Ecological Differentiation and Incipient Speciation in the Fungal Pathogen Causing Rice Blast

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ABSTRACT

Natural variation in plant pathogens has an impact on food security and ecosystem health. The rice blast fungus *Pyricularia oryzae,* which limits rice production in all rice-growing areas, is structured into multiple lineages. Diversification and the maintenance of multiple rice blast lineages have been proposed to be due to separation in different areas and differential adaptation to rice subspecies. However, the precise world distribution of rice blast populations, and the factors controlling their presence and maintenance in the same geographic areas, remain largely unknown. We used genotyping data for 886 isolates from more than 185 locations in 51 countries to show that *P. oryzae* is structured into one recombining and three clonal lineages, each with broad geographic distributions. No evidence was found for admixture in clonal lineages, and crossing experiments revealed that female sterility and early postmating genetic incompatibilities acted as strong barriers to gene flow between these lineages. An analysis of climatic and geographic data indicated that the four lineages of *P. oryzae* were found in areas differing in terms of the prevailing environmental conditions and types of rice

grown. Pathogenicity tests with representatives of the five main rice subspecies revealed differences in host range between pathogenic lineages, highlighting a contribution of specialization to niche separation between lineages, despite co-existence on the same host species. Our results demonstrate that the spread of a pathogen across heterogeneous habitats and divergent populations of a crop species can lead to niche separation and incipient speciation in the pathogen.

Short title: Incipient speciation in the rice blast pathogen

INTRODUCTION

Understanding and controlling natural variation in fungal plant pathogens is of great importance for maintaining food security and ecosystem health. In addition to the overwhelming phylogenetic diversity of pathogens (Burdon, 1993), many pathogens also harbor substantial diversity at the species scale (Taylor and Fisher, 2003). Most fungal pathogens of plants form distinct populations (Taylor et al., 2006), and this population structure is a compound outcome of migration, selection and drift, acting at multiple scales, and patterned by factors such as time (Ali et al., 2014; Pagliaccia et al., 2018), admixture (Robert et al., 2015), geography (Ali et al., 2014; Amend et al., 2009; Marin et al., 2009; Sork and Werth, 2014; Yang et al., 2018), mode of reproduction (Ali et al., 2014; Büker et al., 2013; Gibson et al., 2012; Ropars et al., 2016) and environmental conditions (Walker et al., 2015). The identity of the host plants with which fungal pathogens interact is widely thought to be the the environmental factor structuring the pathogen population with the strongest impact. Many plant pathogens reproduce within or on their host, resulting in assortative mating on the basis of host use and a strong association between adaptation to the host and reproductive isolation (Giraud et al., 2010; Servedio et al., 2011). However, more generally, population structure can result from limited dispersal (i.e. limited gene flow because of distance) or limited adaptation (i.e. limited gene flow because of differences in the capacity to exploit resources), both of which may be due to a wealth of potential factors largely unexplored in plant pathogens. Developing an understanding of how population structure emerges and is maintained

50 in fungal plant pathogens is a major goal in evolutionary microbiology, because it will improve our
51 comprehension of both disease emergence and the origin of fungal biodiversity.

52 *Pyricularia oryzae* (Ascomycota; syn. *Magnaporthe oryzae*) is a model widespread fungal plant
53 pathogen displaying population subdivision. Several lineages, each associated mostly with a single main
54 cereal/grass host, have been characterized within *P. oryzae* (Gladieux et al. 2018a). The lineage
55 infecting Asian rice (*Oryza sativa*) has been characterized in detail. Previous population genomic studies
56 of population structure in the rice-infecting lineage revealed genetic subdivision, with three (Saleh et
57 al., 2014; Tharreau et al., 2009; Zhong et al., 2018) to six (Gladieux et al. 2018b) lineages, with different
58 geographic patterns. The lineages were estimated to have diverged approximately 1000 years ago
59 (Gladieux et al. 2018b; Zhong et al. 2018), and pathogenicity tests suggested the local adaptation of
60 some lineages to the indica or japonica subspecies of rice (Gallet et al., 2016; Liao et al., 2016; Gladieux
61 et al., 2018b). *Pyricularia oryzae* is a heterothallic fungus with a sexual cycle involving mating between
62 individuals of opposite mating-types, with at least one of the partners capable of producing female
63 structures (i.e. "female-fertile"). A single lineage (lineage 1, prevailing in Southeast Asia) displayed a
64 genome-wide signal of recombination, a balanced ratio of mating-type alleles and a high frequency of
65 fertile females, consistent with sexual reproduction (Saleh et al. 2012a; Saleh et al. 2014; Gladieux et
66 al. 2018b). The other lineages had clonal population structures and highly biased frequencies of mating
67 types or fertile females, suggesting strictly asexual reproduction (Saleh et al. 2014; Gladieux et al.
68 2018b). Gene flow remains possible between lineages, because there are rare fertile strains and some
69 lineages have fixed opposite mating types. However, resequencing studies have revealed signatures of
70 recent shared ancestry between clonal lineages and the recombinant lineage from South-West Asia,
71 but not between the different clonal lineages, consistent with a lack of recent gene flow between
72 widespread lineages of opposite mating types (Gladieux et al. 2018b). The loss of female fertility has
73 probably been a key factor in the emergence of clonal lineages, inducing a shift towards strictly asexual
74 reproduction, and protecting populations adapting to new conditions from maladaptive gene flow from
75 ancestral, recombining populations (Giraud et al., 2010). This raises questions about the nature of the

76 factors underlying the emergence of different rice-infecting lineages in *P. oryzae*, and contributing to
77 their maintenance today, because, according to the competitive exclusion principle, different lineages
78 should not be able to co-exist on the same host (Hardin 1960; Giraud et al. 2017; Abbate et al. 2018).
79 Previous studies have helped to cement the status of *P. oryzae* as a model for studies of the population
80 biology of fungal pathogens, but most efforts to understand the population structure of the pathogen
81 have been unable to provide a large-scale overview of the distribution of rice-infecting lineages and of
82 the underlying phenotypic differences, because the availability of sets of genetic markers was limited
83 (Saleh et al., 2014; Tharreau et al., 2009), the number of isolates was relatively small (Gladieux, et al.
84 2018b; Zhong et al. 2018) or because few phenotypic traits were scored (Saleh et al., 2014; Tharreau
85 et al., 2009; Zhong et al., 2018).

86 We provide here a detailed genomic and phenotypic overview of natural variation in rice-
87 infecting *P. oryzae* pathogens sampled across all rice-growing areas of the world. With a view to
88 inferring and understanding the population structure of rice-infecting *P. oryzae*, we analyzed genetic
89 variation, using data with a high resolution in terms of genomic markers and geographic coverage, and
90 measured phenotypic variation for representative isolates. We addressed the following specific
91 questions: (i) How many different lineages of *P. oryzae* infect rice? (ii) Do lineages of *P. oryzae* display
92 footprints of recombination? (iii) Do lineages of *P. oryzae* present evidence of recent admixture? (iv)
93 Are the different lineages sexually competent and compatible? (v) Are the different lineages
94 differentially adapted to host and temperature? (vi) Can we find differences in the geographic and
95 climatic distribution of lineages?

96 **RESULTS**

97 **The rice blast pathogen comprises one recombining, admixed lineage and three clonal, non-admixed**
98 **lineages**

99 We characterized the global genetic structure of the rice blast pathogen, using an Illumina genotyping
100 beadchip to score 5,657 SNPs distributed throughout the genome of 886 *P. oryzae* isolates collected

101 from cultivated Asian rice in 51 countries. The final dataset included 3,686 SNPs after the removal of
102 positions for which quality was low or data were missing. Clustering analyses based on sparse
103 nonnegative matrix factorization algorithms, as implemented in the sNMF program (Frichot et al.,
104 2014), revealed four clusters, hereafter referred to as "lineages" (Figure 1C). The model with *K*=4
105 clusters was identified as the best model on the basis of the cross-entropy criterion, as models with
106 *K*>4 induced only a small decrease in cross-entropy, suggesting that *K*=4 captured the deepest
107 subdivisions in the dataset (Supplementary figure 1). The neighbor phylogenetic network inferred with
108 SPLITSTREE also supported the subdivision into four widely distributed lineages, with long branches
109 separating three peripheral lineages branching off within a central lineage (Figure 1A; 1B). A
110 comparison with previous findings revealed that the three peripheral lineages in the network
111 corresponded to the previously described lineages 2, 3 and 4 (Gladieux et al. 2018b), whereas the
112 central lineage corresponded to the combination of recombining lineage 1 and lineages 5 and 6,
113 represented by only a few individuals in Gladieux et al. (2018b). The central lineage is referred to
114 hereafter as lineage 1, for the sake of simplicity. Lineages 2 and 3 were similar to lineages B and C,
115 respectively, identified on the basis of microsatellite data in a previous study (Saleh et al., 2014 -
116 Supplementary figure 2). Genetic differentiation between the four lineages was strong and significant
117 (Weir and Cockerham's F_{ST}> 0.54), indicating strong barriers to gene flow between the lineages. All
118 genotypes from lineages 2 to 4 had high proportions of membership *q* in a single cluster (*q* > 0.89),
119 whereas shared ancestry with the other three lineages was detected in lineage 1, with 32% of
120 genotypes having *q* > 0.10 in lineages 2-4 (Figure 1C). Admixture may account for the lower F_{ST} observed
121 in comparisons between lineage 1 and the other lineages. We detected no footprints of recombination
122 in lineages 2, 3 and 4 with the pairwise homoplasy index (PHI) test and a null hypothesis of clonality
123 (Table 1), confirming previous findings (Saleh et al. 2014; Gladieux et al. 2018b; Zhong et al. 2018). The
124 rooted genealogies of the clonal lineages suggested that they were of Asian origin, and the patterns of
125 shared multilocus genotypes were consistent with multiple introductions and intense movements of
126 biological material over long distances (Supplementary text 1).

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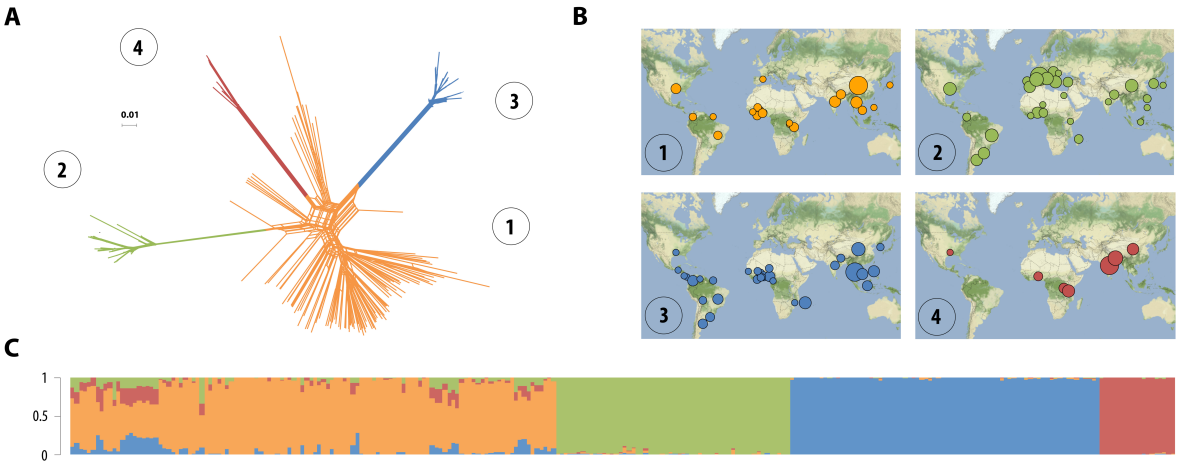


128

129 Figure 1. Population subdivision inferred from 264 *P. oryzae* genotypes, representing 886 isolates, with four identified lineages
130 represented in different colors. A: Neighbor-net phylogenetic network estimated with SPLITSTREE; reticulations indicate
131 phylogenetic conflicts caused by homoplasy. B: Geographic distribution of the four lineages identified with SPLITSTREE and sNMF,
132 with disk area proportional to sample size. C: Ancestry proportions in K=4 clusters, as estimated with sNMF software; each
133 multilocus genotype is represented by a vertical bar divided into four segments indicating membership in K=4 clusters.

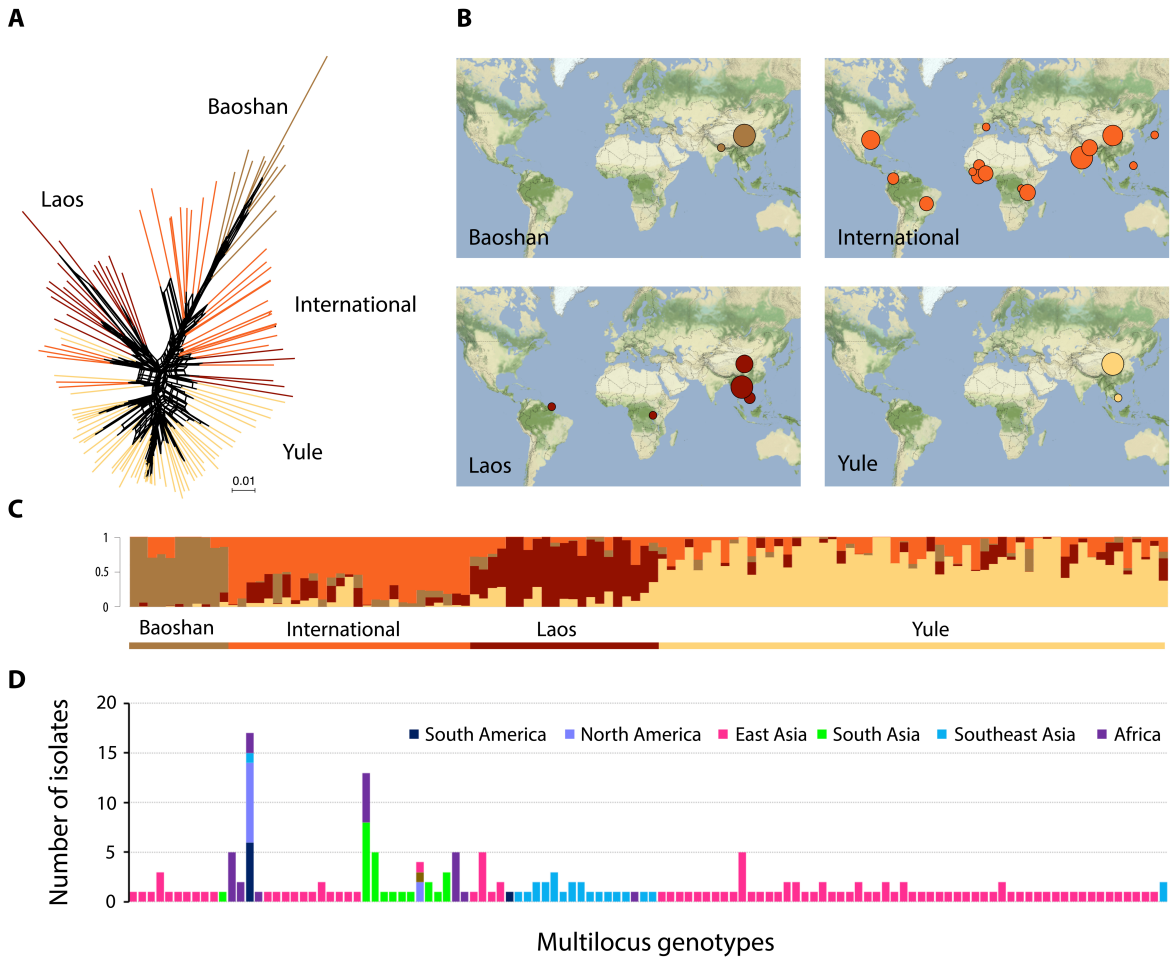
134

135 **Geographic structure and admixture within recombining lineage 1**

136 Lineage 1 was the only lineage for which there was population genetic evidence of recombination in
137 the PHI test (Table 1). Most of the lineage 1 isolates were collected in Asia (79%), but the lineage was
138 present in all continents in which the pathogen was sampled (Europe: one isolate; North America: 10,
139 Central and South America: 7, Africa: 22) (Figure 2). Clustering analysis on this single lineage with sNMF
140 detected four clusters with different geographic distributions (Figure 2). Estimates of differentiation
141 were lower between the clusters within lineage 1 (F_{ST} < 0.49) than between the main lineages (F_{ST} >
142 0.54), consistent with a longer history of restricted gene flow between the main lineages and a more
143 recent history of admixture between clusters within lineage 1 (Supplementary table 1; Figure 1C).

144 Two clusters within lineage 1 (referred to hereafter as *Baoshan* and *Yule*) consisted mostly of
145 isolates sampled from two different sites in Yunnan (Baoshan and Yule, respectively) located about 700
146 km apart. The third cluster consisted mostly of isolates from Laos and South China (referred to

147 hereafter as *Laos*), and the fourth brought together 95% of the isolates from lineage 1 collected outside
148 Asia (referred to hereafter as *International*). In Asia, the *International* cluster was found mostly in the
149 Yunnan province of China, India and Nepal (Figure 2). PHI tests for recombination rejected the null
150 hypothesis of clonality in all four clusters (Table 1). Admixture was widespread in lineage 1 (Figure 2),
151 with most of the isolates (78%) displaying membership proportions $q > 0.10$ for two or more clusters
152 (Figure 2). Only five genotypes were detected in multiple countries (genotype ID [number of countries]:
153 2 [6], 18 [2], 58 [2], 98 [3] and 254 [3], corresponding to 41 isolates in total). All these genotypes
154 belonged to the *International* cluster.



155

156 Figure 2. Population subdivision in lineage 1. A: Neighbor-net phylogenetic network estimated with SPLITSTREE; reticulations
157 indicate phylogenetic conflicts caused by homoplasy. B: Geographic distribution of the four clusters identified with sNMF,
158 with disk area proportional to sample size. C: Ancestry proportions in four clusters, as estimated with sNMF; each multilocus
159 genotype is represented by a vertical bar divided into four segments, indicating membership in K=4 clusters. D: Number of

160 isolates and their geographic origin for each multilocus genotype of lineage 1. Panels C and D share an *x*-axis (each vertical
161 bar represents a different multilocus genotype).

162 Table 1. PHI test for recombination (null hypothesis: clonality) and distribution of mating types and fertile
163 females in lineages, and in clusters within lineage 1.

Lineage/*Cluster*	*p*-value PHI test	Mat1.1/Mat1.2 (%)	Female fertility (%)
1	<0.0001	53/47	43
Baoshan	<0.0001	69/31	67
International	<0.0001	55/45	4
Laos	<0.0001	68/32	37
Yule	<0.0001	40/60	79
2	0.25	97/3	0
3	0.18	3/97	7
4	0.22	97/3	0

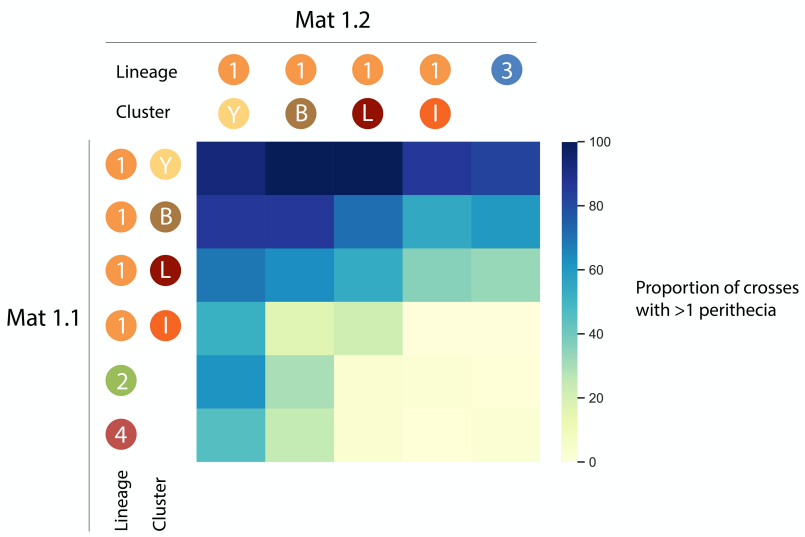
164

165 **Reproductive barriers caused by female sterility and postmating genetic incompatibilities**

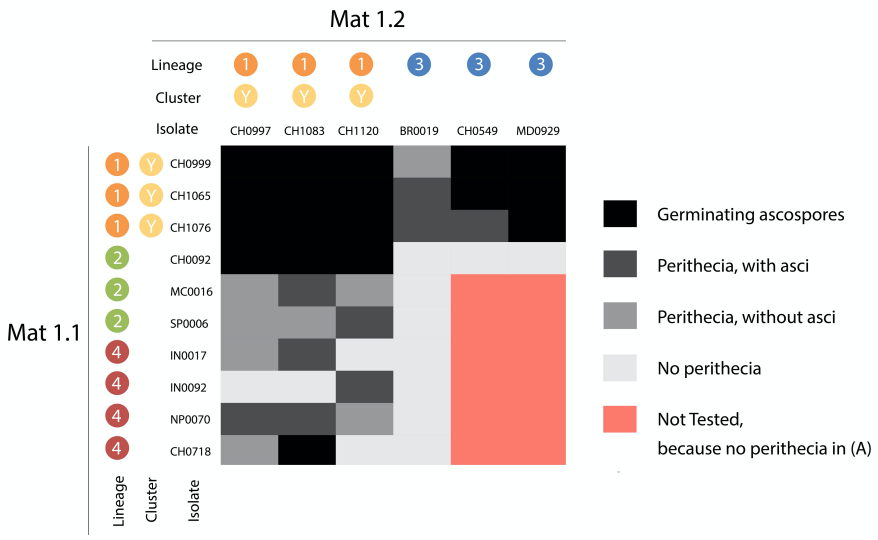
166 Our analysis of genotyping data revealed the existence of a large, ancient, recombinant population
167 from which several clonal lineages have more recently emerged. This raised the question as to why
168 these populations became clonal, with no further gene flow. We addressed this question by
169 determining the capacity of the different lineages to engage in sexual reproduction, by characterizing
170 the distribution of mating types and the ability to produce female structures (i.e. female fertility). Using
171 *in vitro* crosses with tester strains, we found that lineages 2, 3 and 4 were composed almost exclusively
172 of a single mating type (97% of lineage 2 and 4 isolates tested carried the *Mat1.1* allele; 97% of lineage
173 3 isolates carried the *Mat1.2* allele), and had only small proportions (0-7%) of isolates with female
174 fertility (Table 1). By contrast to these clonal lineages, the mating type ratio was more balanced in
175 lineage 1 (52% of *Mat1.1*; Table 1), with *Mat1.1*/*Mat1.2* ratios ranging from 40/60 in the *Yule* cluster
176 to 69/31 in the *Baoshan* cluster. Female fertility rates were higher in three of the clusters in lineage 1

177 (*Yule*: 79%; *Baoshan*: 67%; *Laos*: 37%; Table 1), but that in the International cluster (4% female fertility)
178 was as low as those in the clonal clusters (Table 1). These observations indicate that there is a potential
179 for sexual reproduction between lineages with compatible mating-types and, albeit rare, fertile
180 females, but not between lineages 2 and 4, due to the low female fertility rates and highly biased
181 mating-type ratios.

(A) Production of perithecia



(B) Production of germinating ascospores



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183 Figure 3. Success of crosses between lineages 2-4 and clusters within lineage 1 with (A) proportion of crosses producing at
184 least one perithecium, (B) scoring of ascus formation and ascospore germination for a subset of crosses.

185 We further assessed the likelihood of sexual reproduction within and between lineages, by
186 evaluating the formation of sexual structures (perithecia), the production of asci (i.e. meiotic octads
187 within the perithecia) and the germination of ascospores (i.e. meiospores in meiotic octads) in crosses
188 between randomly selected isolates of opposite mating types from all four lineages (Figure 3A). This
189 experiment revealed a marked heterogeneity in the rate of perithecium formation across lineages.
190 Three clusters in lineage 1 had the highest rates of perithecia formation, with isolates in the *Yule* cluster,
191 in particular, forming perithecia in 93% of crosses with isolates from the same cluster, and in more than
192 46% of crosses with isolates from other lineages (Figure 3A). Isolates from lineages 2, 3 and 4 could
193 only be crossed with isolates from other lineages, given their highly biased mating-type ratios, and the
194 percentage of these crosses producing perithecia was highly variable (ranging from 0% to 83%,
195 depending on the lineages involved; Figure 3A). The rate of perithecium formation was similar in
196 crosses involving the *International* cluster of lineage 1 and in crosses involving clonal lineages 2-4, and
197 none of the crosses attempted between isolates from the *International* cluster led to perithecium
198 formation (Figure 3A). Perithecium dissection for a subset of crosses involving some of the most fertile
199 isolates revealed that most inter-lineage crosses produced perithecia that did not contain asci or
200 contained asci with low ascospore germination rates (Figure 3B). Whereas 100% of crosses between
201 isolates of the *Yule* cluster produced numerous germinating ascospores, ascospore germination rates
202 were only 33%, 56% and 7% in *Yule* x lineage 2, *Yule* x lineage 3 and *Yule* x lineage 4 crosses, respectively.
203 Together, these results indicate that the clonal structure of lineages 2-4 is caused by highly biased
204 mating types and female sterility, and that the three clonal lineages and the *International* cluster of
205 lineage 1 are isolated from each other by strong pre- and early-postmating barriers, including breeding
206 system isolation (differences in mating type and female sterility), and a combination of gametic
207 incompatibility, zygotic mortality or hybrid non-viability. However, three of the clusters in lineage 1 had
208 biological features consistent with an ability to reproduce sexually, suggesting possible sexual
209 compatibility with clonal lineages 2-4 and the *International* cluster.

210 **Specialization to temperature conditions and rice subspecies**

211 Specialization can further reduce gene flow, and may facilitate niche separation between lineages,
212 which should favor the emergence of new lineages and their maintenance on the same host species in
213 conditions of competitive exclusion. The strong reproductive barriers demonstrated in our analysis of
214 crossing experiments would favor the establishment of such specialization, because reproductive
215 isolation facilitates adaptation to new conditions, by preventing maladaptive gene flow from ancestral,
216 non-adapted populations (Giraud et al., 2010). Given the very broad distribution of *P. oryzae* and the
217 strong environmental heterogeneity with which it is confronted in terms of the nature of its hosts and
218 the climate in which it thrives, we evaluated the variation of pathogen fitness over different types of
219 rice and temperature conditions. Our experiments measuring growth and sporulation rates at different
220 temperatures revealed limited differences in performance between lineages, and no pattern
221 consistent with strict specialization according to temperature (Supplementary text 2). We therefore
222 report only the results of pathogenicity tests below.

223 We assessed the importance of adaptation to the host, by challenging 45 rice varieties
224 representative of the five main genetic groups of Asian rice (i.e. the temperate japonica, tropical
225 japonica, aus, aromatic and indica subgroups of *Oryza sativa*) with 70 isolates representative of the
226 four lineages of *P. oryzae* and the four clusters within lineage 1 (Supplementary Figure 3). Qualitative
227 symptoms were noted and analyzed with a proportional-odds model, revealing significant differences
228 between lineages (p-value = 2.2×10^{-16}), and between rice genetic groups (p-value=2.2×10^{-16}), and a
229 significant interaction between these two variables (p-value=2.0×10^{-10}). In comparisons between
230 lineages, lineage 2 differed from the other lineages in generally producing smaller lesions (Figure 4A).
231 In comparisons of rice genetic groups, significantly strong symptoms were observed on temperate
232 japonica rice than on the other types of rice, whereas the varieties of the aromatic genetic group were
233 significantly more resistant to rice blast (Figure 4B). The finding of a significant interaction between
234 lineage and rice genetic group indicates that the effect of the lineage on the proportion of compatible
235 interactions differed between rice types, suggesting adaptation to the host. In particular, an analysis
236 of interactions also revealed that the isolates of the *Yule* cluster were highly pathogenic on most indica

varieties (Supplementary Figure 3), with all isolates from this cluster pathogenic to all indica varieties except IR8. Together, these experiments revealed significant differences in host range between lineages, but with overlaps between these host ranges, indicating that specialization to the host was not strict (Figure 4C-G). Adaptation to the host or small differences in temperature optima could, nonetheless, further increase niche separation and reduce gene flow between lineages, via pre-mating barriers such as immigrant non-viability (i.e., lower rates of contact between potential mates due to the mortality of immigrants; Gladieux et al., 2011; Nosil et al., 2005), and post-mating barriers, such as ecological hybrid non-viability (i.e., lower survival of ill-adapted hybrid offspring).

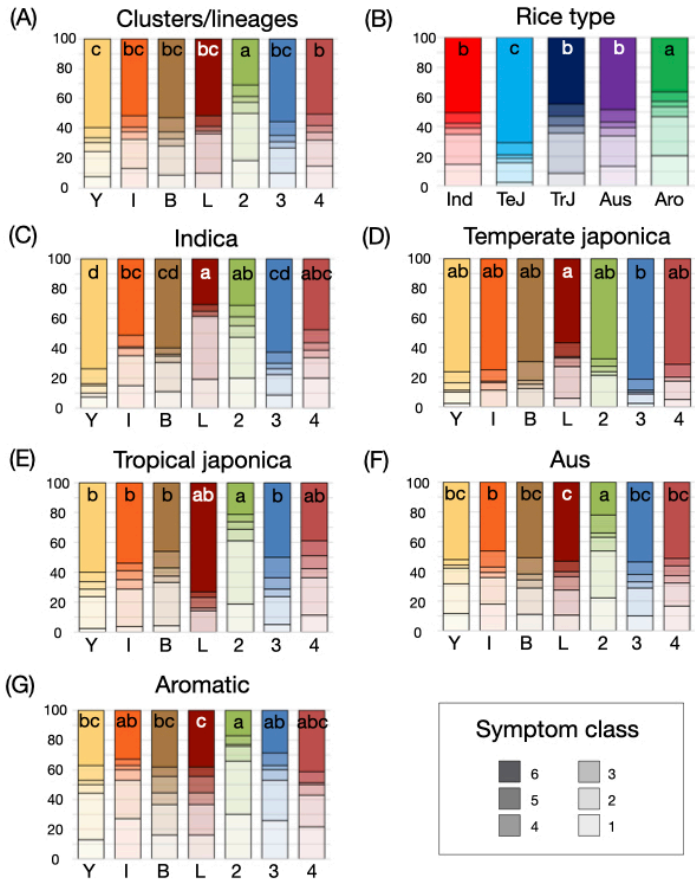

Figure 4: Compatibility between 70 *P. oryzae* isolates and 45 rice varieties, representing five subgroups of rice. A: Proportions of symptom scores as a function of the lineage of origin of isolates, or cluster of origin for isolates from lineage 1; B: Proportions of symptom scores as a function of the type of rice; C-G: Proportions of symptom scores as a function of the lineage of origin of isolates, for each type of rice. Abbreviations: Y, *Yule*; I, *International*; B, *Baoshan*; L, *Laos*; 2, lineage 2; 3,

250 lineage 3; 4, lineage 4; Ind, indica; TeJ, temperate japonica; TrJ, tropical japonica; Aro, aromatic. Small capitals indicate
251 significant differences.

252 **Geographic and climatic differentiation in the distribution of *P. oryzae* lineages**

253 Our tests of adaptation to host and temperature under controlled conditions showed that
254 specialization was not strict, but it remained possible that fitness differences between lineages would
255 be sufficient under natural conditions to induce separation in different ecological niches, and/or that
256 our experimental conditions did not capture the full extent of the phenotypic differences. Here, we
257 tested the hypothesis that lineages thrive in geographically and/or environmentally different
258 conditions, which would provide indirect evidence for specialization to different habitats. We tested
259 this hypothesis by collecting geographic and climatic data for all isolates, approximating to regions or
260 the nearest cities when the exact GPS position of the sampling area was not available (Supplementary
261 table 2). At a larger scale, clonal lineages 2 and 3 were both widespread, with lineage 2 found on all
262 continents, and lineage 3 on all continents except Europe. Lineage 2 was the only lineage sampled in
263 Europe (with the exception of a single isolate from lineage 1), whereas lineage 3 was more widespread
264 in intertropical regions. Lineage 4 was mostly found in South Asia (India, Bangladesh, Nepal), and in
265 the USA and Africa (Benin, Tanzania). The recombining lineage, lineage 1, was found in all continents,
266 but was mostly found in Asia (79% of all isolates and 94% of all genotypes). At a smaller scale, two, or
267 even three different lineages were sampled in the same year in the same city in 11 different countries
268 from all continents. However, in only one instance did two isolates from different lineages have
269 identical GPS positions (isolates US0106 and US0107 sampled from the same field). Thus, lineages had
270 different, but partially overlapping distributions, including some overlap with the distribution of the
271 sexually reproducing lineage (lineage 1).

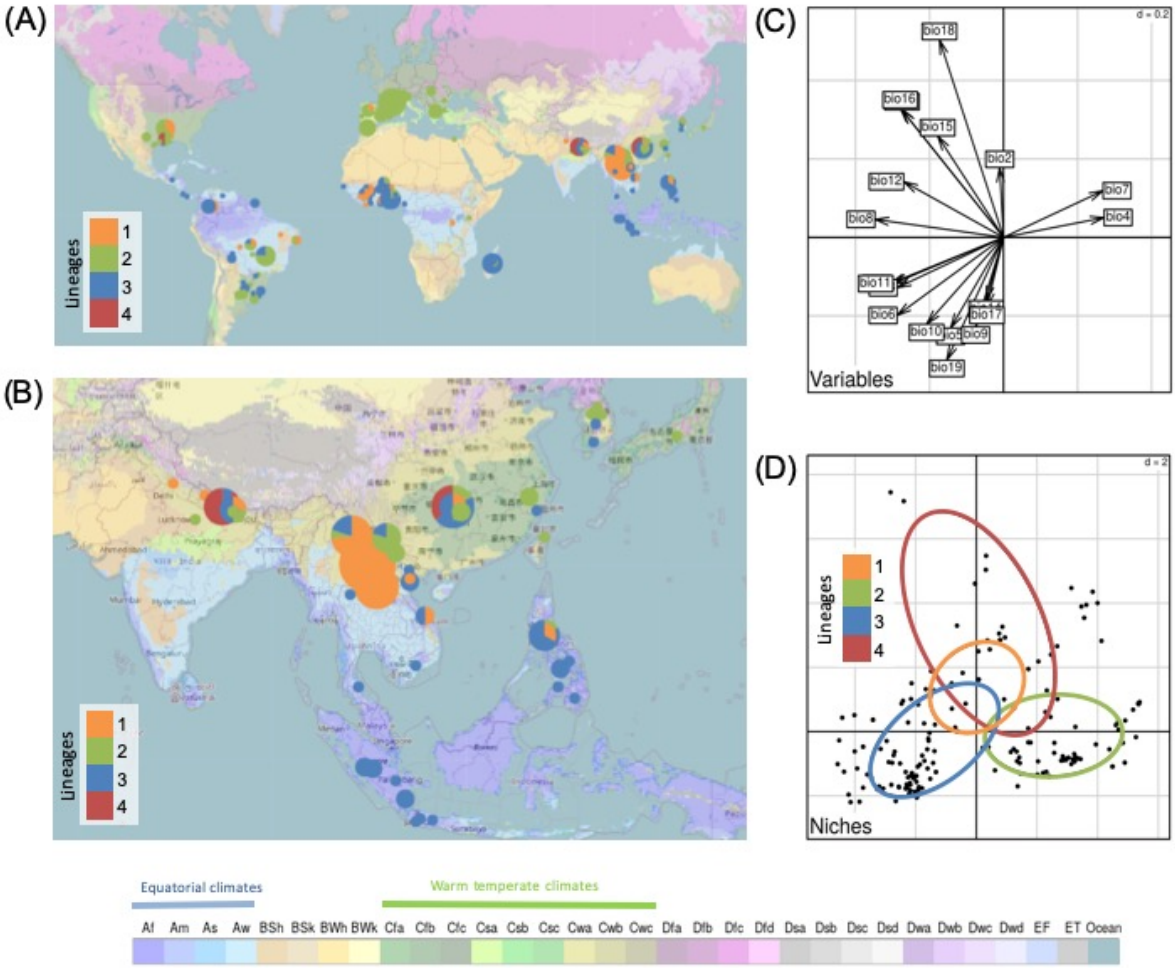

Figure 5. Geographic distribution of four lineages of *P. oryzae* and corresponding climatic data. (A, B) Pie charts representing the distribution of the four lineages in the world (A) and in South, East and Southeast Asia (B), keeping only isolates for which the sampling position was precisely known (i.e., for which the region, city or GPS position was documented). Background map is from OpenStreetMap (CC-BY-SA) and represents the updated Köppen-Geiger climate classification of main climates at world scale as described in Kottek et al. (2006). (C, D) Outlying Mean Index analysis, testing the separation of ecological niches among lineages, with (C) canonical weights of the19 environmental variables included in the analysis, and (D) site coordinates (dots) and realized niches of lineages represented as ellipses. Variable bio13 co-localise with bio16, and variables bio1 and bio3 co-localize with bio11. The 11 temperate variables included in the analysis were (bio1) Annual Mean Temperature, (bio2) Mean Diurnal Range, (bio3) Isothermality [100*(bio2/bio7)], (bio4) Temperature Seasonality [standard deviation*100], (bio5) Max Temperature of Warmest Month, (bio6) Min Temperature of Coldest Month, (bio7) Temperature Annual Range [bio5-bio6], (bio8) Mean Temperature of Wettest Quarter, (bio9) Mean Temperature of Driest Quarter, (bio10) Mean Temperature of Warmest Quarter, (bio11) Mean Temperature of Coldest Quarter. The 8 precipitation variables included in the analysis were (bio12) Annual Precipitation, (bio13) Precipitation of Wettest Month, (bio14) Precipitation of Driest Month, (bio15)

286 Precipitation Seasonality (Coefficient of Variation), (bio16) Precipitation of Wettest Quarter, (bio17) Precipitation of Driest
287 Quarter, (bio18) Precipitation of Warmest Quarter, (bio19) Precipitation of Coldest Quarter.

288 We then investigated whether differences in the geographic range of lineages were associated
289 with differences in climatic variables. By plotting sampling locations onto a map of the major climate
290 regions (Kottek et al. 2006) we were able to identify clear differences in the climatic distributions of
291 lineages 2 and 3, with lineage 2 mostly found in warm temperate climates and lineage 3 found in
292 equatorial climates (Figure 5). We used the outlying mean index (OMI), which measures the distance
293 between the mean habitat conditions used by a lineage and the mean habitat conditions used by the
294 entire species, to test the hypothesis that different lineages are distributed in regions with different
295 climates. Using information for 19 climatic variables (biomes) from the WorldClim bioclimatic variables
296 database (Fick and Hijmans 2017) for all sampling locations, we obtained marginally significant results,
297 in a statistical permutation test on OMI values, for lineages 2, 3 and 4 (*p*-values: L1, *p*=0.6213; L2,
298 *p*=0.0003; L3, *p*=0.0001; L4, *p*=0.0176). The OMI analysis, in which the first two axes accounted for 69%
299 and 25% of the variability, respectively, revealed that lineage 2 was more frequent in regions with a
300 high annual temperature range (biome 7) or a high degree of seasonality (biome 4); lineage 4 was
301 associated with regions with high levels of seasonal precipitation (biomes 13, 16 and 18), and lineage
302 3 was more frequent in regions with high temperatures (biomes 1, 6, 10 and 11) and high levels of
303 isothermality (biome 3), characteristic of tropical climes (Figure 5; Supplementary figure 3).
304

305 **DISCUSSION**

306 We describe here the population structure of the rice blast fungus, and the eco-evolutionary factors
307 underlying the emergence and maintenance of multiple divergent lineages in this widespread
308 pathogen. Our analysis of SNP genotyping data showed that *P. oryzae* could be subdivided into four
309 lineages. Two of the lineages previously detected in an analysis of whole-genome data (lineages 5 and
310 6; Gladieux et al., 2018b), and represented by only a few individuals in our dataset, were assigned to
311 lineage 1 in our analysis, and may correspond to a subdivision of lineage 1. Differences in the number

312 of lineages detected may reflect an ascertainment bias, given that our SNP-genotyping beadchip was
313 designed with a set of genomic sequences that did not include representatives of lineages 5 and 6.
314 Alternatively, the sampling used in the previous study (Gladieux et al., 2018b) may not have been dense
315 enough to reveal the lack of differentiation between lineages 1, 5 and 6, making them appear to be
316 different entities. Consistent with previous findings, our analysis of the genealogical relationships
317 between isolates revealed three clonal groups connected by long branches to a central recombining
318 group. The finding of an internationally distributed cluster in lineage 1 sheds light on the early
319 evolutionary changes contributing to the emergence of clonal lineages in this pathogen. The
320 *International* cluster of lineage 1 displayed genetic footprints of recombination and sexual
321 reproduction (in the form of an excess of homoplasious variants or relatively balanced mating types),
322 but the signal of recombination detected here may be purely historical, because we also found that the
323 clonal fraction and the frequency of sterile females were very high in this cluster. The loss of female
324 fertility may, therefore, be an early and major cause of the shift towards asexual reproduction. Some
325 genotypes assigned to lineage 1 may, in the future, come to resemble other clonal lineages in analyses
326 of genealogical relationships, and form a long branch stemming from the central lineage 1, particularly
327 for genotypes already displaying clonal propagation, such as genotypes 2, 18, 58, 98 and 254. However,
328 according to the principle of competitive exclusion, competition between clonal lineages should only
329 allow hegemonic expansion of the most competitive clonal groups, unless they separate into different
330 ecological niches. It may be this process that ultimately leads to the fixation of a single mating type, as
331 in clonal lineages 2-4.

332 We found that female sterility and intrinsic genetic incompatibilities represented strong
333 barriers to gene flow between the clonal lineages, potentially accounting for their maintenance over
334 time, without fusion, despite the compatibility of mating types (at least between lineages 2 and 4
335 [*Mat1.1*] on the one hand, and lineage 3 [*Mat1.2*] on the other). These barriers to gene flow may have
336 contributed to the establishment of lineage specialization (because reproductive isolation facilitates
337 adaptation by limiting the flow of detrimental ancestral alleles in populations adapting to a new

338 habitat), accounting for the maintenance of the clonal lineages on the same host species, when faced
339 with competitive exclusion. Our analyses also show that, despite widely overlapping large-scale
340 distributions, the different lineages are essentially found in different regions, with different climatic
341 characteristics, when their distributions are observed at a finer scale. Lineage 1 was mostly sampled in
342 Southeast Asia, lineage 4 predominated in India, and lineages 2 and 3 had global distributions, but the
343 geographic ranges of all these lineages overlapped. However, an analysis of climatic data indicated that
344 lineage 2 predominated in temperate climates, where temperate japonica rice is grown, lineage 3
345 predominated in tropical climates, in which indica rice is grown, and lineage 4 predominated in regions
346 with high levels of seasonal precipitation in which the indica and aromatic rice types are the principal
347 rice types grown. Despite the finding of separation in different climatic regions, our experiments
348 revealed no strong differences between lineages in terms of sporulation and mycelial growth on
349 synthetic media at different temperatures. This suggests that, if adaptation to temperature occurs in
350 this pathogen, it was not measured by our experiments, either because it involves traits other than
351 sporulation and hyphal growth, or because the *in vitro* conditions were not suitable to demonstrate
352 differences. The host range varied across lineages, but all host ranges overlapped, indicating that host
353 specialization was not strict, or was not fully captured by our experiments. Adaptation to the host may,
354 nevertheless, decrease gene flow between populations even further. For instance, lineage 2 had a
355 narrow host range, potentially limiting the opportunities for encounters and mating with other groups.
356 Our results also indicate that lineage 1 may pose a major threat to rice production. Eleven of
357 the 12 most multivirulent isolates (lesion type > 2 on more than 40 varieties tested) belonged to lineage
358 1, and the *Yule* cluster, in particular, was highly pathogenic on indica varieties. The propagation of such
359 highly pathogenic genotypes, belonging to a lineage that has both mating types and fertile females,
360 should be monitored closely. The monitoring of lineage 1 is all the more critical because this lineage
361 has an intermediate geoclimatic distribution that overlaps largely with the distributions of the other
362 three lineages, and some of the attempted crosses between clonal lineage 2-4 isolates and recombining
363 lineage 1 isolates produced viable progeny. This finding confirms the possibility of gene flow into this

364 lineage, as previously demonstrated on the basis of admixture mapping (Gladieux et al., 2018b), but it
365 also raises the question of the threat posed by gene flow from lineage 1, which is highly pathogenic, to
366 the other lineages.

367 In conclusion, our study of genetic and phenotypic variation within and between clonal
368 lineages, and within the recombinant lineage of *P. oryzae* suggests a scenario for the emergence of
369 widespread clonal lineages. The loss of female fertility may be a potent driver of the emergence of
370 asexually reproducing groups of clones. The reproductive isolation generated by the loss of sex and the
371 accumulation of mutations due to the absence of sexual purging would facilitate the specialization of
372 some of these clonal groups, leading to the competitive exclusion of the least efficient clonal groups,
373 and, finally, to the propagation of clonal lineages fixed for a single mating type. Our results, thus,
374 demonstrate that the spread of a pathogen across heterogeneous habitats and divergent populations
375 of a crop species can lead to niche separation and incipient speciation in the pathogen.

376

377 **MATERIALS AND METHODS**

378 **Biological material**

379 We chose 886 *P. oryzae* isolates collected on Asian rice between 1954 and 2014 as representative of
380 the global genetic diversity of the fungus. Isolates were selected on the basis of microsatellite data, to
381 maximize the number of multilocus genotypes represented (Saleh et al. 2014 and unpublished data),
382 or based on geographic origin in the absence of genotypic data, to maximize the number of countries
383 represented in the dataset.

384

385 Sixty-eight isolates were selected for experimental measurements of reproductive success,
386 adaptation to host, and growth and sporulation experiments at different temperatures (Supplementary
387 table 2). This subset of isolates included 10 isolates from each of the three clonal lineages (lineages 2-
388 4), 27 isolates from the various clusters within lineage 1 [*Baoshan* (9 isolates), *International* (10), *Laos*

389 (8), and *Yule* (10)] and isolate CH0999, a reference female-fertile strain of the Mat1.1 mating-type
390 (Saleh et al., 2012a).
391
392 Forty-six varieties were chosen as representative of the five main genetic subgroups of Asian
393 rice (Garris et al., 2005): indica (Chau, Chiem chanh, DA11, De abril, IR8, JC92, JC120, Pappaku), aus
394 (Arc 10177, Baran boro, Black gora, DA8, Dholi boro, Dular, FR13 A, JC148, Jhona 26, Jhona 149,
395 Kalamkati, T1, Tchampa, Tepi boro), temperate japonica (Aichi asahi, Kaw luyoeng, Leung pratew,
396 Maratelli, Nep hoa vang, Nipponbare, Sariceltik, Som Cau 70A), tropical japonica (Azucena, Binulawan,
397 Canella de ferro, Dholi boro, Gogo lempuk, Gotak gatik, Moroberekan, Trembese) and aromatic (Arc
398 10497, Basmati lamo, Dom zard, Firooz, JC1, Kaukkyisaw, N12). The Maratelli (temperate japonica) and
399 CO39 (indica) varieties were used as susceptible controls.
400
401 **Genotyping**
402 *Pyricularia oryzae* isolates were genotyped at 5,657 genomic positions with an Illumina Infinium
403 beadchip microarray carrying single-nucleotide polymorphisms identified in 25 previously sequenced
404 *P. oryzae* genomes (Gladieux et al., 2018b). According to our clustering analysis, the 25 isolates
405 sequenced belonged to lineage 1 (n=2 isolates), lineage 2 (n=9), lineage 3 (n=8) or lineage 4 (n=3), and
406 three isolates were not characterized here. The final dataset included 3,686 biallelic SNPs without
407 missing data.
408
409 **Population subdivision and recombination**
410 Among the 886 *P. oryzae* isolates genotyped, we identified 264 different multilocus genotypes, which
411 were used for the analysis of population subdivision. We used the sNMF program to infer individual
412 ancestry coefficients in K ancestral populations. This program is optimized for the analysis of large
413 datasets and does not assume Hardy-Weinberg equilibrium. It is, therefore, more appropriate to deal

414 with inbred or clonal lineages (Frichot et al., 2014). We used SPLITSTREE version 4 to visualize
415 relationships between genotypes in a phylogenetic network, with reticulations to represent the
416 conflicting phylogenetic signals caused by homoplasy. We also used the pairwise homoplasy index (PHI)
417 test implemented in SPLITSTREE to test the null hypothesis of clonality. We then inferred a maximum
418 likelihood genealogy, to investigate further the relationships between genotypes within the clusters for
419 which clonality could not be rejected, and we used the sNMF software to investigate population
420 subdivision further within the cluster for which the null hypothesis of clonality could be rejected. Weir
421 and Cockerham's F_{ST} was calculated with the R package HIERFSTAT, by the WC84 method (Goudet, 2005).
422

423 **Experimental measurement of reproductive success and female fertility**

424 *Pyricularia oryzae* is a heterothallic fungus with two mating types (*Mat1.1* and *Mat1.2*). Sexual
425 reproduction between strains of opposite mating type can be observed in laboratory conditions, and
426 results in the production of ascospores within female sexual structures called perithecia (Saleh et al.
427 2012). On synthetic media, perithecia are formed at the contact zone between parental mycelia.
428 Crosses were carried out on a rice flour agar medium (20 g rice flour, 2.5 g yeast extract, 15 g agar and
429 1 L water, supplemented with 500 000 IU penicillin G after autoclaving for 20 min at 120°C), as
430 described by Saleh et al. (2012b). We assessed reproductive success by determining the production of
431 perithecia produced after three weeks of culture at 20°C under continuous light. Perithecia can be
432 formed by the two interacting partners or by one partner only. Isolates forming perithecia are
433 described as female-fertile. We measured female fertility for 220 isolates (listed in Supplementary table
434 2), by monitoring perithecium production in crosses involving the tester strains CH0997 (Mat1.2) and
435 CH0999 (Mat1.1). We further assessed the presence of asci and germinating ascospores in perithecia
436 for a subset of crosses, by excising perithecia with a scalpel and counting the number of germinated
437 filaments for each individual ascus after overnight incubation on water agar. The subset of crosses
438 included 10 Mat1.1 isolates (lineage 1: CH0999, CH1065, CH1076; lineage 2: CH0092, MC0016, SP0006;

439 lineage 4: IN0017, IN0092, NP0070, CH0718) and 6 Mat1.2 isolates (lineage 1: CH0997; CH1083,
440 CH1120; lineage 3: BR0019, CH0549, MD0929).
441
442 **Pathogenicity tests**

443 Compatibility between *P. oryzae* isolates and rice plants from the five main genetic subgroups of rice
444 (indica, temperate japonica, tropical japonica, aus and aromatic) was measured in controlled
445 conditions. Seventy isolates were inoculated on 46 varieties. Inoculations were performed as described
446 by Gallet et al. (2016). Conidial suspensions (25 000 conidia.mL^{-1}) in 0.5% gelatin were sprayed onto
447 three-week-old rice seedlings (> 6 plants/variety). The inoculated plants were incubated for 8 hours at
448 27°C and 100% humidity and then for seven days with a day/night alternation (13 hours at 27°C/11
449 hours at 21°C), before scoring symptoms. Inoculation and symptom scoring were performed three
450 times, and the maximum of the three scores obtained was used in calculations. Lesion type was rated
451 from 1 to 6 (Gallet et al. 2016) and the disease area was assessed visually on leaves.
452
453 **Mycelial growth and sporulation rate at different temperatures**

454 Mycelial growth and sporulation rate were measured for 42 isolates at five different temperatures
455 (10°C, 15°C, 20°C, 25°C and 30°C). For each isolate, Petri dishes containing PDA medium were
456 inoculated with mycelial plugs placed at the center, and incubated in a growth chamber with a fixed
457 temperature. Mycelium diameter was measured along two perpendicular axes at different time points.
458 At the end of the experiment, conidia were collected by adding 5 mL of water supplemented with 0.01%
459 Tween 20 to the Petri dish and rubbing the surface of the mycelium. Conidia were counted in a
460 hemocytometer. Three biological replicates were performed for each isolate, at each temperature.
461
462 **Statistical analyses of climatic and phenotypic data**

463 Ecological niche separation: OMI (outlying mean index), or marginality, is used to study niche

separation and niche breadth. OMI gives the same weight to all samplings, whether rich or poor in species and individuals, and is particularly suitable in cases in which sampling is not homogeneous. OMI measures the deviation between the mean environmental conditions used by one lineage and the mean environmental conditions used by all lineages. OMI analysis then places the lineages in environmental conditions maximizing their OMI. Environmental values, consisting of 19 biome values (WorldClim bioclimatic variables – Fick and Hijmans 2017), were retrieved for all sampling locations. Climatic values were normalized before the analysis. A contingency table was generated to associate the number of isolates from each lineage with each sampling location. Only isolates with a precise location (known region, city or GPS position of sampling) were included. A random permutation test, with 10000 permutations, was used to assess the statistical significance of marginality for each lineage.

Pathotyping: Ordinal symptoms scores were analyzed with a proportional-odds model accounting for ordered categorical responses with a clm() function implemented in the ordinal R package (version 2018.8-25). The significance of the factors was analyzed by ANOVA. Pairwise comparisons of significant factors were performed after computing least-squares means with LSMEANS version 2.30-0 in R, with Tukey adjustment.

Mycelium growth: Mycelium growth was analyzed separately for each temperature, with a linear mixed-effects model. The significance of the factors was analyzed by ANOVA. Pairwise comparisons of significant factors were performed after computing least-squares means with LSMEANS version 2.30-0 in R.

Sporulation: The median number of spores calculated for the various repetitions of the experiment was analyzed with a negative binomial generalized linear model. Pairwise comparisons of significant factors were performed by computing least-squares means.

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