Differential loss of effector genes in three recently expanded pandemic clonal lineages of the rice blast fungus

Sergio M. Latorre, C. Sarai Reyes-Avila, Angus Malmgren, Joe Win, Sophien Kamoun and Hernán A. Burbano

Abstract

Background: Understanding the mechanisms and timescales of plant pathogen outbreaks requires a detailed genome-scale analysis of their population history. The fungus Magnaporthe (Syn. Pyricularia) oryzae—the causal agent of blast disease of cereals—is among the most destructive plant pathogens to world agriculture and a major threat to the production of rice, wheat, and other cereals. Although M. oryzae is a multihost pathogen that infects more than 50 species of cereals and grasses, all rice-infecting isolates belong to a single genetically defined lineage. Here, we combined the two largest genomic datasets to reconstruct the genetic history of the rice-infecting lineage of M. oryzae based on 131 isolates from 21 countries.

Results: The global population of the rice blast fungus consists mainly of three well-defined genetic groups and a diverse set of individuals. Multiple population genetic tests revealed that the rice-infecting lineage of the blast fungus probably originated from a recombining diverse group in Southeast Asia followed by three independent clonal expansions that took place over the last ~200 years. Patterns of allele sharing identified a subpopulation from the recombining diverse group that introgressed with one of the clonal lineages before its global expansion. Remarkably, the four genetic lineages of the rice blast fungus vary in the number and patterns of presence and absence of candidate effector genes. These genes encode secreted proteins that modulate plant defense and allow pathogen colonization. In particular, clonal lineages carry a reduced repertoire of effector genes compared with the diverse group, and specific combinations of presence and absence of effector genes define each of the pandemic clonal lineages.

Conclusions: Our analyses reconstruct the genetic history of the rice-infecting lineage of M. oryzae revealing three clonal lineages associated with rice blast pandemics. Each of these lineages displays a specific pattern of presence and absence of effector genes that may have shaped their adaptation to the rice host and their evolutionary history.

Keywords: Fungi, Pathogens, Plants, Rice, Cereals, Genomes, Population history, Effectors, Infectious diseases, Pandemics

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Background

Plant diseases are a persistent threat to food production due to a notable increase in the emergence and spread of new pathogens [1, 2]. Understanding the mechanisms and timescales associated with new epidemics is essential for both basic studies and the implementation of effective response measures [3]. A fundamental component of this knowledge is a detailed genome-scale understanding of the population structure and dynamics of global plant pathogen populations [4–6]. Population genetic information drives the selection of isolates for activities as diverse as basic mechanistic research and plant germplasm screening for disease resistance. It also helps to pinpoint the origin of pandemic strains and the evolutionary potential of different pathogen populations [7–12]. A thorough understanding of the global population structure is essential for any surveillance program that aims at rapidly detecting pathogen incursions into new geographical areas. In addition, the recent knowledge gained in the biology of pathogen effectors—secreted molecules that modulate host responses—brings yet another dimension to the population genetics framework, as it enables the reconstruction of the evolutionary history of virulence traits and helps guide the deployment of disease-resistant cultivars [7, 13–16].

Fungal plant pathogens account for ~10–80% of crop losses in agriculture and are viewed as a major threat to global food security [1, 2, 17, 18]. Cereal crops like rice, oat, millet, barley, and wheat have provided the foundation of modern agriculture and the success of human-kind. Today’s agriculture is facing the challenge of ensuring global food security for an ever-expanding world population, which is estimated to exceed 9 billion within the next 30 years [19]. The ascomycete fungus *Magnaporthe* (Syn. *Pyricularia*) oryzae, the causal agent of blast disease of cereals, is often ranked as the most destructive fungal pathogen, causing losses in rice production that, if mitigated, could feed several hundred million people [1, 20]. Despite its Linnean name, *M. oryzae* is a multihost pathogen that can also cause the blast disease on other cereal crops, notably on wheat where it has recently spread from South America to Bangladesh resulting in destructive outbreaks [8, 21, 22]. *M. oryzae* reproduces mainly asexually and field isolates of *M. oryzae* are haploid. Asexual reproduction is the predominant mode of reproduction in almost all rice-growing regions; however, population genetics evidence has identified sexually reproducing populations in Southeast Asia, indicating that *M. oryzae* likely lost sexual reproduction outside of its center of origin [23].

Comparative genomics analyses provided insights into the population structure and host-specialization of *M. oryzae* [24–26]. This pathogen consists of a complex assemblage of genetically distinct lineages that tend to be associated with particular host genera [26]. Remarkably, all rice-infecting isolates belong to a single genetic lineage that is thought to have originated from isolates infecting foxtail millet (*Setaria italica* and *Setaria viridis*). *M. oryzae* host-specific lineages exhibit limited gene flow but recurrent gene gain/loss particularly in regions of the genome linked to transposable elements [24, 25]. As in many other plant pathogens, effector genes exhibit a high degree of presence and absence polymorphisms and signatures of adaptive evolution (e.g., higher rate of non-synonymous over synonymous mutations) [25]. Loss of so-called AVR effector genes—activators of host immunoresponses—can dramatically impact the fitness of the blast fungus by enabling virulence on resistant host genotypes [22, 27, 28].

Although the genome sequence of the *M. oryzae* strain 70-15 was at the time of its publication the first fungal plant pathogen genome to be described [29], it took about a decade before comparative genomics analyses of this pathogen started to be reported [24, 25, 30]. Until recently, understanding of the population genomics structure of the rice blast fungus has remained limited. In 2018, two studies reported whole genome sequences from non-overlapping sets of globally distributed rice-infecting *M. oryzae* isolates [31, 32]. Both studies suggested the presence of a diverse Southeast Asian population and two major clonal groups. However, due to sampling or analytical limitations the two studies reached different conclusions about the composition of worldwide populations, i.e., the number of genetic groups and the processes that gave rise to them.

Here, we performed a combined analysis that builds on the studies of Gladieux et al. [31] and Zhong et al. [32] to reconcile the two datasets and increase the number of examined *M. oryzae* individuals to 131 isolates from 21 countries. This has enabled us to assess the global genetic structure of rice-infecting *M. oryzae* more comprehensively than the prior separate analyses of the two datasets. We discovered that the global population of the rice blast fungus consists mainly of three well-defined genetic groups and a diverse set of individuals. Multiple population genetic tests revealed that the rice blast fungus probably originated from a recombinating population in Southeast Asia followed by three independent clonal expansions that took place over the last ~100–200 years. Patterns of allele sharing identified a subpopulation from the recombinating group that introgressed with one of the clonal lineages before its global expansion. Remarkably, the genetic lineages of the rice blast fungus vary in the number and patterns of presence and absence of secreted protein predicted as effectors. In particular, the clonal lineages are defined by specific sets of effectors that may have shaped their adaptation to the rice host and their evolutionary history.
Results and discussion

The global population structure of rice-infecting *Magnaporthe oryzae* consists of three well-defined genetic groups and a diverse set of individuals

To assess the global population structure of rice-infecting *M. oryzae*, we used a total of 131 genome sequences from Gladieux et al. (*N* = 43) [31] and Zhong et al. (*N* = 88) [32]. The combined use of samples from these two studies increases not only the number of *M. oryzae* samples but also their geographical spread (Additional file 1: Fig. S2B-C and Additional file 2: Table S1). We identified a total of 39,862 single-nucleotide polymorphism (SNPs) (see the “Methods” section). For subsequent analyses, we only used SNPs ascertained in all samples (“full information”) (*N* = 11,478 SNPs).

We first sought to investigate the number of distinct genetic groups in our global sample of *M. oryzae* given previous discrepancies in the number of clades or lineages identified in the two studies. We identified three well-defined groups and a diverse set of individuals based on two lines of evidence. First, we used *f*3-outgroup statistics [33] to evaluate the pairwise relatedness between *M. oryzae* samples relative to an outgroup. The *f*3-outgroup statistics measure the amount of shared evolutionary history between samples, which can be interpreted as shared genetic drift (always relative to an outgroup). We summarized the results of all tests by performing hierarchical clustering based on pairwise shared genetic drift comparisons, i.e., z-scores derived from *f*3-outgroup statistic tests (Fig. 1). Additionally, we calculated pairwise Hamming genetic distances between all samples and summarized the information using principal component analysis (PCA). The samples clustered again in three distinct groups and one diverse set of individuals using PC1, 2, and 3, which together explained more than 90% of the variance (Additional file 1: Fig. S1A). We assessed the robustness of these clusters using Silhouette scores, which indicate how similar an individual is to its own cluster compared to other clusters [34]. We found that the best mean Silhouette scores were obtained when the dataset was divided into four clusters (Additional file 1: Fig. S1B).

Since our two approaches consistently revealed the presence of four groups, we named them groups I, II, III, and IV. Whereas groups II and III are geographically widespread, group I is mainly located in Southeast Asia and group IV in the Indian subcontinent (Fig. 2). The correspondence between our classification and previously described nomenclatures can be found in Additional file 3: Table S2. Our grouping very likely recapitulates the four lineages proposed by Saleh et al. based on microsatellite data [35]. Although it is not possible to directly link the microsatellite data with our analysis, we linked the correspondence between groups indirectly through the analysis presented in Gladieux et al., the same group that previously performed the microsatellite analysis. Zhong et al. [32] divided their dataset in three groups (I–III) but did not identify group IV, since their dataset only included one individual from this group. In addition to groups I–IV, Gladieux et al. [31] identified two additional lineages based on a set of phylogenetic analyses. The combined analysis presented

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**Fig. 1.** Genetic clustering of *Magnaporthe oryzae* reveals three defined groups and a diverse set of individuals. The pairwise relatedness between *M. oryzae* samples (X and Y) was estimated using *f*3-outgroup statistics of the form *f*3(X, Y; outgroup), which measures the amount of shared genetic history (genetic drift) between X and Y after the divergence from an outgroup (*M. oryzae* strain from *Setaria*). The hierarchical clustering is based on *f*3-scores resulting from *f*3-outgroup statistic calculations. Darker colors indicate more shared drift.
here showed that these additional lineages from Gladieux et al. are within the genetic diversity of group I, thus splitting of group I is not warranted.

Global population of rice-infecting *Magnaporthe oryzae* probably arose from a recombining Southeast Asian population followed by clonal expansions

To determine the evolutionary origin of the four *M. oryzae* groups identified in this study, we used a set of statistics that evaluate genetic diversity, recombination, and population differentiation. Initially, we visualized the relationships among samples using a phylogenetic network, which are more appropriate for visualizing reticulate evolution (Fig. 3a) [36]. We found that group I exhibited a high degree of reticulation. In contrast, the phylogenetic network showed long internal branches with terminal star-shape phylogenetic configurations almost devoid of reticulations for the well-defined groups II, III, and IV (Fig. 3a). Such configurations are typical of expanding populations after genetic bottlenecks, driven, for instance, by clonal expansions [37]. We, therefore, queried whether genetic diversity levels and recombination rates support clonality in groups II, III, and IV. Two lines of evidence support clonality in these groups compared with the diverse group I: (i) reduced nucleotide diversity measured as $\pi$ (n) [38] (Fig. 3b) and (ii) lower detectable recombination events calculated using the four-gamete test [39] (Fig. 3c). The reduced levels of diversity in groups II, III, and IV in conjunction with their star-like phylogenies are tell-tale signs of populations that have experienced a strong reduction of diversity followed by a population expansion. Reductions in diversity followed by population expansion are typical of both demographic bottlenecks or founder effects (i.e., the establishment of a new population from a reduced number of individuals). Independent of the exact nature of the demographic processes and evolutionary forces that gave rise to the changes in population size, the
diversity and phylogenetic patterns that we observed are mostly driven by the population expansion phase. To calculate a proxy for recombination, we used the four-gamete test, which puts a bound to the minimum number of recombination events in a sample [39]. Although it is known that this test underestimates recombination events, it is a simple and useful proxy for differences in recombination between populations. Our results showed that groups II, III, and IV have on average ~5-fold less recombination events than the diverse group I. In agreement with our analysis, metainformation obtained by Gladieux et al. and Zhong et al. [31, 32] showed that in almost all cases, only one mating type was present in groups II, III, and IV, whereas the two mating types were segregating in the diverse group I (Additional file 1: Fig. S2 and Additional file 2: Table S1). In sum, we conclude that groups II, III, and IV are likely clonal lineages, while group I consists of genetically diverse and recombining individuals (Fig. 3a–c). The original microsatellite-based study by Saleh et al. [35] reported a high level of genetic variability in group IV; however, both our analyses and the ones carried out by Gladieux et al. [31] supported the clonal nature of this group.

To further investigate the relationships and demographic history of *M. oryzae* groups, we measured population differentiation among groups and leveraged the site frequency spectrum (SFS) for each group individually. To measure population differentiation, we used \( F_{ST} \) [40] and found that when clonal groups II, III, and IV are compared among them, their \( F_{ST} \) distances were the highest. Although a fraction of the allele frequency differences that resulted in high \( F_{ST} \) values could have been driven by selection, the fact that on average \( F_{ST} \) values are much higher among clonal groups likely reflects a long history of independent drift. In contrast, whenever the diverse group I is compared with any of the clonal groups, the \( F_{ST} \) distances decreased, suggesting that group I is a common source of genetic diversity for all clonal lineages (Fig. 3d).

Subsequently, for every group, we investigated their corresponding SFS using Tajima’s \( D \) [41], as this statistic records changes in allele frequencies driven, for instance, by variation in population sizes. We found that Tajima’s \( D \) values for all clonal lineages were negative (Fig. 3e). A demographic interpretation of negative Tajima’s \( D \) values is consistent with population bottlenecks or founder effects followed by population expansions and a concurrent accumulation of rare alleles. Negative Tajima’s \( D \) values are consistent with star-like phylogenies, as new mutations that occurred during the expansion phase accumulate in terminal branches lowering Tajima’s \( D \) values. The inspection of the SFS also revealed an excess of high-frequency derived alleles, a feature of the SFS found mostly in rapidly adaptive populations, and that is particularly strong in asexual organisms or in organisms where meiotic

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**Fig. 3.** *Magnaporthe oryzae* population structure is driven by recombination and global clonal expansions. a Phylogenetic network showing the three well-defined groups (green, blue, and red) and the diverse set of individuals (orange) from Fig. 1. b Within-population comparisons of nucleotide diversity measured as \( \pi \). c Recombination proxy calculated by dividing the number of violations of the four-gamete test by the total number of SNPs. d Genetic distances between groups measured as fixation indices (\( F_{ST} \)). The box colors depict the pairwise comparisons between groups. e Tajima’s \( D \).
recombination happen infrequently [42] (Additional file 1: Fig. S3). By using multiple outgroups, we discarded that our observation is caused by misassignment of the ancestral allele. We believe, instead, that the excess of high-frequency-derived alleles might be driven by a process dubbed genetic draft, i.e., the random association of alleles with genetic backgrounds of different fitness (Gillespie, 2000). Thus, although the SFS is mainly driven by genetic drift during the population expansion phase—as manifested by the negative Tajima’s D—linked selection via genetic draft contributes to the fate of neutral alleles. Further theoretical work is needed to quantify the role of genetic draft in clonal populations of *M. oryzae*.

Overall, our results are consistent with a model where Southeast Asia is a likely center of origin of rice-infecting *M. oryzae* and in which three distinct clonal lineages arose from this ancestral population. These findings are consistent with previous models of the evolution of the rice lineage of *M. oryzae* [35].

*Magnaporthe oryzae* rice-infecting clonal lineages are estimated to have arisen in the last 200 years

To estimate the divergence time of the clonal expansions of *M. oryzae*, we first used a Bayesian phylogenetic analysis leveraging the sample collection dates for tip-calibration [43, 44]. To carry out the analysis, we first removed the diverse group I and used only the three clonal lineages, as the recombining group violates the assumptions of phylogenetic reconstruction. We used a concatenation approach including SNPs in the input pseudo-alignment. We also codified the amount of invariant sites in the configuration file, since the exclusion of invariant sites will lead to deeper divergence times (Additional file 1: Fig. S6). We estimated an evolutionary rate of 2.16e−8 substitutions/site/year (1.80e−8 – 2.55e−8 HPD 95%), which was similar and contains in its HPD 95% a previously calculated rate (1.98e−8 substitutions/site/year) [31]. Our approach of including only the clonal lineages permitted the reconstruction of a robust phylogeny and a more accurate estimation of divergence times, as reflected in the high posterior probabilities supporting the nodes and the narrow HPD 95% confidence intervals of node ages (Fig. 4). The topology of the tree—clearly separating all clonal lineages—and the divergence time estimates were robust when we tested the effect of the small sample size of clonal group IV (Additional file 1: Fig. S7). This contrasts with previous studies that included individuals from the diverse recombining group I in the phylogenetic analysis and produced broader HPD 95% confidence intervals (Fig. 5 [31]).

The phylogenetic reconstructions revealed that all three clonal expansions occurred relatively recently over the last 200 years (123–242) (Fig. 4). These expansions happened concomitantly with an increase of the effective population size of all clonal lineages (Additional file 1: Fig. S4).

To assess the robustness of the phylogenetic reconstruction, we carried out two additional analyses. First, we used a full phylogenetic method that takes into account incomplete lineage sorting. Instead of sampling all possible gene trees, the method computes a tree directly from the markers integrating over all possible gene trees [45] (Additional file 1: Fig. S8). Additionally, we used a coalescent-based method for multi-locus unlinked data that infers the quartet trees for all subsets of isolates and then combines the quartets in a single tree [46, 47] (Additional file 1: Fig. S9). Both analyses confirmed the monophyly of each clonal group.

Patterns of allele frequency sharing identify introgression between a subpopulation of the diverse group I and clonal lineage II

Since the identification of admixture between populations facilitates the reconstruction of the evolutionary history of populations, we investigated the admixture history of *M. oryzae* using D-statistics [48, 49]. This test employs counts of site patterns, which are patterns of alternative alleles at a given genomic position, and evaluates whether these site patterns support one of two alternative discordant topologies. The D-statistics will return a value of zero if the two discordant phylogenies are supported equally, whereas positive or negative values indicate asymmetric support and, therefore, introgression. We test the three possible configurations of the following form: D (outgroup, diverse group I; clonal lineage X, clonal lineage Y) (tree insets in Fig. 5a–c). While for clonal lineages II, III, and VI, we used a strain representative for each clonal lineage, we performed a test for every one of the 22 members of the diverse group I. The test will retrieve positive values when the diverse group I is closer to clonal lineage Y and negative values when the diverse group I is closer to clonal lineage X. We found that group II has drifted farther apart from the diverse group I than the two other clonal lineages, as manifested from positive D-statistics when group II was included (as clonal lineage X) in the comparisons (Fig. 5b, c). This accumulation of genetic drift is consistent with the fact that group II was the clonal lineage that diverged earliest from the recombining diverse group (Fig. 4). We retrieved positive D-statistics in tests including almost all individuals of the diverse group I, with the exception of two individuals collected in China—*CH1016* [31] and *HB-LTH18* [32]—that showed strong signals of genetic introgression with the clonal lineage II, as manifested by negative D-statistic values (Fig. 5b, c). Since we detected introgression between these two Chinese samples and all members of group II regardless of their geographic origin (Additional file 1:...
Fig. 5. Patterns of allele frequency sharing identify introgression between a Chinese *Magnaporthe oryzae* subpopulation and clonal lineage II. D-statistics using three different phylogenetic configurations (depicted as colored inset trees). a) D (outgroup, orange; blue, red). b) D (outgroup, orange; green, red). c) D (outgroup, orange; green, blue). In all cases, a *M. oryzae* strain from wheat was used as an outgroup and a fixed individual was selected as representative from each clonal lineage (blue, orange, red). Points represent D-statistic tests for each of the 22 individuals assigned to the diverse clade (orange), and lines depict 95% confidence intervals. Purple dots in b and c correspond to Chinese individuals CH1016 and HB-LTH18, which are the closest individuals to the green clonal lineage.

Fig. 4. Clonal expansions of *Magnaporthe oryzae* took place in the last 200 years. Bayesian tip calibrated phylogenetic tree using individuals belonging to clonal lineages. Average, and HPD 95% confidence intervals are shown in calendar years. The Bayesian posterior probability is shown in red for nodes leading to the clonal lineage expansions.
Fig. S10A-B), we inferred that the admixture should have taken place before the clonal expansion that gave rise to group II about 197–294 years ago (Fig. 4). Previous attempts to detect interlineage recombination were not statistically robust and plagued with false positives [31]. In contrast, D-statistics provide a statistically robust framework that reliably permits distinguishing between introgression and incomplete lineage sorting using genome-wide SNPs [48, 49].

To further investigate the extent and location of the introgression between group II and the two Chinese Group I individuals (CH1016/HB-LTH18), we segmented the genomes of the two Chinese individuals based on their similarity at segregating sites to either group I or group II (Additional file 1: Fig. S11B). This analysis revealed that the genome-wide percentage of group II-like fragments in the Chinese individuals is 44.58%, including a ~4 Mb region in chromosome 3 (Additional file 1: Fig. S11B). To test whether those fragments are a good proxy for the percentage of introgression, we carried out two additional tests. First, we repeated the D-statistic test presented in Fig. 5b and supplementary Fig. 11A, but this time, removing the candidate introgressed fragments. In contrast to the outcome of the test with whole-genome data, this time the test did not indicate introgression, i.e., it was not different from zero (Additional file 1: Fig. S11C). Second, we estimated the proportion of introgression by using a f4-ratio test [50] with the following setup: (group III, group II, group I (without introgressed Chinese individuals), outgroup)/(group III, group II, Chinese introgressed individuals, outgroup). This test estimated the mixture proportion to be ~31.68%, a lower but similar value to the overall percentage of identified group II-like fragments in the Chinese individuals.

**Lineages of Magnaporthe oryzae show distinct patterns of presence and absence of effector genes**

In *M. oryzae*, regions of the genome containing effector genes exhibit a high rate of structural variation as illustrated by presence and absence polymorphisms [25]. We investigated the distribution of known and predicted effector genes within the population structure framework we defined for the rice lineage of *M. oryzae*. We mapped the genome sequences of the 131 isolates to the sequences of 178 known and candidate effectors predicted from the genomes of *M. oryzae* from hosts as diverse as rice, wheat, finger millet, foxtail millet, oat, and *Digitaria* spp. [51]. This pan-effectorome set enabled us to capture as much effector gene diversity as possible. In total, 134 effectors were identified in the 131 isolates (Additional file 4: Table S3). Remarkably, the number of effectors per isolate varied from 108 to 125 with clonal lineages carrying a reduced repertoire of effector genes compared with the diverse genetic group (Fig. 6a, b). This indicates that clonal-expansion-driven bottlenecks not only reduced the overall genetic diversity of all pandemic clonal lineages but are associated with a less diverse repertoire of dispensable genes such as effectors. In pathogenic bacteria, a reduction in the effectiveness of purifying selection has been associated with an increase in gene loss [52]. Moreover, gene loss is particularly prevalent in clonal pathogenic bacteria and has been postulated as a source of phenotypic variation in these otherwise genetically similar species [53]. The association between gene loss and reduced purifying selection in bacteria is a consequence of their strong deleterional bias, i.e., bacteria with reduced effective population size experience genome reduction [54]. In contrast, eukaryotes with small effective population sizes have larger genomes [55] and filamentous plant pathogens are notorious for having repeat-driven genome expansions associated with a “two-speed” architecture [56, 57]. This relation is, however, more complex in the rice blast fungal phylum Ascomycota, where both genome expansions and reductions have been observed [58]. It remains to be tested if the concurrent loss of genetic diversity and dispensable/non-core genes is a widespread consequence of clonality-driven bottlenecks, or if clonal expansions are driven by (adaptive) phenotypic novelty resulting from gene loss.

We next mapped the distribution of the subset of 69 effectors that display presence and absence polymorphisms across all strains (Fig. 6c). The resulting matrix clearly shows that there are distinct patterns of presence and absence of effectors across the genetically defined groups. For example, a set of four effectors (Avr-Rmg8, FR13.00013421, TH16.00119491, and CD156.00121281) are absent in group III. Likewise, PWL3, INA168.g3317, and FR13.00107561.M are absent in groups III and IV, GY11.00110071.M is absent in group II, and FR13.00028961 is absent in group IV (Fig. 6c, Additional file 5: Table S4).

To determine which effectors have the strongest association with the defined genetic structure, we conducted two separate analyses based on the presence and absence effector repertoire per isolate. First, a PCA and effector loadings analysis revealed a set of 13 effectors that explained 90% of the variance of both PC1 and PC2 (Additional file 1: Fig. S12A-B). Similarly, by using extremely randomized trees (a classification machine learning technique), we identified a set of 16 effectors that explained 90% of the variance (Additional file 1: Fig. S12C). Although the two methods produced different rankings of the impact of each effector gene, we found an overlap of 92.3% between the top 13 effectors found in the two subsets. In both cases, the top effectors reproduced the separation of the isolates in the described genetic clusters (Additional file 1: Fig. S12D-E). A close
inspection of this group of top effectors, which were selected in an unbiased way, revealed that they are differentially (almost) present or (almost) absent in the four *M. oryzae* genetic groups (Additional file 5: Table S4). Thus, this group of effectors might have played an important role in the initial adaptation of...
M. oryzae clonal expansions to different rice subspecies and varieties.

The matrix in Fig. 6c indicates that patterns of presence and absence of effector genes reflect different timescales in the evolution of the clonal lineages of M. oryzae. AVR effectors, such as AVR-Pia and AVR-Pii, show a patchy distribution within the clonal lineages. Their recurrent deletion in M. oryzae populations has generated virulent races [27]. This may reflect the fact that their matching resistance genes have been repeatedly bred and deployed into rice cultivars. Other candidate effectors that display a similar patchy distribution may be candidate AVR effectors that are detected by one of the dozens of blast resistance genes that have been bred into rice cultivars.

Our finding that the clonal lineages of rice-infecting M. oryzae display distinct repertoires of effectors raises a number of interesting questions. It is possible that this reflects the distinct genotype of the founding individual of the given clone. It is also possible that the absence of a given AVR effector(s) has facilitated the spread of the clonal lineage to otherwise resistant host genotypes as previously noted in M. oryzae [22, 25, 30, 59]. In the future, it would be interesting to test the extent to which effectors that define the clonal lineages are detected by particular resistance genes. For example, AVR-Rmg8, which is known in wheat blast isolates to mediate avirulence on Rmg8 containing wheat varieties, may also be detected by a rice resistance gene. Future experiments will tease out the degree to which the distinct effector repertoires of the clonal lineages of M. oryzae reflect their adaptation to the rice host and their evolutionary history. Such analyses will require new genomic resources that permit a more accurate identification of effectors in canonical chromosomes and mini-chromosomes [60, 61]. To this aim, it will be fundamental to generate multiple reference genomes sequenced with long-read technologies in conjunction with a de novo assembly. Multiple reference genomes sequenced with long-read technologies in conjunction with a de novo assembly may be candidate AVR effectors that are detected by one of the dozens of blast resistance genes that have been bred into rice cultivars.

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Methods

Datasets and mapping

We used M. oryzae Illumina reads from two recent sequencing studies (43 samples from Gladieux et al. [31], and 88 samples from Zhong et al. [32] (Additional file 2: Table S1)). Raw sequencing reads were downloaded and mapped to the M. oryzae reference genome (GUY-11 PacBio assembly [62]) using bwa-mem V.0.7.12 [63] with default parameters.

Variant identification and filtering

De novo variants were identified using GATK V.3.8.0 [64]. The following set of filters were applied: QD < 5.0; QUAL < 5000.0; MQ < 20.0; −20 < ReadPosRankSum < 2.0; −2.0 < MQRankSum < 2.0; −2.0 < BaseQRankSum < 2.0. In all subsequent analyses, we used only biallelic SNPs present in all samples (“full information”).

Population structure analyses

To assess the global population structure of M. oryzae, we first determined patterns of allele sharing using f3-outgroup statistics [33]. We performed the test using the program qp3Pop from the AdmixTools package [50]. The test was used to establish the pairwise relatedness between M. oryzae samples (X and Y) after divergence from an outgroup: f3(X, Y; outgroup). We used a deeply diverged Setaria-infecting M. oryzae strain SA05-144 [25] as outgroup. We calculated z-scores for every possible pairwise sample comparison included in the f3-statistics test (N = 8515). Subsequently, we carried out hierarchical clustering using the function hclust from the R package stats [65]. As input, we used a distance matrix generated from the f3-statistics-derived z-scores (Fig. 1a).

Additionally, we determined the level of population structure using genetic distances coupled with dimensionality reduction methods. We calculated pairwise Hamming distances using Plink V.1.9 [66]. Such distances were used as input for principal component analysis (PCA) using the function prcomp from the R package stats [65] (Additional file 1: Fig. S1A). To assess the robustness of the clusters, PCA coordinates were used to compute silhouette scores using the function silhouette from the R package cluster [67]. We calculated mean silhouette scores for different numbers of clusters (K = 2–6) and found that the highest mean silhouette scores were obtained when K = 4. We also used Discriminant Analysis of Principal Components (DAPC) [68], implemented in the adegenet R package. The analysis was carried out by capturing the variance in the 10 first PCs. The Bayesian information criterion (BIC) indicated
that the best number of groups was $K = 4$ (Additional file 1: Fig. S1C-D). We used the grouping of individuals in four clusters for subsequent analyses.

**Population genetics analyses**

We constructed a neighbor network using the program *SplitsTree* V.4.14.6 [36]. As a proxy for recombination within each of the clusters, we used the four-gamete test [39] as implemented in *RminCutter* [69]. To this aim, we created consensus *fasta* sequences from the contigs 1 to 7 using the filtered vcf file with *bcftools* V. 1.3.1 [70]. The summary statistic was calculated by dividing the total number of violation events of the four-gamete test by the total number of SNPs. Nucleotide diversity ($\pi$), fixation indices $F_{ST}$, and Tajima’s $D$ values were calculated using *vcftools* V.0.5.15 [71]. We calculated the unfolded site spectrum (SFS) for each genetic group using custom scripts. Ancestral alleles were ascertained requiring concordance between a *Setaria* - and a wheat-infecting out-group strain (SA05-144 [25] and BTJP-4(12) [72]).

We computed $D$-statistic values [48] as follows:

$$D(O, T; X, Y) = \frac{(p_0 - p_T)(p_X - p_Y)}{(p_O + p_T - 2p_O p_T)(p_X + p_Y - 2p_X p_Y)}$$

where $p_O$, $p_T$, $p_X$, and $p_Y$ are frequencies of randomly selected alleles in populations (Outgroup, (T)est, $X$, and $Y$) at each locus. The reported 95% confidence intervals were calculated as $D \pm (SE \times 1.96)$ where the standard error was computed using a jackknife weighted by the number of SNPs for each 5 Mb block in the genome [73]. We performed the calculations using *popstats* [74].

**Genomic segmentation analysis**

Based on the $D$-statistic results, two isolates from the diverse group I (CH1016 and HB-LTH18) showed genome-wide introgression evidence with the clonal lineage II. In order to identify which regions of the genomes of CH1016 and HB-LTH18 show higher nucleotide similarity to clonal lineage II than to members of the diverse group I, we performed a window-based similarity analysis. These regions, especially if they overlap between CH1016 and HB-LTH18, will be strong candidates for being introgressed from the clonal lineage II. Consequently, we performed window-based pairwise nucleotide similarity comparisons between an example isolate of clonal lineage II (TW-PT3-1) and the two Chinese individuals (CH1016 and HB-LTH18). To this end, we divided the seven chromosomes in 400 windows, each of which had the same number of SNPs. To ascertain the basal level of similarity among clonal lineage II individuals, we compared our example clonal lineage II isolate TW-PT3-1 with another clonal lineage II isolate (BR0026). Finally, to ascertain the nucleotide similarity between clonal lineage II and non-introgressed individuals from the diverse group I, we compared our example clonal lineage II isolate TW-PT3-1 with diverse group I isolates CH0532 and CH0333.

**Phylogenetic analysis**

We first carried out a Bayesian tip-dated phylogenetic analysis. To perform this analysis, we first removed individuals from the diverse group I, as these recombining group of individuals do not comply with the assumptions of any phylogenetic analysis (Fig. 2c). We kept only biallelic variant positions to perform a Markov chain Monte Carlo-based phylogenetic reconstruction using *BEAST* V.2.4.8 [75]. We used the isolates’ collection dates (Additional file 2: Table S1) as prior information for the estimation of divergence times. We used *ModelTest-NG* [76] to assess the best suitable substitution model. Based on the lowest Akaike information criterion (AIC), we selected the general time-reversible model. Since the calculation was performed with non-recombining individuals from the same species, we used a strict clock rate with a prior value of 1.98e–8 substitutions/site/year, which was the rate ascertained in Gladieux et al. [31]. To test the hypothesis of a non-clocklike data, we estimated the coefficient of variation in a model relaxed clock log normal model to be 0.0042, suggesting strong evidence for a clock-like data [77]. In order to reduce the effect of demographic history assumptions, and to calculate the dynamics of the population size through time, we also chose a Coalescent Extended Bayesian Skyline approach [78]. Invariant sites were explicitly considered in the model by adding a “constantSiteWeights” tag in the XML configuration file. We combined the output of four independent MCMC chains. Each chain had a length of 10 million iterations and was logged every 1000 iterations. We only used chains with overall ESS values above 200 and summarized a maximum clade credibility tree with *TreeAnnotator*. We summarized effective population size through time using an Extended Bayesian Skyline Plot (Additional file 1: Fig. S4). Configuration and log files are provided in our repository (see code and data availability).

To assess the robustness of the phylogenetic reconstruction to different sources of tree discordances, we carried out several additional analyses. First, to illustrate the effect of recombination in the phylogeny, we included all the individuals from the genetic group I, who displayed signatures of sexual recombination (Fig. 2c) and computed a new phylogeny following the same approach employed for the clonal groups (Additional file 1: Fig. S5). To evaluate the effect of unequal sample sizes
on the topology and divergence times of the clonal groups, we downsampled both genetic groups II and III to \( N = 7 \) (the number of isolates from the genetic group IV). Subsequently, we repeated the phylogenetic analysis using only the 131 \( M. \) oryzae isolates described in Additional file 2: Table S1, we mapped the publicly available genomic short-read sequences from these isolates to a reference set of diverse effector candidate sequences. We used the recently reported database from Petit-Houdenot and colleagues [51], which is composed by 195 candidates with similarity to both AVR and MAX effectors from isolates infecting a wide variety of hosts (e.g., rice, wheat, foxtail millet, oat, and other Digitaria species). We reduced the redundancy of the reference by removing highly similar sequences (\( \geq 90\% \) identity). The final reference set included 178 coding DNA sequences for candidate effectors (Additional file 6: Table S5) from different \( M. \) oryzae lineages infecting hosts such as rice, wheat, oat, millet, and wild grasses. The coordinates of the reference effector genes corresponding to \( M. \) oryzae PacBio genome GUY-11 (GenBank accession GCA_002368485.1) are shown in Supplementary Table 4. We used elongation factor 2 mRNA sequence (GenBank accession XM_003714691.1) from \( M. \) oryzae as a positive control for presence of a gene, and a secreted protein gene CoMC69 from the fungus Colletotrichum orbiculare as a negative control for absence of a gene in the reference for short-read mapping.

Mapping was performed with \textit{bwa-mem} V.0.7.15 [80]. An effector was deemed present if more than 80\% of its sequence was recovered with a minimum depth of 3x, using \textit{SAMtools} V1.6 [81].

To summarize effector content per isolate, we built a presence and absence matrix indicating presence and absence of effector genes with 1 and 0, respectively (Fig. 6a, b and Additional file 7: Table S6). For subsequent analyses, we excluded effector genes that were either present or absent in all lineages, as they are uninformative for clustering algorithms. This filtering resulted in a presence and absence matrix that contains a set of 69 informative effectors. We organized the columns of this matrix according to the den-drogram of genetic groups (Fig. 1), while the rows were sorted using hierarchical clustering with the function \textit{hclust} from \textit{R stats} package [65].

To determine which effectors have the strongest association with the defined genetic structure of \( M. \) oryzae, we conducted PCA and loading analysis using the presence and absence matrix per isolate as input. Analyses were carried out with the \textit{princomp} function of the \textit{R stats} package [65]. Then, by multiplying the absolute value of X and Y coordinates of each loading vector in PC1 and PC2, we assessed the strength of importance per effector (Additional file 1: Fig. S12A). We selected a subset of effectors that contains the 13 most important effectors (i.e., loading vectors with the highest magnitudes), as they together explained 90\% of the variance (Additional file 1: Fig. S12C). We recalculated the PCA using only this subset of 13 effectors, which resulted in an increase from 44.8 to 73.8\% (total increase of 29\%) of the variance explained by PC1 and PC2 together.

A similar analysis was also carried out using the extremely randomized trees algorithm implemented in the \textit{Python scikit-learn} module [82], with 100 trees per forest, and trained using all the effector presence and absence data. The feature importances were extracted from the trained model. This process was repeated 2500 times to ensure consistency, and the mean effector importance for reconstructing the population structure was calculated in order to rank the effectors. Using this method, 90\% of the variance was explained by 16 effectors (Additional file 1: Fig. S12C).

**Effector genes repertoire**

To determine the effector gene repertoire for each of the 131 \( M. \) oryzae isolates described in Additional file 2: Table S1, we mapped the publicly available genomic short-read sequences from these isolates to a reference set of diverse effector candidate sequences. We used the recently reported database from Petit-Houdenot and colleagues [51], which is composed by 195 candidates with similarity to both AVR and MAX effectors from isolates infecting a wide variety of hosts (e.g., rice, wheat, foxtail millet, oat, and other Digitaria species). We reduced the redundancy of the reference by removing highly similar sequences (\( \geq 90\% \) identity). The final reference set included 178 coding DNA sequences for candidate effectors (Additional file 6: Table S5) from different \( M. \) oryzae lineages infecting hosts such as rice, wheat, oat, millet, and wild grasses. The coordinates of the reference effector genes corresponding to \( M. \) oryzae PacBio genome GUY-11 (GenBank accession GCA_002368485.1) are shown in Supplementary Table 4. We used elongation factor 2 mRNA sequence (GenBank accession XM_003714691.1) from \( M. \) oryzae as a positive control for presence of a gene, and a secreted protein gene CoMC69 from the fungus Colletotrichum orbiculare as a negative control for absence of a gene in the reference for short-read mapping.

Mapping was performed with \textit{bwa-mem} V.0.7.15 [80]. An effector was deemed present if more than 80\% of its sequence was recovered with a minimum depth of 3x, using \textit{SAMtools} V1.6 [81].

To summarize effector content per isolate, we built a presence and absence matrix indicating presence and absence of effector genes with 1 and 0, respectively (Fig. 6a, b and Additional file 7: Table S6). For subsequent analyses, we excluded effector genes that were either present or absent in all lineages, as they are uninformative for clustering algorithms. This filtering resulted in a presence and absence matrix that contains a set of 69 informative effectors. We organized the columns of this matrix according to the dendrogram of genetic groups (Fig. 1), while the rows were sorted using hierarchical clustering with the function \textit{hclust} from \textit{R stats} package [65].

To determine which effectors have the strongest association with the defined genetic structure of \( M. \) oryzae, we conducted PCA and loading analysis using the presence and absence matrix per isolate as input. Analyses were carried out with the \textit{princomp} function of the \textit{R stats} package [65]. Then, by multiplying the absolute value of X and Y coordinates of each loading vector in PC1 and PC2, we assessed the strength of importance per effector (Additional file 1: Fig. S12A). We selected a subset of effectors that contains the 13 most important effectors (i.e., loading vectors with the highest magnitudes), as they together explained 90\% of the variance (Additional file 1: Fig. S12C). We recalculated the PCA using only this subset of 13 effectors, which resulted in an increase from 44.8 to 73.8\% (total increase of 29\%) of the variance explained by PC1 and PC2 together.

A similar analysis was also carried out using the extremely randomized trees algorithm implemented in the \textit{Python scikit-learn} module [82], with 100 trees per forest, and trained using all the effector presence and absence data. The feature importances were extracted from the trained model. This process was repeated 2500 times to ensure consistency, and the mean effector importance for reconstructing the population structure was calculated in order to rank the effectors. Using this method, 90\% of the variance was explained by 16 effectors (Additional file 1: Fig. S12C).

**Supplementary information**

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Additional file 1: Fig. S1. Principal component analysis (PCA) reveals four defined groups. Fig. S2. Relation between genetic groups and sample mating type. Fig. S3. The unfolded Site Frequency Spectrum (SFS). Fig. S4. Recent increase of population size in clonal lineages of Magnaporthe oryzae. Fig. S5. Effect of recombination on the Magnaporthe oryzae phylogeny construction. Fig. S6. Estimated Time to Most Recent Common Ancestor (TMRCA) for clonal lineages with / without invariant sites. Fig. S7. Effect of sample size on the topology of the tree and on the estimation of divergence times. Fig. S8. Phylogenetic inference using Single Nucleotide Polymorphisms using SNAPP. Fig. S9. Phylogenetic inference using SVDquartets. Fig. S10. Two Chinese individuals display consistent introgression with the clonal lineage II. Fig. S11. Ancestry-based genomic segmentation of Chinese
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Authors’ contributions
SML, SK, and HAB conceived the study. SML, CSR, AM, JW, SK, and HAB designed and performed data analyses. SML, SK, and HAB wrote the paper. All authors read and approved the final manuscript.

Authors’ information
Sergio M. Latorre: @smlatorreo
C. Sarai Reyes-Avila: @SaraiReyesAvila
Angus Malmgren: @AngusMalmgren
Joe Win: @jewin
Sophien Kamoun: @kamounlab
Hermán A. Burbano: @hermanaburbano

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Availability of data and materials
The datasets and scripts generated during and/or analyzed during the current study are available in the Gitlab repository, https://gitlab.com/smlatorreo/genetic_history_of_rice-infecting_magnaporthe_oryzae [83].

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Research Group for Ancient Genomics and Evolution, Max Planck Institute for Developmental Biology, Tuebingen, Germany. 2The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, Norwich, UK. 3Natural History Museum of Denmark, University of Copenhagen, Copenhagen, Denmark. 4Centre for Life’s Origin and Evolution, Department of Genetics, Evolution and Environment, University College London, London, UK.

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