High Molecular Weight DNA kit (Macherey-Nagel, Düren, Germany) for the
636 parental strain LCP06136, with mechanical disruption of about 30 mg of lyophilized mycelium with
637 two tungsten beads (3 mm diameter) for 5 min at 30 Hz. The Nanopore library was prepared with the
638 SQK-LSK109 ligation sequencing kit, starting with 1.5 µg DNA, and sequencing was performed in-
639 house with a MinION combined with a MinIT (version 19.06.9), a 72 hour run, and with the
640 Fastcalling basecalling algorithm. The genome of the LCP06136 strain was sequenced alone in a R9
641 flow cell, whereas the 24 offspring genomes were sequenced in a 12-plex with the Rapid Barcoding
642 Sequencing kit SQK-RBK004. We assessed run quality using Porechop v0.2.3_sequan2.1.1 (Wick *et*
643 *al.* 2017), and when necessary, demultiplexed the Nanopore raw reads with the default parameters.

644 *De novo* assemblies of the genomes were constructed from both Illumina and Nanopore reads. For the
645 LCP06136 parental genome, the raw Nanopore reads were trimmed and assembled with Canu v1.8
646 (Koren *et al.* 2017) with the option genomeSize=28m. The assembly obtained with Canu was polished
647 twice with Illumina reads (Dumas *et al.* 2020) using Pilon v1.24 (Walker *et al.* 2014) with the default
648 settings. For each round of polishing, Bowtie2 (Langmead and Salzberg, 2012; Langmead *et al.* 2019)
649 was used to align the Illumina trimmed reads with the assembly for polishing with a maximum length
650 (-X) of 1000bp. The Illumina reads were first trimmed with Trimmomatic v0.36 (Bolger, Lohse &

651 Usadel, 2014) with the following options: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:10
652 TRAILING:10 SLIDINGWINDOW:5:10 MINLEN:50. Redundant contigs were removed based on a
653 self-alignment using NUCmer version 3.1 (Kurtz *et al.* 2004). For other parental strains (LCP06037,
654 LCP06039, LCP06043, LCP06059), available Illumina reads (Dumas *et al.* 2020) were trimmed with
655 Trimmomatic v0.36 (Bolger *et al.* 2014) the same way as previously described, and assembled with
656 SOAPdenovo2 v2.04 (Luo *et al.* 2012) using the corresponding default size 23 k-mer length. The
657 assemblies obtained were then scaffolded using Ragout v2.3 with the LCP06136 and the LCP06133
658 genomes as references (Kolmogorov *et al.* 2018). For the 24 offspring genomes, the raw Nanopore
659 reads were trimmed and assembled both with Canu v1.8 (Koren *et al.* 2017) and with Flye version 2.9
660 (Kolmogorov *et al.* 2019), and then merged using Quickmerge (Solares *et al.* 2018). The merged
661 assemblies obtained were polished once with a concatenation of Illumina reads of the parental strain
662 LCP06136 and LCP06133 (Dumas *et al.* 2020) in the same way as for the LCP06136 parental
663 genome. The obtained assemblies were scaffolded using Ragtag v2.1.0 (Alonge *et al.* 2022). We
664 evaluated the quality of the final assemblies with Quast v5.0.2 (Gurevich *et al.* 2013) and their
665 completeness with BUSCO v5.3.2 (Manni *et al.* 2021) using the eurotiales_odb10 lineage dataset.
666 Based on the quality and completeness, we selected 11 out of the 24 offspring strain assemblies for
667 further analyses.

668 Transposable elements (TEs) were annotated using the REPET package
669 (<https://urgi.versailles.inra.fr/Tools/REPET>). The TEdenovo pipeline (Flutre *et al.* 2011) was used to
670 detect repeated elements in LCP06136 and LCP06133 (<https://doi.org/10.57745/SIP7CH>) genomes,
671 and to provide consensus sequences. These consensus sequences were classified with PASTEC v1.3
672 (Hoede *et al.* 2014), based on the Wicker hierarchical TE classification system (Wicker *et al.* 2007)
673 and manually curated. The resulting bulk library of consensus sequences was then used to annotate TE
674 copies in the six whole genomes (namely, LCP06136 and LCP06133, LCP06037, LCP06039,
675 LCP06043, LCP06059) using the TEannot pipeline (Quesneville *et al.* 2005). Repeat-induced point
676 mutations (RIP) in assemblies were detected using the web-based tool The RIPper (Van Wyk *et al.*
677 2019).

678 All final assemblies were formatted (contigs being renamed and ordered in descending order of size),
679 and repeats of the LCP06133 and LCP06136 parental genomes were masked with RepeatMasker
680 v4.1.2 (Smit et al 2013) after *de novo* repeat detection using RepeatModeler v2.0.2 (Smit and Hubley
681 2008). We performed gene annotation on the masked assemblies of the parental strain LCP06136
682 using the Funannotate v1.8.9 pipeline (Palmer and Stajich 2020), with Braker v2.1.6 (Brůna *et al.*
683 2021; Hoff et al. 2016; Hoff *et al.* 2019; Stanke *et al.* 2006; Stanker *et al.* 2008), which uses a
684 combination of the *ab initio* gene predictors Augustus and GeneMark-ET (Lomsadze et al. 2014) with
685 NCBI blast (Altschul *et al.* 1990) and blast+ (Camacho *et al.* 2009). We ran BRAKER twice, first
686 using the BUSCO dataset of proteins eurotiales_odb10 (Manni *et al.* 2021) with the ProtHint pipeline
687 (Brůna et al. 2020; Buchfink et al. 2015; Gotoh et al. 2014; Iwata and Gotoh, 2012; Lomsadze *et al.*
688 2005), and then using the RNA-seq dataset from Punt *et al.*(2020) and mapped using STAR v2.5.4b
689 (Dobin *et al.* 2013) with the default settings. We combined the results of the two BRAKER runs with
690 TSEBRA (the Transcript Selector for BRAKER; <https://github.com/Gaius-Augustus/TSEBRA>).

691 We studied synteny between assemblies by investigating collinearity of one-to-one orthologs along
692 whole genome alignments, using nucmer v3.1 (Kurtz *et al.* 2004), with 500pb as the minimum match
693 length. We visualized synteny between genomes by plotting one-to-one ortholog links using Circos
694 v0.69-6 (Krzywinski *et al.* 2009).

695 **Phenotyping**

696 We selected four traits for their applied interest in cheesemaking that are easily measurable with
697 precision and at high throughput, for which we assessed the phenotypes in all the isolated offspring
698 obtained for the five analyzed crosses. The growth rate was measured using a ScanStation
699 (Intersciences, Mourjou, France), an incubator for temperature-controlled growth (set at at 22.5°C)
700 taking pictures regularly for each of 100 Petri dishes as well as analyzing images. We spread 5 µL of
701 standardized spore suspension (250 spores/inoculation) on each Petri dish, containing malt medium
702 (Biokar BK045 HA) at 15 g.L⁻¹ in source water (Cristalline, Chelles, France), sterilized at 120°C
703 during 15 minutes. The ScanStation took pictures of the Petri dishes right side up on a black

704 background, for five days every 30 minutes, *i.e.* 240 measures per strain (Figure 5 D). The ScanStation
705 estimated the colony area and diameter for each picture, by counting the number of pixels
706 corresponding to the fungal colony. Because the colony diametral growth was near linear when plotted
707 against days (Supplementary Figure 6), we used, as a measure of growth rate, the slope of the least
708 square linear regression computed with the *lm* function of the base package R software v4.3.2
709 (<http://www.r-project.org/>). The coefficients of determination (r^2), measuring the fit of the data to a
710 line, are presented in the Supplementary Table 5 and the linear regression of an offspring with a
711 median r^2 in the Supplementary Figure 6. To ensure the robustness of the linear fit, strains with
712 coefficients of determination below 0.98 were not considered in the QTL analysis of this trait (21 of
713 885, *i.e.* 2.4% of strains filtered out).

714 The colony color was measured after growth on raw sheep milk powder medium (Biocoop, Paris,
715 France) at 21% in source water, sterilized at 105°C for five minutes, and mixed with 1.7% agar in
716 source water, sterilized at 121°C for 15 minutes. After 13 days of growth, two pictures were taken by
717 the ScanStation under standardized conditions of light and on a white background (Figure 5 F). We
718 recorded for each strain the colony color decomposition using RGB (red, green and blue) and HSB
719 (hue, saturation, and brightness) with ImageJ v1.52n (Schneider 2012; Supplementary Table 6). In the
720 RGB system, the levels of red, green and blue are represented by a range of integers from 0 to 255
721 (256 levels for each color). In the HSB system, hue ranges from 0 to 360 (0 is red, 120 is green, 240 is
722 blue), saturation and brightness from 0 to 100% (0% saturated is neutral grey, 100% saturated is the
723 full color ; 0% brightness is black, 50% brightness is normal, 100% brightness is white). In order to
724 obtain uncorrelated components from the RGB data, we divided each value by the sum of the three
725 components. For each of these parameters, we recorded average values across all the colony pixels for
726 each strain. For QTL detection, we chose *a posteriori*, among these color parameters, those whose
727 distributions and heritabilities were optimal for QTL analysis (*i.e.* largest and uncorrelated variance);
728 we thus retained the RGB proportion, as well as HSB.

729 The lipolytic and proteolytic activities of *P. roqueforti* strains were measured *in vitro* following
730 Dumas *et al.* (2020): for each strain, 50 μ l of standardized spore suspensions (ca. 2,500

731 spores/inoculation) were inoculated at the top of a test tube containing agar and tributyrin for lipolytic
732 activity measure (10 mL.L⁻¹, ACROS Organics) or semi-skimmed cow milk for the proteolytic activity
733 measure (40 g.L⁻¹, Casino). The lipolytic and proteolytic activities were estimated by the degree of
734 compound degradation, which changes the media from opaque to translucent. We measured the
735 distance between the initial mark and the hydrolyzed, translucent front, after 7, 14, 21 and 28 days of
736 growth at 20°C in the dark (Figure 5 E). As lipolysis curves were nearly linear when plotted against
737 days, we estimated the lipolysis rate with a linear regression computed with the R base package
738 function *lm*. Proteolysis profiles appeared less linear but with no clear sigmoid nor other regression
739 patterns, so we also chose to fit linear models for more simplicity. The coefficients of determination
740 (r^2), measuring the fit of the data to a line, are presented in the Supplementary Table 5 and an example
741 of a linear regression of an offspring with a median r^2 in the Supplementary Figure 6.

742 For extrolite production, we grew fungal cultures in 24-well sterile microplates containing 2 mL of
743 yeast extract sucrose (YES) agar medium buffered at pH 4.5 with phosphate-citrate buffer and
744 characterized by a high C/N ratio to favor extrolite production as previously described (Frisvad &
745 Filtenborg, 1983). For each strain, 1 μ L of a calibrated spore suspension (10⁶ spores.mL⁻¹) prepared
746 from a 7-day culture was inoculated in the centre of the well. Two replicates per strain were performed
747 for extrolite analyses. The plates were incubated at 25°C in the dark for seven days and then stored at -
748 20°C until extrolite analysis. For extrolite extractions, we used an optimized “high-throughput”
749 extraction method (Lo *et al.* 2023; Crequer *et al.* 2024). Briefly, 2g-aliquots (the entire YES culture
750 obtained from a well) were homogenized after thawing samples with a sterile flat spatula then 12.5 mL
751 of acetonitrile (ACN) supplemented with 0.1% formic acid (v/v) was added, samples were vortexed
752 for 30 sec followed by 15 min sonication. Extracts were again vortexed before being centrifuged for
753 10 min at 5000g at 4°C. The supernatants were directly collected and filtered through 0.45 μ m PTFE
754 membrane filters (GE Healthcare Life Sciences, UK) into amber vials and stored at -20°C until
755 analyses. Extrolite detection and quantification were performed using an Agilent 6530 Accurate-Mass
756 Quadrupole Time-of-Flight (Q-TOF) LC/MS system equipped with a Binary pump 1260 and degasser,
757 well plate autosampler set to 10°C and a thermostated column compartment. Filtered 2 μ L aliquots

758 were injected into a ZORBAX Extend C-18 column (2.1x50 mm and 1.8 μm , 600 bar) maintained at
759 35°C with a flow rate set to 0.3 mL.min⁻¹. The mobile phase A contained milli-Q water + 0.1% formic
760 acid (v/v) and 0.1% ammonium formate (v/v) while mobile phase B was ACN + 0.1% formic acid.
761 Mobile phase B was maintained at 10% for 4 min, followed by a gradient from 10 to 100% for 18 min.
762 Then, mobile phase B was maintained at 100% for 5 min before a 5-min post-time. Samples were
763 ionized in positive (ESI+) electrospray ionization modes in the mass spectrometer with the following
764 parameters: capillary voltage 4 kV, source temperature 325°C, nebulizer pressure 50 psig, drying gas
765 12 L.min⁻¹, ion range 100-1000 m/z. Target extrolite characteristics used for quantifications are given
766 in Supplementary Table 7 and included commercially available extrolites produced by *Penicillium*
767 species, namely andrastin A, eremofortins A & B, (iso)-fumigaclavin A, mycophenolic acid and
768 roquefortine C. Andrastin A, eremofortins A & B and (iso)-fumigaclavin A standards were obtained
769 from Biovotica (Goettingen, Germany), while all others were from Sigma-Aldrich (St Louis, MO,
770 USA). All stock solutions were prepared in dimethyl sulfoxide (DMSO) at 1 mg.mL⁻¹ in amber vials.
771 As the PR toxin was not commercially available, a previously produced purified stock solution (Gillot
772 *et al.* 2017) without known concentration was used to ensure MPA (mycophenolic acid) and PR toxin
773 were separated (as they have the same mass) as well as linearity of PR toxin quantification. For these
774 analyses, metabolite identification was performed using both the mean retention time \pm 1 min and the
775 corresponding ions listed in Supplementary Table 7. We used a matrix-matched calibration curve (r^2
776 >0.99 for all extrolites except 2 >0.96) to confirm linearity the relation between signal area and
777 extrolite concentration with final concentrations ranging from 1 to 10000 ng.mL⁻¹ according to the
778 target metabolite and method performance was carried out as previously described (Gillot *et al.* 2017).

779 **Statistical analysis on phenotypes**

780 For phenotype distributions, we performed tests of normality with the *shapiro.test* function of the base
781 package of R software v4.3.2 (<http://www.r-project.org/>). We ran tests for unimodality with the
782 *dip.test* function of the Diptest package v0.75-7 (Maechler 2016) and tests for bimodality with the
783 *bimodality_coefficient* function of the Mousetrap package v3.2.1 (Kieslich *et al.* 2019) with a
784 threshold of 0.555 according to Pfister *et al.* 2013. We performed the trimodality and amodality tests

785 with the *is.trimodal* and *is.amodal* functions, respectively, of the LaplacesDemon v16.1.6 package
786 (Hall *et al.* 2021). We computed the average heterosis as $((F1 - MP) / MP) * 100$, F1 and MP being the
787 trait mean values of the progeny and the parents, respectively.

788 We computed the matrix of Pearson and Spearman correlation coefficients with the R stats package
789 v4.3.2 (<http://www.r-project.org/>). We produced the violin plots using vioplot v0.4.0 (Adler *et al.*
790 2022), ggplot2 v3.4.3 (Wickham 2016) and reshape2 v1.4.4 (Wickham 2007) R packages. We
791 performed the principal component analysis (PCA) using the function *PCA* from the R base package,
792 assigning trait means to missing values. Plots were drawn using FactoMineR v2.8 (Lê *et al.* 2002),
793 factoextra v1.0.7 (Kassambara and Mundt 2020) and corrplot v0.92 (Wei and Simko 2021) packages.

794

795 **Phenotype heritability**

796 Because we could not perform replicated measurements of phenotypes for each strain for lipolysis,
797 proteolysis, growth rate and color, we only estimated the narrow sense heritability (h^2) of these
798 phenotypes across the five progenies by estimating the slope of linear regressions of the five F1
799 progenies mean trait values on the mean values of the parental pairs using the *lm* function from the R
800 base package. Extrolite production was determined for three crosses (SxN, RxN and RxL), with two
801 replicates for each strain; we therefore estimated broad-sense heritability (H^2) for these phenotypes for
802 each cross based on variability among replicates, in addition to narrow-sense heritability as for the
803 other phenotypes. Broad sense heritability was determined by performing a one-way ANOVA with the
804 *lm* function of the stats v4.2.1 package in R.

805 **Genotyping based on indels**

806 We genotyped 389 offspring from the cross RxN between LCP06136 (Roquefort; MAT1-1;
807 <https://www.ebi.ac.uk/SAMEA103939766>) and LCP06133 (non-Roquefort; MAT1-2;
808 SAMEA103939763) with 200 markers with a polymorphism of amplification size between the two
809 parental strains, due to the presence of indels (Supplementary Table IND). Primers were designed (i)
810 to yield an amplicon size between 150 to 410 bp and a difference ranging from six to 20 bp between

811 alleles, (ii) to be compatible in multiplex and (iii) to be evenly distributed along the assembly of the
812 reference genome available at the beginning of the study (FM164; EMBL accession numbers
813 HG792015 to HG792062, Cheeseman & Ropars *et al.* 2014). Indels were detected after a whole
814 assembly alignment (blast v2.9.0; Altschul *et al.* 1990) of the two parental strains with the gap and
815 extension penalties set to 1. After adding 250 base pairs upstream and downstream from the hit
816 position, we performed a multi-alignment using MAFFT (Kato 2002) with the whole genomes of the
817 parental strains of the other crosses. We only kept a consensus sequence generated using consambig
818 (Rice *et al.* 2000) and designed primers using Primer3 (Untergasser *et al.* 2012).

819 To reduce the cost of fluorescence primers, we only used four universal fluorescent primers (M13(-
820 21), D8S1132f, D12S1090f, DYS437f), labeled with different fluorescent dyes (FAM, ATTO550,
821 ATTO565, YAKIMA Yellow; Eurofins, Ebersberg, Germany). The specific primers attached during
822 PCR to universal fluorescent primers thanks to a tail added to each specific primer matching an edge
823 of the universal primers (Schuelke 2000; Missiaggia and Grattapaglia 2006). For each locus, the PCR
824 amplification thus required three primers: the forward fluorescent universal primer, the forward
825 specific primer with a 5' tail corresponding to the chosen universal primer and the regular specific
826 reverse primer. The 200 markers were genotyped in 25 multiplex groups of 8 locus organized
827 according to their amplicon size and their color fluorescent dye (Supplementary Table 8). For each
828 multiplex, two separate sets of quadruplex PCR reactions were performed to minimize the interaction
829 between primers and then pooled into octoplexes for the genotyping step. The PCR mix contained 0.16
830 μM of each of the fluorescent universal primer and of the reverse specified primer and 0.04 μM of the
831 5' tail forward primer in a final 15 μl reaction volume (2x QIAGEN Multiplex PCR Master Mix with
832 3 mM Mg^{2+} , 10x primer mix with 1.6 or 0.4 μM of each primer, 3 μl of DNA diluted 50 fold) using the
833 same PCR cycle conditions as for the microsatellites loci previously described in the recombinant
834 offspring detection paragraph, except that it started with a hybridization temperature of 62°C instead
835 of 60°C. Genotyping and analysis were also done as previously described in the recombinant offspring
836 detection paragraph.

837 **Genotyping based on GBS**

838 For all five crosses, we genotyped the obtained progeny (1073 offspring in total) at the AGAP CIRAD
839 platform with genotyping-by-sequencing using the *ApeKI* enzyme. DNAs were purified after digestion
840 on QIAquick columns (Qiagen, Les Ulis, France) and their qualities were controlled using a
841 TapeStation instrument (Agilent Technologies, Les Ulis, France). Sequencing was performed on an
842 Illumina HiSeq 4000 (2x150 bp). The following numbers of recombinant offspring were genotyped by
843 GBS: 384 for the cross RxN, 157 for the cross RxS, 185 for the cross RxL, 176 for the cross SxN and
844 171 for the cross LxN.

845 Demultiplexed reads were mapped to the LCP06136 high-quality genome for the crosses RxL and
846 RxS and to the LCP06133 high-quality genome for the crosses RxN, LxN and SxN using *Bowtie2*
847 version 2.3.4.1 (Langmead and Salzberg 2012; Langmead *et al.* 2019). In *Bowtie2*, we set the
848 maximum length (-X) to 1000 and used the preset “very-sensitive-local”. We used SAMtools v1.7
849 (Danecek *et al.* 2021) to sort and filter out duplicate reads and reads with a mapping quality score
850 above ten for SNP calling. Single nucleotide polymorphisms (SNPs) were called using the GATK
851 v4.1.2.0 Haplotype Caller (McKenna *et al.* 2010), generating a gVCF file per strain with option ploidy
852 2 to detect potential duplicated regions in progenies, as we had found indels with two alleles in some
853 offspring. GVCFs were combined using GATK CombineGVCFs, genotypes with GATK
854 GenotypeGVCFs, and biallelic SNPs were selected after filtration using GATK SelectVariants. We
855 filtered SNPs using GATK VariantFiltration and options QUAL <30, DP < 5, QD < 20.0, FS > 60.0,
856 MQ < 40, 0.05<AF<0.95, SOR > 3.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, DP>5.0 and
857 GQ<10. We generated the genotype matrix based on the VCF file with *Bcftools* v1.11 (Li 2011;
858 Danecek *et al.* 2021). Markers with no data or with no differences between the two parental genomes
859 were filtered out. To reconstruct offspring genotypes, we performed SNP calling in the diploid mode
860 in order to detect double copies of translocated regions. Markers corresponding to the translocated
861 regions were in fact called as heterozygous in some offspring; they were therefore placed in offspring
862 at the two parental locations, which were each assigned the corresponding parental allele.

863 After performing crosses and isolating ascospores, we detected sectors of two slightly different
864 morphotypes in subsequent cultures of the parental strain LCP06039 aiming at phenotype

865 measurements. However, genetic data indicated that the cross was performed with a pure strain and
866 not a mixture: (i) the indel genotypes of the two morphotypes were assessed and were identical, and
867 (ii) the GBS genotypes of the two morphotypes were not typed directly, but we did not detect genome-
868 wide segregation distortion in the progeny in GBS data for this cross, while a cross involving a
869 mixture of strains with different genotypes should result in over-representation of the alleles by the
870 pure parent; on the contrary, the segregation distortions detected in some genomic regions
871 corresponded to over-representations of the strain showing two morphotypes rather than its under-
872 representation. We therefore considered that a single strain contributed to the cross and that the two
873 morphotypes were the result of plasticity or a few somatic mutations in a single strain. We therefore
874 built the genetic map and ran a QTL analysis for the LxN cross. For analyses involving parental traits,
875 and therefore potentially affected by the presence of two morphotypes (*i.e.* for heritability estimates
876 and transgression detection) we computed estimates using all three possible values for the LCP06039
877 parent strain phenotype: the means of the trait values for the two morphotypes or the value of one of
878 the two morphotypes. This did not affect conclusions as the two morphotypes were actually very close
879 for all trait measures.

880 **Experiment on the stability of genotypes across replication**

881 As we identified a genomic region with two alleles present in multiple offspring in the RxN cross with
882 associated QTLs, we wanted to check whether this particular genotype was stable during strain culture
883 and across multiple replication steps. We therefore cultivated, on malt agar, the two parental strains
884 from the RxN cross and the 11 offspring for which genomes were sequenced with Nanopore, six with
885 alleles of both parents for this genomic region, four with the LCP06136 parental allele and one with
886 the LCP06133 allele. We cultivated the strains for one week on malt agar and replated them on a new
887 plate by transplanting some conidia from the edge of the colonies. We performed 19 replication steps.
888 In the end, we re-genotyped the lines with the six indel markers present in the rearranged region.

889 **Linkage map and QTL detection**

890 Obtained offspring phenotypes are presented in Supplementary Table 9 and offspring genotype are
891 presented in Supplementary Tables 10, 11, 12, 13 and 14 for SxN, LxN, RxN, RxS and RxL crosses
892 respectively. The genetic map and statistical tests for QTL association were performed using the R
893 software v3.5.1 (<http://www.r-project.org/>) with the ASMap v1.0-5 package (Taylor and Butler 2017)
894 for the genetic map and qtl v1.60 package (Broman *et al.* 2003) for statistical tests. For the genetic
895 map, we used unique segregating markers with less than 10% missing data and markers with
896 segregation distortion with significant Chi Square p-values adjusted with Bonferroni corrections.
897 Individuals with more than 30% of missing data were filtered out (Table 1), concerning up to 14% of
898 the offspring of a cross. Linkage groups and marker ordering were determined with the *mstmap*
899 function of AsMap package, with parameter “bychr” set as FALSE and a p.value of 10^{-12} . Markers with
900 less than 20% missing data and less than 95% of one parental allele were then reintegrated in linkage
901 groups with the *pull.cross* function with “max.rf” and “min.lod” parameters set at 0.1 and 20,
902 respectively. We reconstructed marker order in linkage groups with the *mstmap* function, the
903 parameter “bychr” set as TRUE, and a p.value < 10^{-12} . We estimated genetic distances between markers
904 with the AsMap *quickEst* function and the Kosambi's mapping function. We compared the obtained
905 order to the reference genome order using the *compareorder* function of the qtl package with error
906 probability set at 0.005. We changed the order only if it improved the LOD score and minimized the
907 linkage group length. We represented the relationships between chromosome physical positions and
908 genetic maps using ALLMAPS in JCVI utility libraries v1.3.6 (Tang *et al.* 2015), adapted to include
909 genetic features.

910 We performed single QTL detection using the *scanone* function of the qtl package v1.6 with the
911 threshold at 5% being estimated with 1000 permutations. We used the *addqtl* function of the qtl
912 package to mask the first set of QTLs and thus detect additional QTLs that may have been hidden by
913 the main ones. We estimated confidence intervals for QTL using the *lodint* function of the qtl package
914 with default parameters. We identified QTL interactions using the *scantwo* function with the Haley-
915 Knott method using a threshold at 5% estimated by 1,000 permutations. We performed analyses of
916 variance (ANOVAs) using a linear model with the *fitqtl* function of the qtl package, keeping

917 interactions between loci only when they were significant in both *scantwo* and the full model
918 AVOVAs. We extended QTL confidence intervals including the first or last marker of a linkage group
919 to the start or end of the corresponding chromosome. For comparisons of QTL interval positions
920 between crosses, we transposed the intervals determined with the positions on the Roquefort parent
921 genomes (for RxL and RxS crosses) onto the non-Roquefort genome. To do this, we extracted genome
922 sequences using bedtools getfasta v2.30.0 (Quinlan and Hall 2010) and mapped the extracted
923 sequences onto the non-Roquefort parent genome using minimap2 v2.17-r941 (Li 2018). The
924 representation of the aligned QTL intervals was constructed using the karyoplotR v1.24.0 package
925 (Gel and Serra 2017).

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936

937 **Author contribution**

938 TG, TC, AB, DR, CC and MP designed the study. TG, DR, ECo and MP acquired funding. TC, AS
939 and SLP performed crosses, isolated strains and extracted DNAs, supervised by TG. TC, MLP and SB
940 performed experiments for phenotyping lipolysis, proteolysis and growth rates supervised by CC, DR
941 and MP. TC, ASn and SLP performed genotyping and genome sequencing, supervised by TG. ECr
942 and GC performed experiments for estimating the production of extrolites supervised by MC. TC and
943 ASn performed the experiment on the stability of genotypes across replication and the Oxford

944 Nanopore sequencing, supervised by TG. TC, ECr, ASi and RRdIV performed the genomic analyses,
945 supervised by TG, MFO and AB. TC, TG and ECr wrote the manuscript. All authors revised the
946 manuscript.

947 **Data availability**

948 All accession numbers will be available upon manuscript acceptance.

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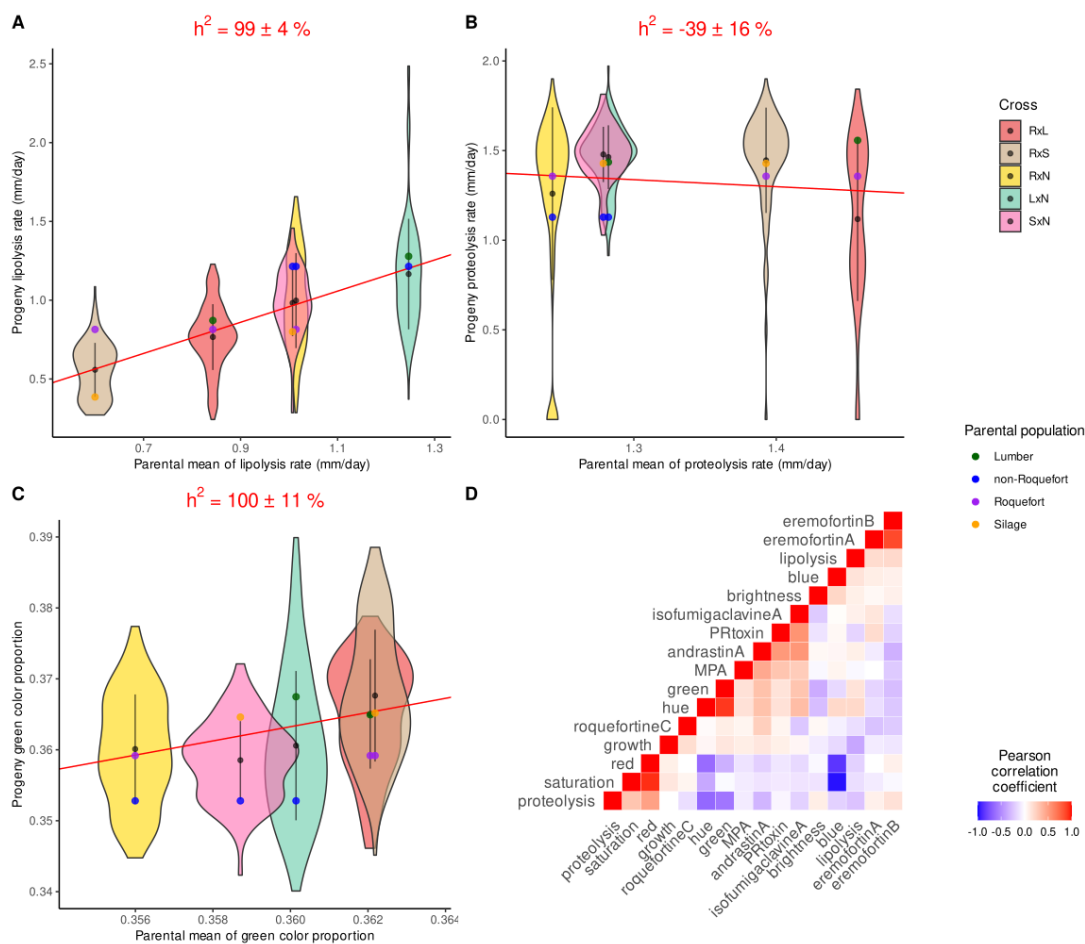
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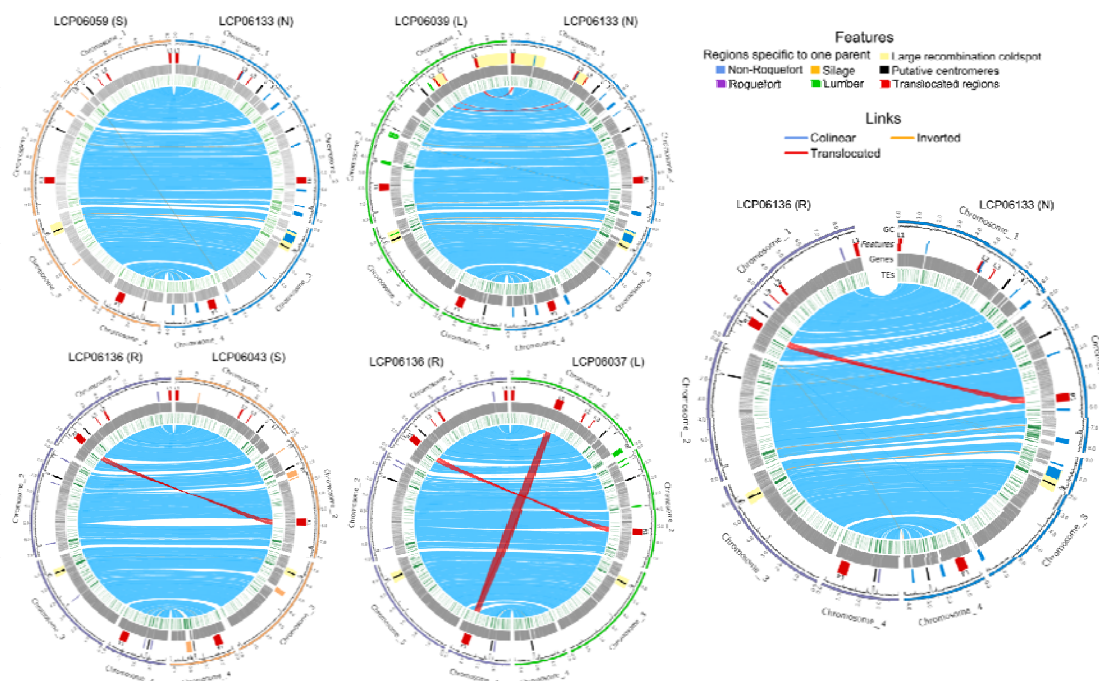
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1415

1416 Figure 1: Distributions of phenotypes in the five progenies in *Penicillium roqueforti* as a function of
 1417 their parental means, for (A) the lipolysis rate (mm/day), (B) the proteolysis rate (mm/day) and (C) the
 1418 relative green color channel of the progeny; the parental means are plotted along the x axis (RxL in
 1419 red, RxS in beige, RxN in yellow, LxN in turquoise and SxN in pink). The black point and line
 1420 represent the mean and standard deviation, respectively, for each progeny. The colored points
 1421 represent the parental values (lumber in green, non-Roquefort in blue, Roquefort in purple, silage in
 1422 orange). The red line represents the linear regression across progenies, from which the slope is used to
 1423 estimate the heritability (h^2) value given at the top. (D) Pearson correlation coefficients, as a color
 1424 gradient, between trait values across offspring and crosses.

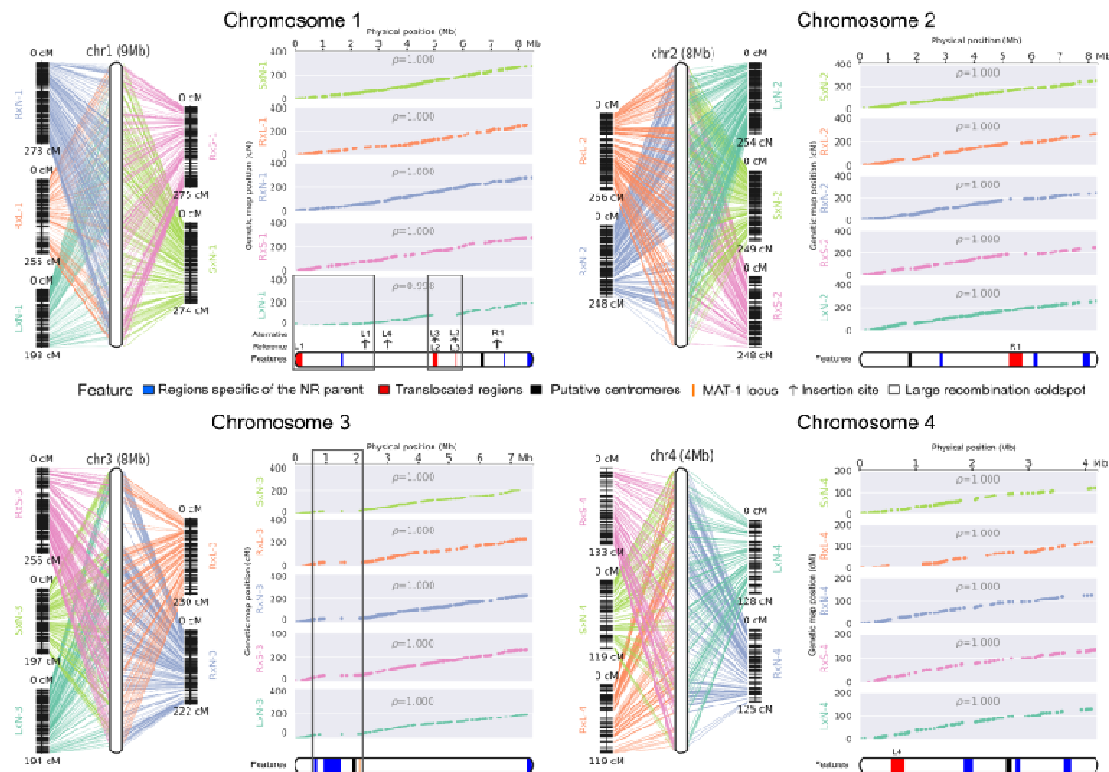
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1427 Figure 2: Genome comparisons between parental strains for each cross. Chromosomes are represented
1428 with colors corresponding to the population-of-origin of the strains: light orange, blue, purple and
1429 green for silage (S), non-Roquefort, (N) Roquefort (R) and lumber (L) populations, respectively. From
1430 outside to inside, the tracks represent the i) GC content, ii) translocated regions of more than 50kb
1431 between parental genomes in red (with their IDs, the first letter indicating the population-of-origin of
1432 the parent in which the translocation occurred), specific regions that are lacking in other genomes (*i.e.*
1433 acquired horizontally transferred regions) with their color indicating the populations in which they are
1434 present, putative centromeres in black, and large recombination cold spots in light yellow, iii)
1435 predicted genes in grey and iv) transposable elements in green. Orthologs between genomes are linked
1436 one to each other, with blue, orange and red links, indicating synteny, inversions or >50kb
1437 translocations, respectively.

1438



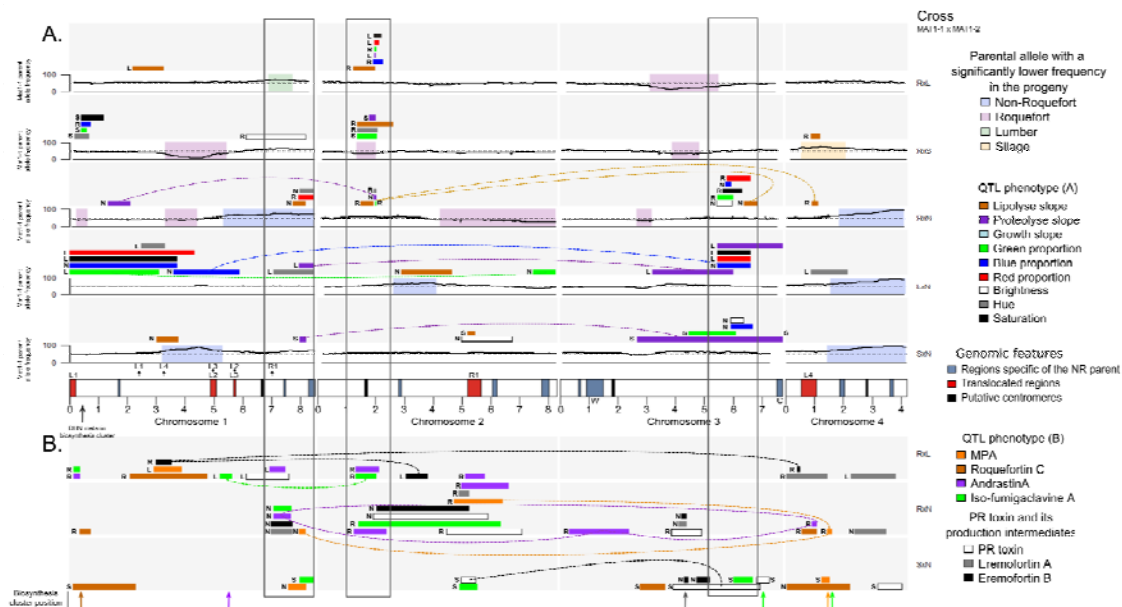
1439

1440 Figure 3: Genetic and physical maps for the four chromosomes of *Penicillium roqueforti*, obtained by
 1441 analysing five inter-population crosses. Each panel corresponds to an individual chromosome, line
 1442 graphs on the left represent linkage groups (one color per cross, with cross IDs as in Table 1; note that
 1443 different parents were used for the silage and lumber populations between crosses); the lines link
 1444 markers to chromosomes of the reference genome (LCP06133 non-Roquefort parent). In each panel,
 1445 plots on the right represent the relationship between genetic and physical distance, with one plot per
 1446 cross and the same color code as in left panels. At the bottom of each panel, the reference chromosome
 1447 is represented, with filled black, red and blue rectangles representing the putative centromeres, the
 1448 translocated regions and the horizontally transferred regions of more than 50kb when present,
 1449 respectively. The red translocated regions are labelled as in Figure 2. The orange bar in chromosome 3
 1450 represents the mating-type locus position. The empty black rectangles indicate large recombination
 1451 cold spots situated between translocated or parent-specific regions.

1452

1453

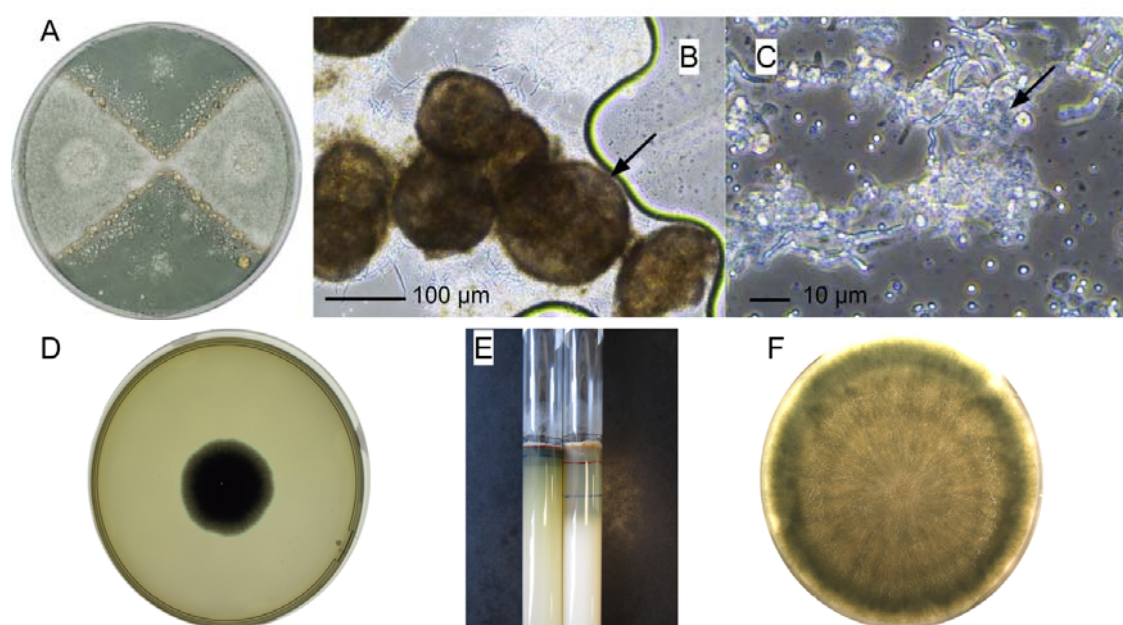
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1456 Figure 4: Representation of identified quantitative trait loci (QTLs) and segregation distortion along
 1457 the four chromosomes of *Penicillium roqueforti* in five progenies. Only QTLs explaining more than
 1458 5% of the total variance are represented. The color of the bars indicates the considered trait while the
 1459 letter indicates the parent having the alleles increasing the trait value. Dotted lines link the QTLs with
 1460 significant interactions and color indicates the phenotype. The cross ID is indicated on the right, the
 1461 first parent carrying the MAT1-1 mating type. (A) QTLs identified for phenotypes linked to lipolysis,
 1462 proteolysis, growth and colony color for the five crosses (in lines, indicated on the right with the same
 1463 code as in Table 1, note that different parents were used for the silage and lumber populations between
 1464 crosses). Black curves represent the proportion in progenies of the allele from the parent carrying the
 1465 MAT1-1 mating type. Transparent colored rectangles on curves indicate regions with significant
 1466 segregation distortion, their color corresponding to the under-represented parental allele: blue, purple,
 1467 green and yellow for non-Roquefort, Roquefort, lumber/spoiled food and silage/spoiled food parents,
 1468 respectively. The x-axis represents genomic physical positions. At the bottom, the four chromosomes
 1469 of the reference genome (LCP06133) are represented, with rectangles representing genomic features:
 1470 large horizontally transferred regions specific to the reference genome in blue, and translocated
 1471 regions in the reference genome in red (with same IDs as in Figure 2). The location of the horizontally

1472 transferred regions (W for *Wallaby* and C for *CheesyTer*) are indicated. The location of the
1473 dihydroxynaphthalene (DHN) melanin production cluster is indicated by a black arrow. The large
1474 vertical empty black rectangles indicate pleiotropic QTL regions, with effects on multiple traits. (B)
1475 QTLs identified for extrolite production (with colors corresponding to the different toxins), analyzed
1476 in three crosses (SxN, RxN and LxN). The locations of the gene clusters of the toxin biosynthesis
1477 pathways are indicated by arrows with the same color code (the PR toxin cluster being in gray,
1478 eremofortin A and B being intermediates).
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1482 Figure 5: Observations of *Penicillium roqueforti* crosses and examples of phenotypic trait
1483 determination. (A) Petri dish with a cross between the LCP06136 (MAT1-1; Roquefort; at the top and
1484 bottom) and LCP06173 (MAT1-2; non-Roquefort; at left and right) strains. Sexual structures are
1485 formed at the contact zones between the two strains. (B) Cleistothecia (sexual structures of *Penicillium*
1486 *roqueforti*) shown by black arrow. (C) Asci containing ascospores shown by the black arrow. (D) Petri
1487 dish used for estimating colony growth rate, by counting the number of pixels occupied by the fungal
1488 colony (one offspring grown on malt medium for 120 hours). (E) Two lipolysis test tubes showing the
1489 lysis dynamics, for two different strains, with marks on the tubes indicating the limit of lipid medium

1490 degradation at different times (blue, red and blue marks, drawn at 0, 7 and 14 days, respectively). (F)

1491 Petri dish on which an offspring grew on raw sheep milk medium during 13 days for color measure.

1492

1493 Table 1: The five crosses performed using six strains of *Penicillium roqueforti* from different populations (R for Roquefort, N for non-Roquefort, L for
 1494 lumber/food spoiler, S for silage/food spoiler) with opposite mating types (MAT1-1 and MAT1-2), with the number of isolated and phenotyped offspring and
 1495 the number of marker obtained from GBS genotyping. LCP: « Laboratoire de Cryptogamie, Paris ». *The strain LCP06039 was found to be a mixture of two
 1496 morphotypes ("dark" and "light"), which was detected after the cross had been performed. ¤These offspring have been genotyped with indels, in addition to
 1497 GBS, as all other offspring.

| Cross | MAT1-1 | | MAT1-2 | | Ascospores isolated | Offspring GBS-genotyped | Offspring growth-phenotyped | Offspring color-phenotyped | Offspring lipolysis phenotyped | Offspring proteolysis phenotyped | Offspring phenotyped (mycotoxins) | Offspring used for map construction | Number of marker |
|-------|---------------------|-----------|---------------------|----------|---------------------|-------------------------|-----------------------------|----------------------------|--------------------------------|----------------------------------|-----------------------------------|-------------------------------------|------------------|
| | Population | Strain | Population | Strain | | | | | | | | | |
| RxN | Roquefort | LCP06136 | non-Roquefort | LCP06133 | 389 | 384¤ | 307 | 311 | 387 | 382 | 274 | 358 | 2462 |
| RxS | Roquefort | LCP06136 | silage/food spoiler | LCP06043 | 157 | 157 | 157 | 157 | 157 | 156 | 0 | 148 | 2902 |
| RxL | Roquefort | LCP06136 | lumber/food spoiler | LCP06037 | 185 | 185 | 129 | 131 | 150 | 141 | 120 | 159 | 2943 |
| SxN | silage/food spoiler | LCP06059 | non-Roquefort | LCP06133 | 176 | 176 | 92 | 92 | 150 | 150 | 75 | 160 | 2860 |
| LxN | lumber/food spoiler | LCP06039* | non-Roquefort | LCP06133 | 171 | 171 | 91 | 91 | 150 | 151 | 0 | 165 | 2618 |

1498

1499

1500 Table 2: The five crosses performed using six strains of *Penicillium roqueforti* from different
 1501 populations (R for Roquefort, N for non-Roquefort, L for lumber/food spoiler, S for silage/food
 1502 spoiler), the linkage group and the total, the number of markers used for genetic map
 1503 construction, the total length of the linkage group, the average and maximum spacing in cM
 1504 between markers, the mean recombination rate in cM/Mb, and the reference chromosome
 1505 and genome size in Mb.

| Cross | Linkage group | Number of markers | Mean number of crossing over | Length (cM) | Average spacing (cM) | Maximum spacing (cM) | Mean recombination rate (cM/Mb) | Reference size (Mb) |
|---------------------|---------------|-------------------|------------------------------|-------------|----------------------|----------------------|---------------------------------|---------------------|
| SxN | L.1 | 213 | 2.74 | 274 | 1.3 | 8.8 | 32.21 | 8.51 |
| | L.2 | 242 | 2.49 | 249 | 1 | 12.8 | 29.97 | 8.31 |
| | L.3 | 189 | 1.97 | 197 | 1 | 8.8 | 25.51 | 7.72 |
| | L.4 | 94 | 1.19 | 120 | 1.3 | 8.8 | 28.47 | 4.22 |
| | overall | 738 | 8.39 | 841 | 1.1 | 12.8 | 29.25 | 28.75 |
| LxN | L.1 | 161 | 1.98 | 199 | 1.2 | 9.2 | 23.39 | 8.51 |
| | L.2 | 248 | 2.55 | 255 | 1 | 9.2 | 30.69 | 8.31 |
| | L.3 | 164 | 1.95 | 195 | 1.2 | 6.7 | 25.25 | 7.72 |
| | L.4 | 103 | 1.28 | 128 | 1.3 | 8.6 | 30.36 | 4.22 |
| | overall | 676 | 7.75 | 776 | 1.2 | 9.2 | 26.99 | 28.75 |
| RxN | L.1 | 466 | 2.78 | 279 | 0.6 | 7.3 | 32.79 | 8.51 |
| | L.2 | 435 | 2.48 | 248 | 0.6 | 8.7 | 29.85 | 8.31 |
| | L.3 | 378 | 2.22 | 223 | 0.6 | 9.6 | 28.88 | 7.72 |
| | L.4 | 179 | 1.25 | 126 | 0.7 | 8.7 | 29.89 | 4.22 |
| | overall | 1458 | 8.74 | 875 | 0.6 | 9.6 | 30.43 | 28.75 |
| RxS | L.1 | 191 | 2.74 | 275 | 1.4 | 11.7 | 32.32 | 8.51 |
| | L.2 | 242 | 2.47 | 248 | 1 | 13.9 | 29.85 | 8.31 |
| | L.3 | 227 | 2.55 | 256 | 1.1 | 12.4 | 33.15 | 7.72 |
| | L.4 | 115 | 1.33 | 133 | 1.2 | 7.5 | 31.55 | 4.22 |
| | overall | 775 | 9.10 | 913 | 1.2 | 13.9 | 31.75 | 28.75 |
| RxL | L.1 | 186 | 2.64 | 264 | 1.4 | 14.9 | 31.03 | 8.51 |
| | L.2 | 295 | 2.66 | 267 | 0.9 | 13.5 | 32.14 | 8.31 |
| | L.3 | 181 | 2.30 | 231 | 1.3 | 9.5 | 29.91 | 7.72 |
| | L.4 | 129 | 1.19 | 119 | 0.9 | 12.9 | 28.23 | 4.22 |
| | overall | 791 | 8.79 | 881 | 1.1 | 14.9 | 30.64 | 28.75 |
| Crosses mean | L.1 | 243.4 | 2.58 | 258.2 | 1.18 | 10.38 | 30.348 | 8.51 |
| | L.2 | 292.4 | 2.53 | 253.4 | 0.9 | 11.62 | 30.5 | 8.31 |
| | L.3 | 227.8 | 2.20 | 220.4 | 1.04 | 9.4 | 28.54 | 7.72 |
| | L.4 | 124 | 1.25 | 125.2 | 1.08 | 9.3 | 29.7 | 4.22 |
| | overall | 887.6 | 8.55 | 857.2 | 1.04 | 12.08 | 29.812 | 28.75 |