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Sex at the origin: an Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually

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Abstract

Sexual reproduction may be cryptic or facultative in fungi and therefore difficult to detect. *Magnaporthe oryzae*, which causes blast, the most damaging fungal disease of rice, is thought to originate from southeast Asia. It reproduces asexually in all rice-growing regions. Sexual reproduction has been suspected in limited areas of southeast Asia, but has never been demonstrated in contemporary populations. We characterized several *M. oryzae* populations worldwide both biologically and genetically, to identify candidate populations for sexual reproduction. The sexual cycle of *M. oryzae* requires two strains of opposite mating types, at least one of which is female-fertile, to come into contact. In one Chinese population, the two mating types were found to be present at similar frequencies and almost all strains were female-fertile. Compatible strains from this population completed the sexual cycle *in vitro* and produced viable progenies. Genotypic richness and linkage disequilibrium data also supported the existence of sexual reproduction in this population. We resampled this population the following year, and the data obtained confirmed the presence of all the biological and genetic characteristics of sexual reproduction. In particular, a considerable genetic reshuffling of alleles was observed between the 2 years. Computer simulations confirmed that the observed genetic characteristics were unlikely to have arisen in the absence of recombination. We therefore concluded that a contemporary population of *M. oryzae*, pathogenic on rice, reproduces sexually *in natura* in southeast Asia. Our findings provide evidence for the loss of sexual reproduction by a fungal plant pathogen outside its centre of origin.

Keywords: female fertility, *Magnaporthe oryzae*, mating type, population genetics, recombination, sexual reproduction

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Introduction

The ability of organisms to adapt to environmental conditions is tightly linked to their mode of reproduction. Sexual reproduction shuffles existing genetic material, generating selectable genetic variation (Otto & Lenormand 2002; Otto 2009). Segregation and recombination then eliminate deleterious mutations and fix beneficial mutations (Fisher 1930; Muller 1932, 1964), increasing the efficiency of natural selection. This process is particularly important for species confronted with changing environments, such as pathogens encountering new hosts or new agricultural practices, as demonstrated both theoretically (Otto & Michalakis 1998) and experimentally (Goddard *et al.* 2005; Grimberg & Zeyl 2005; Perlstein *et al.* 2007; Zhan *et al.* 2007). Conversely, an

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absence of recombination ensures that beneficial associations between loci are maintained. This makes it possible to retain local adaptations, once they have occurred (Fisher 1930; Maynard Smith 1978; Feldman *et al.* 1980; Charlesworth & Barton 1996). Many fungal pathogens get the best of both worlds by alternating recombination and vegetative reproduction. This strategy enables them to adapt to changes in agricultural practice and threatens the durability of control measures (McDonald & Linde 2002).

Fungal plant pathogens can easily overcome the resistance of new resistant cultivars: they escape plant surveillance systems, which are often determined by single resistance genes, by modifying a single so-called 'avirulence' gene (Jones & Dangl 2006). In these gene-for-gene interactions, recombination facilitates the adaptation of the pathogen, by combining favourable mutations at multiple avirulence loci. Many of the strategies widely used in breeding for resistance, such as the use of combinations of several resistance genes (pyramiding), are unlikely to be effective in populations displaying sexual reproduction, because a multivirulent strain may easily arise through recombination followed by selection. Thus, deciphering the mode of reproduction of pathogens is an important step towards the definition of durable control strategies (Zeyl 2009).

It is difficult to detect sexual reproduction in populations in which sex is cryptic or facultative. Sexual organs may be hard to observe, as sexual reproduction may be restricted to limited spatial areas or periods of time. High genotypic diversities and low levels of linkage disequilibrium (LD) measured with molecular markers can be used to search for genetic signatures of recombination (Balloux *et al.* 2003; De Meeûs & Balloux 2004; Halkett *et al.* 2005; Arnaud-Haond *et al.* 2007). This requires that appropriate population samples are available, i.e., substantial samples from recombinant populations. Recent studies based on biological or molecular approaches and including well-studied human pathogens have suggested that several fungi long thought to be exclusively asexual actually reproduce sexually (Taylor *et al.* 1999), in either controlled conditions (Hull *et al.* 2000; Horn *et al.* 2009; O'Gorman *et al.* 2009) or natural populations (Burt *et al.* 1996; Matute *et al.* 2006). Other studies have reported the presence of mating-type genes in the genomes of strictly clonal species and in species for which no sexual cycles has been described, probably reflecting a recent loss of the ability to reproduce sexually (e.g. Wong *et al.* 2003; Galagan *et al.* 2005; Fisher 2007; Hoff *et al.* 2008; López-Villavicencio *et al.* 2010). However, the occurrence of sexual reproduction in contemporary populations *in natura* remains difficult to prove unequivocally.

Magnaporthe oryzae is the fungus responsible for blast disease in rice and other grasses; it is considered a model phytopathogenic fungus species (Wilson & Talbot 2009). The genetic structure of *M. oryzae* populations pathogenic on rice suggests that reproduction is clonal in most rice-growing areas (Colombia, USA, Europe, Korea, Japan: Zeigler 1998 for a review; Morocco: El Guilli *et al.* 2005; Madagascar: Andriantsimialona & Tharreau 2008). Moreover, despite careful investigation, perithecia (the organs in which meiosis takes place) have never been reported in the field (Zeigler 1998). However, sexual reproduction has been shown to occur *in vitro* between strains sampled *in natura* (Silué & Nottéghem 1990; Hayashi *et al.* 1997). As in other heterothallic Ascomycetes, the sexual cycle of this fungus requires two strains of opposite mating types (MAT1/-MAT2), at least one of which must be female-fertile (able to produce perithecia). Female fertility is thus a key biological characteristic underlying the mode of reproduction of *M. oryzae*. In this species, female-fertile strains have been collected from rice only very rarely, and only in the Yunnan province of China, northern Thailand and northern India (Zeigler 1998; Kumar *et al.* 1999; Mekwatanakarn *et al.* 1999). Southern China and northern India are the two domestication and diversification centres of Asian cultivated rice, *Oryza sativa* (Londo *et al.* 2006). Genetic diversity analyses have identified the Himalayan foothills as the most likely centre of origin of the *M. oryzae* populations pathogenic on rice (Zeigler 1998; Tharreau *et al.* 2009). Based on these findings, it has been suggested that this fungus may reproduce sexually in south Asia, close to the Himalayan foothills (Zeigler 1998; Tharreau *et al.* 2009). Worldwide migrations would have been accompanied by a decrease in the diversity of this species (Zeigler 1998; Tharreau *et al.* 2009) and a loss of its ability to reproduce sexually. Bottlenecks accompanying migrations and/or selective effects owing to differences in fitness between strains of the two mating types (Soubabère *et al.* 2000; Couch *et al.* 2005) may have led to the fixation of a single mating type in invaded areas, imposing an obligate asexual mode of reproduction on pathogen populations outside the centre of origin. The testing of these hypotheses requires an initial demonstration that *M. oryzae* reproduces sexually in its putative centre of origin. Previous studies have suggested that *M. oryzae* may reproduce sexually in this region, based on the observation of both mating types and of female-fertile strains in restricted areas of Asia (Zeigler 1998). Both these criteria are essential for sexual reproduction to occur, but they are not sufficient to prove that sexual reproduction is still occurring in this area. Only Kumar *et al.* (1999) have provided genetic evidence of sexual reproduction in a *M. oryzae* population pathogenic on

rice. Kumar *et al.* (1999) detected female-fertile strains and both mating types in two Indian populations. As expected, genotyping detected identical multilocus genotypes (MLGs) owing to clonal reproduction. However, no significant LD was observed after clone correction, suggesting that some recombination had occurred. Unfortunately, significant LD was detected the following year, and the authors concluded that 'It cannot be inferred from the data whether the recombination events producing the present Himalayan populations are ongoing' (Kumar *et al.* 1999). It therefore remains to be determined whether sexual reproduction is currently occurring in rice-specific *M. oryzae* populations.

In this study, we combined biological and population genetics approaches to determine whether sexual reproduction was occurring in certain Asian populations. We also contrasted the features of these populations with those of supposedly clonal populations from other continents. We first searched for biological signatures of sexual reproduction in nine populations from around the world, by determining the relative proportions of the two mating types and the frequency of female-fertile strains. We then searched for molecular signatures of recombination, using microsatellite markers and determined whether the genetic characteristics of the nine populations studied could have resulted exclusively from clonal reproduction, by comparing the observed data with simulations for exclusively asexual populations.

Materials and methods

Strain collection, isolation and storage

We used the CIRAD *Magnaporthe oryzae* collection to analyse mating type and female fertility. This collection is comprised of strains isolated from cultivated rice, in 55 countries, over the last 40 years. For detailed population studies, strains were collected, between 1994 and 2009, from cultivated rice in nine populations (the term 'population' is used to refer to all the fungal strains obtained from the same field, on the same date, for the same rice variety) from five countries: China, USA, Madagascar, France and Colombia (Table 1). One population (CH1) was sampled in two consecutive years (2008 and 2009). The TH population was isolated from a barley seed lot from Thailand. Barley has been shown to be susceptible to several *M. oryzae* genetic subgroups specializing on different host plants, including rice. The TH population was shown to have all the genetic and pathogenicity characteristics of populations of *M. oryzae* attacking rice (D. Tharreau, unpublished), and could therefore be considered a 'rice' population. These populations represented a total of 456 strains (23–108 strains per population), which were considered representative of all continents and of the genetic diversity already observed in a worldwide collection of more than 2000 rice-infecting strains (Tharreau *et al.* 2009). Fungal strains were isolated from infected plant material placed in humid chamber at 21 °C for 1–2 days, and genetically pure fungal strains were obtained by monospore isolation. Fungal strains were grown on rice flour medium, as previously described (Silué & Nottéghem 1990), and were stored on filter paper at −20 °C, as described by Valent *et al.* (1986).

Crosses for mating type and female fertility determination

Mating type and female fertility were determined as previously described (Nottéghem & Silué 1992). Each

Table 1 Genetic diversity and multilocus linkage disequilibrium in the studied populations

Origin	Population	*N*	*G*	*CG*	*CG:N* (%)	*STG:N* (%)	N_a	H_e	$\bar{r}_D$	*Comp* (%)
Thailand	TH	27	20	18	67	55	4.1	0.47	0.14	58.1
China	CH1-2008	24	21	18	75	80	5.2	0.63	0.16	27.2
China	CH1-2009	83	76	63	76	86	7.1	0.64	0.07	0.7
China	CH2	38	32	21	55	64	3.8	0.50	0.21	47.1
China	CH3	23	14	14	61	43	4.1	0.50	0.25	83.1
China	CH4	25	5	4	16	13	1.5	0.08	0.62	100
Colombia	CL	31	9	2	6	11	1.5	0.06	NT	100
France	FR	23	12	4	17	22	1.6	0.09	0.30	100
USA	USA	37	20	6	16	31	3.2	0.56	0.57	97.8
Madagascar	MD	95	18	10	11	3	2.1	0.07	0.31	97.1

N, sample size (number of genotyped strains); *G*, number of multilocus genotypes; *CG*, number of clonal groups (calculated with e-Burst, see Methods) ; *CG:N*, clonal richness; *STG*, Stoddart and Taylor's genotypic diversity index standardized by dividing by sample size; N_a, mean number of alleles per locus; H_e, gene diversity; $\bar{r}_D$, multilocus association index, calculated from data corrected for mutation (see Methods); *Comp*, Proportion of compatible pairs of loci; $\bar{r}_D$ was significantly different from 0 in all populations (as assessed with MULTILOCUS 1.3 after 1000 randomizations).

strain to be tested was cocultured on rice flour medium with two reference female-fertile strains of each mating type. Reference strains are strains of known mating type that produce and induce the production of perithecia when cocultured with strains of the opposite mating type. We assessed perithecium production along the line of contact between the strain tested and the reference strain with which it was cocultured, after incubation under continuous fluorescent light for 21 days at 20 °C. If two lines of perithecia were produced (i.e. a line of perithecia borne by the test strain and another borne by the reference strain), the tested strain was considered female-fertile and of the opposite mating type to the reference strain. If a single line of perithecia was produced (sexual organs borne by the reference strain only), the tested strain was considered female-sterile and of the opposite mating type to the reference strain.

DNA extraction and microsatellite amplification

DNA was extracted as previously described (Adreit *et al.* 2007) and stored at −20 °C. We genotyped all strains with 17 microsatellites developed for routine population genetics studies (Adreit *et al.* 2007), selected from a set of about 300 microsatellite markers developed for genetic mapping (Kaye *et al.* 2003; Wang *et al.* 2005; Sreewongchai *et al.* 2009). The chosen markers were evenly distributed on the seven *M. oryzae* chromosomes (Table S1, Supporting information). The microsatellites were amplified by PCR (QIAGEN multiplex PCR kit), and the products obtained were separated and analysed on a 16-capillary ABI Prism 3130XL machine (Applied Biosystems, Foster City, CA, USA). For this analysis, we mixed 1 μL of amplified products with 15 μL Formamide GeneScan-500LIZ size marker (Applied Biosystems). GeneMapper® (Applied Biosystems) was used for allele calling and assignment. The reproducibility of genotyping between runs was ensured by the inclusion of two control strains in each run. Individuals with more than three missing data were eliminated, leaving 406 strains for use in the population genetics analyses.

Genetic diversity and clonal structure

The mean number of alleles per locus, N_a, and gene diversity, H_e (Nei 1987), were calculated for each population with Genepop 4 (Raymond & Rousset 1995). H_e, which is frequently used to estimate expected heterozygosity, provides a measurement of unbiased gene diversity (the probability that two alleles chosen at random from a sample are identical, regardless of sample size). It is therefore suitable for use even with haploid organisms. The number of MLG (*G*) and the *G:N* ratio (*N* = number of genotyped strains) were calculated for each population with Multilocus 1.3 (Agapow & Burt 2001). Differences between two MLGs concerning only one of the 17 loci are more likely to have arisen through mutation than through recombination. We accounted for this by considering two MLGs identical at all but one locus to belong to the same clonal group (*CG*). We used eBurst 3.8 (Feil *et al.* 2004) to detect MLGs differing at only one locus and to group these MLGs into the same *CG*. The *CG:N* ratio was also calculated, to determine the fraction of the sample consisting of different MLGs, corrected for mutation. As *CG:N* could be biased by differences in sample size between the nine populations (Grünwald *et al.* 2003), we also estimated Stoddart and Taylor's genotypic diversity index *STG* (Stoddart & Taylor 1988). This index ranges from 1 in clonal populations to *N* (sample size) in recombining populations. Grünwald *et al.* (2003) suggested that the best method of scaling was to divide *STG* by the expected maximum number of MLGs (g_{max}). However, the large differences in allelic diversity between the populations studied here tended to bias g_{max}. We therefore standardized *STG* by dividing by sample size.

Statistical tests were carried out with the R package version 2.12.0 (R Development Core Team, 2010).

Multilocus and pairwise linkage disequilibrium

Multilocus LD was estimated by calculating the association index $\bar{r}_D$ in Multilocus 1.3 (Agapow & Burt 2001). The widely used association index I_A is based on the variance of the distances between all pairs of individuals (the number of loci by which each pair of individuals differs) and was developed to estimate the degree of deviation from random mating in a population (but see De Meeûs & Balloux 2004 for a discussion on the limitations of this index for detecting recombination). $\bar{r}_D$ is derived from I_A, a slight modification rendering it independent of the number of loci scored (Agapow & Burt 2001). It varies between 0 (random mating) and 1 (complete LD). Multilocus and pairwise $\bar{r}_D$ were calculated from data corrected for *CG* (see above). Their significance was assessed on the basis of 1000 randomizations. The significance of allelic associations between pairs of loci was also assessed by carrying out Fisher's exact test with Genepop 4 software (Raymond & Rousset 1995). Finally, we used Multilocus 1.3 to calculate the proportion of pairs of loci that were compatible (four-gamete test adapted for multiple alleles).

Computer simulations

It is not straightforward to determine whether recombination occurs in a population on the basis of summary

statistics, partly because these statistics are affected by factors other than recombination. They also reflect historical population structure and current rates of mutation, drift and migration (De Meeûs & Balloux 2004). We assessed the likelihood of the values of $\bar{r}_D$ and *CG:N* obtained for the populations studied being generated in the absence of recombination, by simulating asexual populations evolving in conditions generating levels of genetic diversity similar to those observed. In particular, we investigated the effects of high effective population size and migration. Simulations were carried out with quantiNEMO 1.0.2 software (Neuenschwander *et al.* 2008), modified to account for asexuality. We simulated asexual individuals characterized by 17 microsatellite loci following a stepwise mutation model (SMM), with a mutation rate $\mu = 10^{-5}$. The simulations were designed to be compatible with the high level of genetic diversity observed. Such high diversities could be accounted for by high effective population sizes. We tested two values for effective population size: $N_e = 1000$ and $N_e = 10\,000$ individuals. Migration would also be expected to increase within-population diversity. The spores of *M. oryzae* have a limited dispersal range (Nottéghem 1977), and natural populations from neighbouring fields are generally highly structured in invaded areas (Tharreau *et al.* 2009). However, little is known about the scale and intensity of gene flow between populations in the putative centre of origin of this species. We ensured that our simulations were conservative, by simulating nine populations connected by migration. For each parameter set, we simulated nine populations of equal size, connected to each other by migration, in an island model with a migration rate $m = 0.5$. The stepping-stone model of migration and a higher mutation rate ($\mu = 10^{-4}$) were also tested and yielded similar results (not shown). Only simulations with $\mu = 10^{-5}$ and $N_a = 25$ are shown here.

For each simulation, we followed the establishment of an equilibrium between mutation and drift. We sampled 30 individuals, at random, from each population, every 1000 generations and used them to estimate H_e for each population. In this way, we were able to demonstrate the stabilization of H_e at the end of each simulation (i.e. 60 000 generations). At this time point, we sampled 30 individuals at random from each simulated population, for the estimation of *CG:N* and $\bar{r}_D$ (with clone correction). The mean and standard deviation of *CG:N* and $\bar{r}_D$ were then calculated over 18 simulated populations (two replicates of nine populations).

Simulations were also performed to follow changes in the number of MLGs and pairwise LD between two consecutive clonal generations. We generated data sets for the 17 microsatellite markers considered for nine simulated clonal populations of 1000 individuals each. We used a high mutation rate ($\mu = 10^{-4}$) and a migration rate of $m = 0.5$ (island model). Once an equilibrium between mutation, migration and drift was reached, we sampled 24 individuals from two consecutive generations within each of the simulated populations. The results for these nine simulations were compared with real data for the CH1 population. We accounted for differences in sample size between the CH1-2008 and CH1-2009 populations by performing 30 random resamplings of 24 individuals in the 2009 population. We determined the number of MLGs and the number of *CG*s identical in the two consecutive generations (for simulated data sets) or years (for real resampled data sets). We also assessed the significance of the LD between pairs of loci, in each sample, for each generation (for simulated data sets) or year (real resampled data sets), by carrying out Fisher's exact test in GENEPOP 4 (Raymond & Rousset 1995). Bonferroni correction was applied to adjust the significance threshold for multiple testing (136 possible pairs). We then calculated the percentage of pairs in LD maintained between two consecutive generations or years.

Results

Geographic distribution of mating type and female fertility

We analysed the worldwide geographic distribution of mating types of 3800 *M. oryzae* strains (collected as single isolates from different locations) and that of female-fertile strains for a subset of 2805 strains. Both mating types were found in most regions of the World, with the exception of the Mediterranean region (MAT1 strains only; Fig. S1a, Supporting information). We identified a total of 227 female-fertile strains, 225 of which were sampled in Asia. Only two female-fertile strains were identified outside south Asia, both in South America (French Guiana). Thus, female-fertile strains were present almost exclusively in Asia, and their frequencies were highest close to the Himalayas (204/225 female-fertile strains; Fig. S1b, Supporting information). Moreover, the two mating types were equally frequent in this region, identifying the Himalayan foothills as the best place to search for evidence of sexual reproduction in *M. oryzae*.

Biological evidence of sexual reproduction

Biological and population genetics investigations were conducted on population data (data for at least 20 strains isolated from the same field, on the same date). The relevant geographic scale for populations of *M. oryzae* is the rice field, because this species mostly

disperses over short distances (a few metres, Nottéghem 1977). We characterized nine populations collected around the world (Fig. 1, Table 1): five from the Himalayan foothills and four from other continents (Europe, America and Africa).

Strains of the two mating types were found in all the Asian populations tested, and in the US population, whereas a single mating type was detected in the other populations (Fig. 1). Female-fertile strains were found only in Asian populations, but at different frequencies: <20% in the Chinese populations CH2, CH3 and CH4, and >50% in CH1 (2008 and 2009) and in the Thai population TH (Fig. 1). Thus, only the Asian populations had the biological characteristics required for sexual reproduction. Furthermore, for these populations, *in vitro* crosses between strains of opposite mating types from the same population (CH1-2008 or TH) led to the completion of the entire sexual cycle, including the production of viable progenies. In one of the crosses between two CH1 strains, we checked that the mating-type locus and nine unlinked microsatellite markers followed the expected 1:1 segregation in the progeny (data not shown). All these offspring had different MLGs (data not shown).

Genetic signatures of recombination

We characterized the genetic diversity of all populations (Table 1), using 17 microsatellite markers. Considering all the individuals in the nine populations studied, the number of alleles at each locus varied from 3 (Pyrms637b-638b) to 29 (Pyrms261-262 and Pyrms 683B-684B; Table S1, Supporting information). The genetic diversity of populations was assessed by calculating unbiased gene diversity (H_e) and the mean number of alleles per locus (N_a). Neither of these variables is affected by the mode of reproduction in haploid organisms (Balloux *et al.* 2003; Halkett *et al.* 2005). The

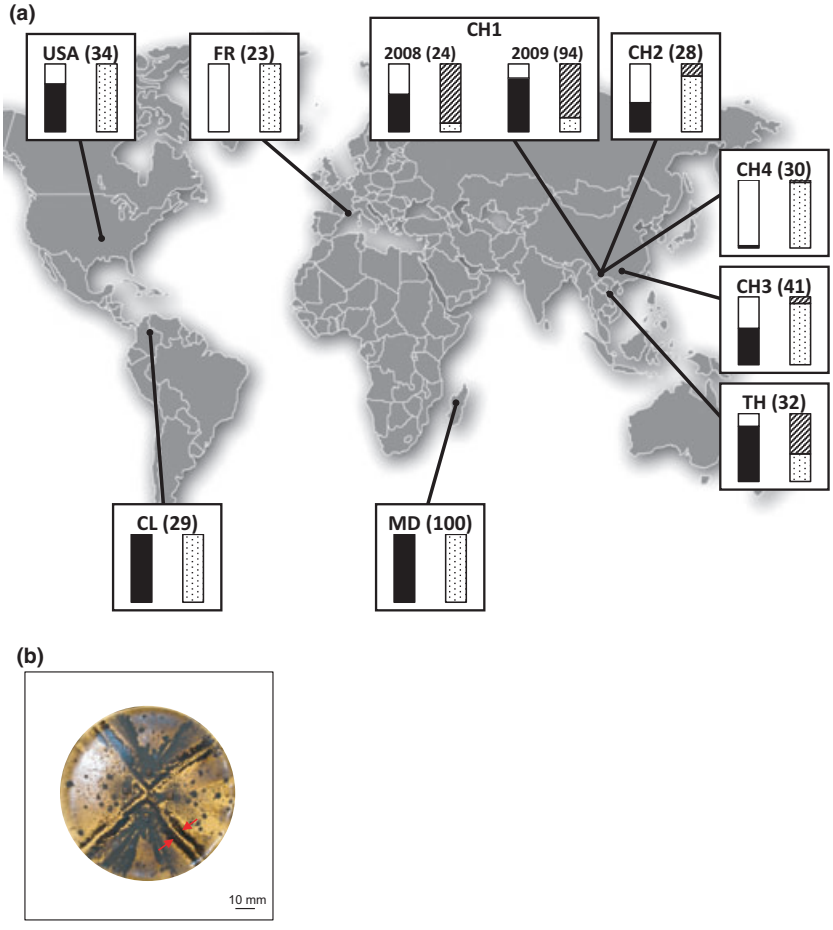

Fig. 1 Mating types and female fertility in nine *M. oryzae* populations. (a) Percentage of MAT1 (white) and MAT2 (black) strains and of female-fertile (hatched) and female-sterile (dotted) strains in each population, determined by crossing *in vitro*. The sample size is indicated in brackets. (b) Coculture of female-fertile strains of opposite mating types (here from the CH1 population) on a Petri dish. After 20 days of incubation at 20 °C, two lines of perithecia (indicated by red arrows) are formed between the two strains.

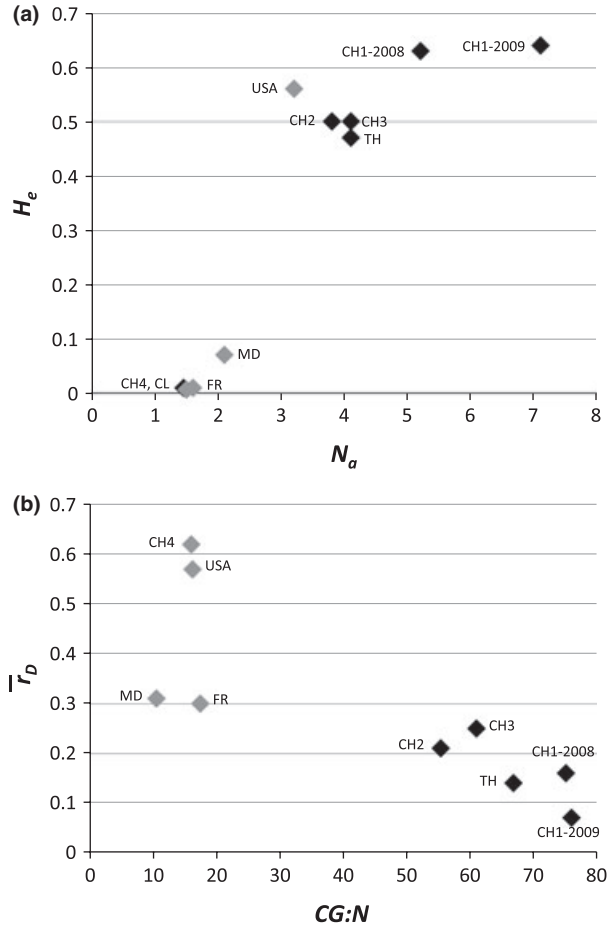

Fig. 2 Diversity and recombination in the 10 populations studied (Asian populations in black, populations from other parts of the world in grey). (a) Gene diversity, H_e as a function of mean allelic diversity, N_a. (b) Multilocus association index $\bar{r}_D$ (calculated from data corrected for mutation, see Methods), as a function of *CG:N* (expressed in %). The CL population is not represented on panel b because the multilocus $\bar{r}_D$ could not be estimated for this population. CG, clonal group.

populations studied clearly fell into two groups defined on the basis of these diversity indices (Table 1, Fig. 2a): CH1 (2008 and 2009), CH2, CH3, TH and USA clearly had a significantly higher gene diversity (Student's *t* test for H_e: $P = 0.01$) and a significantly larger number of alleles (Student's *t* test for N_a: $P = 6 \times 10^{-3}$) than the other four populations.

The degree of clonality, assessed by calculating *CG:N* and *STG:N*, differed significantly between populations (Table 1, Fig. 2b). With the exception of CH4, all populations from the putative centre of origin had a significantly higher *CG:N* than the other populations (mean = 58% vs. 13% in the other populations; Student's *t* test $P = 2 \times 10^{-5}$). Estimates of genotypic diversity based on the standardized Stoddart and Taylor's index *STG:N* were consistent with the results obtained with *CG:N*. Genotypic diversity exceeded 50% in the Asian populations CH1, CH2 and TH, whereas it was below 32% in CH4 and non-Asian populations. The CH3 population was intermediate, with a *STG:N* of 43%.

Consistent with these findings, in the populations from the putative centre of origin (other than CH4), multilocus LD was weak, with an $\bar{r}_D$ index of 0.07–0.25 (vs. 0.30–0.62 in the other populations; Student's *t* test $P = 0.04$). Despite these significant differences between Asia and the other continents, $\bar{r}_D$ was significantly different from 0 in all populations ($P < 0.008$ after 1000 randomizations), making it possible to reject the hypothesis of panmixia. Differences in $\bar{r}_D$ between populations seemed to follow a more continuous distribution than the clearly bimodal distribution of *CG:N* values. This might reflect the sensitivity of $\bar{r}_D$ to factors other than recombination, such as migration and population size (De Meeûs & Balloux 2004). We also determined the number of loci required to discriminate between the various MLGs in the different populations, because sexual reproduction would be expected to generate sufficient allelic combinations for the detection of many MLGs with small numbers of loci (Delmotte *et al.* 2002). In populations from the putative centre of origin (other than CH4), the identification of 62–88% of the observed MLGs was possible with six of the 17 loci, whereas only 41–46% of MLGs could be distinguished with the same number of loci on the other populations (Fig. S2, Supporting information). These results strongly suggest that recombination occurred in the CH1 (2008 and 2009), CH2, CH3 and TH populations.

Investigations of pairwise LD provide information about the intensity and extent of recombination in genomes. Pairwise $\bar{r}_D$ values were calculated for data corrected for mutation (see Methods), for populations in which a sufficiently large number of pairs (i.e. non-monomorphic pairs) could be tested: the CH1, CH2, CH3, TH and USA populations. We also tried to investigate the pairs displaying significant association in these populations. The proportion of the 136 possible pairs displaying significant pairwise LD was low (7–38%) in the CH1-2008, CH2, CH3 and TH populations, but reached 82% in the USA population (Fig. S3, Supporting information). Furthermore, pairwise $\bar{r}_D$ values were centred on 0.2 in the Asian populations tested vs. 0.6 in the USA population. By observing pairs of loci, it is also possible to infer whether the different genotypes observed result from mutation only (compatible pairs; see Methods). All pairs of loci would be expected to be compatible in clonal populations, whereas very few pairs of loci would be expected to be compatible in recombining populations. We found that 97–100% of the pairs of loci were compatible in the

CH4, FR, MD and USA populations. The proportion was lowest in the CH1 population (27% in 2008 and 1% in 2009) and intermediate in the TH, CH2 and CH3 populations (47–83%). The USA population is a good asexual 'control' population for comparison with the Asian populations, avoiding the confounding effect of genetic diversity. Indeed, none of the strains sampled from this population was female-fertile, but the genetic diversity of this population (as assessed by determining H_e and N_a) was similar to that of the Asian populations. This diversity resulted from the coexistence of genetically unrelated clones within the USA population, probably due to multiple introductions of strains of different origins. The indices used to detect recombination (*CG:N*, $\bar{r}_D$, pairwise $\bar{r}_D$ and proportion of compatible pairs) clearly distinguished the USA population from the Asian populations (with the exception of CH4; Table 1). These results confirm that the non-Asian and CH4 populations have the genetic features expected of populations displaying clonal reproduction, whereas most Asian populations, including CH1 in particular, have genetic characteristics compatible with sexual reproduction.

Evidence of sexual reproduction provided by computer simulation

We investigated whether the values of *CG:N* and $\bar{r}_D$ observed in most Asian populations could be reached without recombination, by simulating clonal populations connected to each other by migration, with a migration rate $m = 0.5$, for 17 microsatellite loci with a mutation rate $\mu = 10^{-5}$ (Table S2, Supporting information). The genetic diversity of these simulated clonal populations (N_a and H_e) was similar to that observed for the Asian populations. Even with a high effective population size ($N_e = 10\,000$), which would probably decrease the effects of genetic drift, the *CG:N* index reached at equilibrium was always significantly lower (14–43%, Kruskal–Wallis test $P = 6 \times 10^{-3}$) than that for the Asian populations. Consistently, $\bar{r}_D$ was always significantly higher (0.33–0.40, Kruskal–Wallis test $P = 3 \times 10^{-3}$) in the simulated populations than in the Asian populations. In the TH and CH1 populations, the observed values of *CG:N* (67% and 75%, respectively) and $\bar{r}_D$ (0.14 and 0.16, respectively) were very different from the values obtained in simulations (*CG:N* = 14 ± 4% and 50 ± 8% for $N_e = 1000$ and 10 000, respectively; $\bar{r}_D = 0.33 \pm 0.12$ and 0.40 ± 0.18 for $N_e = 1000$ and 10 000, respectively; Fig. 3, Tables 1 and S2, Supporting information). The observed values for the CH2 and CH3 populations were less different from the values obtained in the simulations: *CG:N* = 55% and 61%, respectively and $\bar{r}_D = 0.21$ and 0.25, respectively. The simulations showed that the high *CG:N* and low $\bar{r}_D$ values obtained for most of the populations from the putative centre of origin of the species were unlikely to have been achieved by strict asexuality, supporting the notion that sexual reproduction occurs in Asia.

Resampling a sexually reproducing population

The CH1 population collected in 2008 provided the best evidence for contemporary sexual reproduction, with the two mating types present at similar frequencies, a high frequency of female-fertile strains (79.2%; Fig. 1), the highest value of *CG:N* and the lowest level of LD (*CG:N* = 75%, $\bar{r}_D = 0.16$; Table 1, Fig. 2b). The sample collected from the same field in 2009 presented similar characteristics, indicating the occurrence of sexual reproduction (Figs 1 and 2b, Table 1). The 2008 and 2009 samples of the CH1 population were not differentiated ($F_{ST} = 0.006$), confirming that they belonged to the same population. Sexual reproduction, by reshuffling allelic combinations at different loci, would be expected (i) to generate new MLGs across generations and (ii) to disrupt significant associations between pairs of loci. Conversely, in strictly clonal populations, we would expect (i) MLGs to persist across generations and (ii) the persistence of significantly linked pairs of loci across generations. A larger number of MLGs persisted across two simulated consecutive generations in the clonal simulations (7.2 ± 1.9 standard deviation) than between the two samples of the CH1 population (in which one MLG persisted). These results are consistent with significant allele shuffling between the 2 years. We then used the same simulated data to focus on changes in statistical associations between pairs of loci between the two sampled years. The percentage of locus pairs disrupted between two consecutive generations (NS/S) and the percentage of pairs linked only in the second generation (NS/S) were not significantly different in the simulated populations and the CH1 population (Fig. 4). However, the percentage of pairs remaining significantly linked in both generations (S/S) was significantly lower in the CH1 population (0.4 ± 0.5% standard deviation) than would be expected under strict asexual reproduction (25 ± 8% standard deviation; Student's t test $P = 3.10^{-5}$). Conversely, pairs of loci remaining significantly unlinked (NS/NS) were the most frequent in the CH1 population, accounting for a mean of 89% (±2% standard deviation) of the pairs. This proportion was significantly lower in simulated clonal populations (mean of 46 ± 9% standard deviation; Student's t test $P = 6.10^{-8}$). Thus, the CH1 population deviates significantly from the null hypothesis of an absence of recombination, and this deviation is

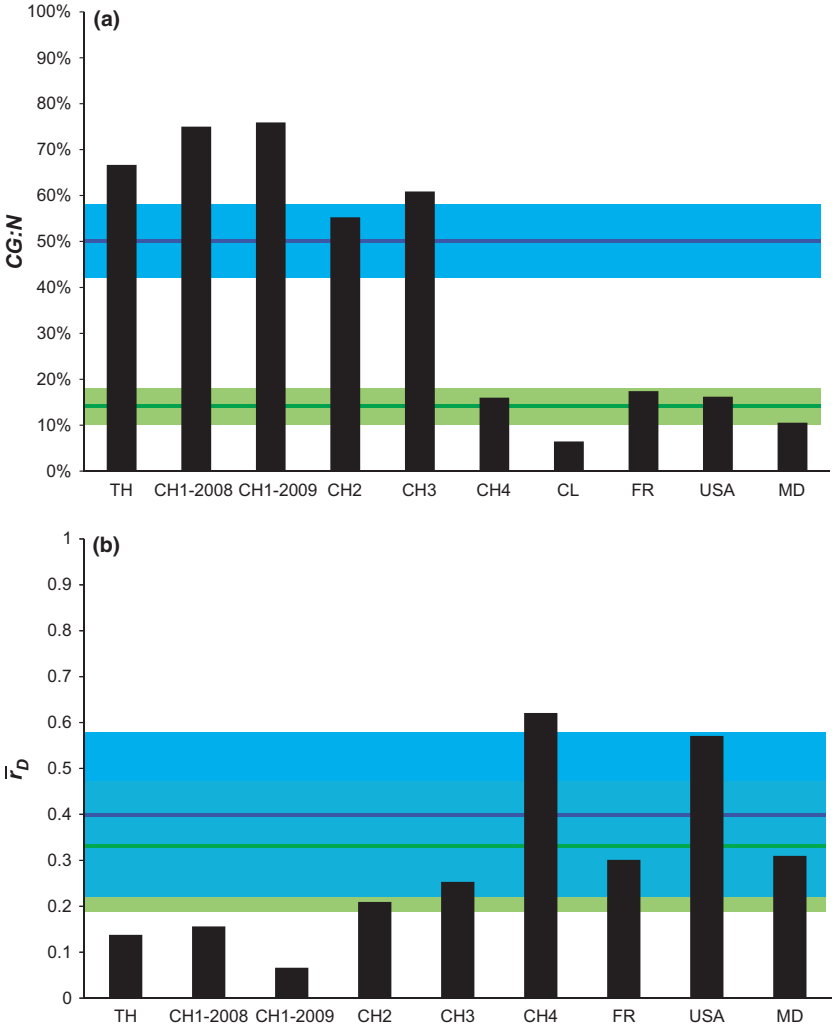

Fig. 3 Genotypic diversity and linkage disequilibrium in simulated clonal populations, in comparison with observed data. (a) *CG:N* in each studied population (bars). (b) Multilocus $\bar{r}_D$ (calculated from data corrected for mutation, see Methods) for each of the populations studied except for CL (bars). For simulated populations, two replicates of nine simulated clonal populations with a mutation rate $\mu = 10^{-5}$, an initial $N_a = 25$, a migration rate $m = 0.5$ (island model of nine connected populations) and a population size $N_S = 1000$ (green) or $N_S = 10\,000$ (blue), were studied. *CG:N* and $\bar{r}_D$ were calculated on random samples of 30 individuals taken from each of the 18 simulated populations after 60 000 generations (lines and filled areas representing the mean and standard deviation, respectively). CG, clonal group.

unlikely to be due to sampling bias or to other factors, such as high mutation rates or migration. Moreover, no significant correlation was observed between the 2008 and 2009 values for pairwise LD, as estimated by calculating the pairwise $\bar{r}_D$ (Spearman's rho nonparametric test: $P = 0.39$; Fig. S4, Supporting information), confirming a lack of maintenance of pairwise associations over time in this population. These results provide strong evidence for genome-wide recombination in the CH1 population between the two sampling years.

Discussion

This study is the first to combine several different, complementary approaches (reproductive biology, population genetics and computer simulations) to explore possible differences in the mode of reproduction of *M. oryzae* between populations at its centre of origin and in invaded areas. Contemporary sexual reproduction has never previously been unequivocally demonstrated for this species. The most successful previous attempt at demonstrating contemporary sexual reproduction in *M. oryzae* was the study by Kumar *et al.* (1999). Using the multilocus association index I_A, these authors identified two populations from north India with no significant LD. However, this result was not confirmed with samples collected the following year. The absence of LD observed in the first year of sampling may reflect recent or ancient recombination. Alternatively, it may simply result from sampling bias, as recent studies have shown that I_A is sensitive to evolutionary forces other than recombination (such as genetic drift and migration; De Meeûs & Balloux 2004), and that potential bias in sampling is likely to mimic the recombination signature detected by measuring LD (Prugnolle & De Meeûs 2009). Such biased sampling is likely to have occurred in Kumar's study, because samples of different rice varieties (and sometimes from different years) were pooled for analysis, and *M. oryzae* population structure depends strongly on rice variety (Chen *et al.* 1995).

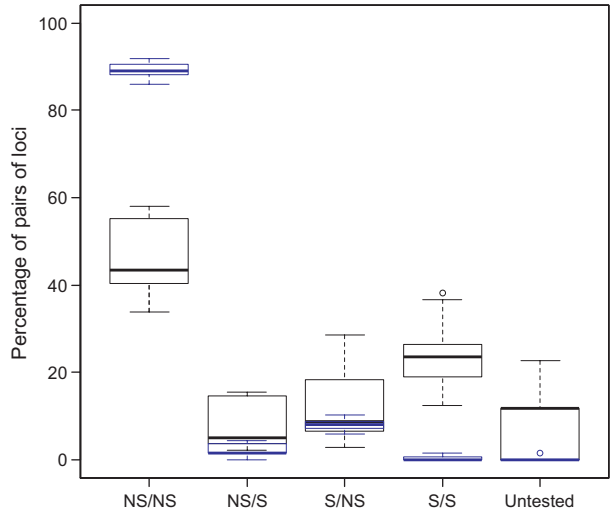

Fig. 4 Comparisons of pairwise linkage disequilibrium (LD) between two consecutive generations in simulated clonal populations and in the CH1 population. The different categories on the *x*-axis represent pairs of loci not significantly linked in the two consecutive years (NS/NS), not significantly linked in the first year but significantly linked the following year (NS/S), significantly linked in the first year but not in the following year (S/NS), significantly linked in the two consecutive years (S/S) and untested. White boxplots represent the percentage of locus pairs in each category calculated from random samples of 24 individuals from two consecutive generations in nine simulated clonal populations of 1000 individuals each, at mutation–migration–drift equilibrium. Blue boxplots represent the percentage of locus pairs in each category calculated from the CH1-2008 sample and 30 random resamplings of 24 individuals from the CH1-2009 sample.

Our analysis of a worldwide strain collection confirmed previous observations (Zeigler 1998; Tharreau *et al*. 2009) that strains of both mating types are found in Asia, and that female-fertile strains are almost entirely restricted to this area. Only two female-fertile strains were found outside this region, both in French Guiana. However, these strains are unlikely to reflect the presence of contemporary sexual reproduction, as historical and genotypic data indicate that these strains were probably imported from south Asia by Hmong migrants in the 1970s (J. L. Nottéghem, personal cummunication). This study represents a step forward by confirming the co-occurrence of both mating types and female-fertile strains in Asia at the population scale. The presence of these features in populations from the Himalayan foothills is indicative of the occurrence of sexual reproduction in this region (Taylor *et al*. 1999; Zeyl 2009).

In addition to biological data, we used several population genetics tools to test for the occurrence of recombination, for further characterization of the mode of reproduction of the populations studied. All our analyses were consistent with recombination in the CH1, CH2, CH3 and TH populations, with the strongest evidence obtained for the CH1 population. Simulations of clonal populations with parameter ranges likely to maximize genetic diversity (i.e. high allelic diversity, large population size and high migration rate) showed that the low values of $\bar{r}_D$ and the high values of *CG:N* observed in the Asian populations could not be attained with a strictly clonal mode of reproduction. The CH1 population, in particular, had the highest *CG:N*, the highest genotypic richness and the lowest $\bar{r}_D$ with respect to simulated clonal populations and the most diverse clonal population sampled (USA). For the CH1 population, which was collected in the Yunnan province of China, we also investigated whether recombination was still occurring, by comparing samples collected in two consecutive years. We used the simulated clonal data sets to test the null hypothesis of the persistence of MLGs and significant pairwise allelic associations between two consecutive generations in the absence of recombination, even in the presence of high levels of genetic diversity. A single MLG was sampled in both years, and <1% of pairs of loci remained linked in both years, strongly suggesting that sexual reproduction is ongoing in the CH1 population. Signatures of recombination in Asian populations might also have resulted from parasexuality. This process is thought to occur in *M. oryzae* (Zeigler *et al*. 1997), but should generate only rare recombination events. It is therefore unlikely to have generated the reshuffling of alleles observed between 2008 and 2009 in the CH1 population. Sexual reproduction is thus the hypothesis most likely to account for all of the biological and genetic characteristics of the CH1 population. This work provides strong, albeit indirect evidence that sexual reproduction occurs in this species in Asia. The discovery of perithecia in the field would provide definitive evidence for contemporary sexual reproduction in the population concerned. However, the discovery of sexual structures would provide no information about the relative importance of the sexual and asexual modes of reproduction. For the management of control strategies, it is actually more important to determine the extent to which recombination occurs in the field than to find sexual stages of the fungus. It remains possible that *M. oryzae* reproduces sexually outside of the growing season, on rice straw or a secondary host plant.

The detection of sexual reproduction, and hence recombination, in field populations of plant pathogens is important, because the choice of control strategy, particularly as concerns the use of resistant varieties and pesticides, depends largely on the reproduction system of the pathogen (McDonald & Linde 2002). Recombination increases the probability of favourable allelic

combinations being generated, selected and rapidly spread, thereby compromising classical control strategies (McDonald & Linde 2002). Most of the strategies based on varietal resistance commonly proposed for the control of rice blast fungus (e.g. lineage exclusion and pyramiding; Bonman *et al.* 1992; Zeigler *et al.* 1994) are likely to fail if the pathogen displays sexual reproduction (McDonald & Linde 2002). For example, the lineage exclusion strategy is based on the observation that isolates from the same clonal lineage have genes controlling cultivar specificity (avirulence genes) in common. It has been suggested that the use of combinations of different resistance genes effective against different lineages might make it possible to exclude all lineages (Zeigler *et al.* 1994). This strategy implicitly assumes that there is no genetic exchange between lineages, because such exchanges would generate new combinations of avirulence genes, resulting in multivirulent strains, which would then be selected. Our results suggest that there is a need to reconsider blast management strategies, at least in some areas of south Asia. Variety management based on the use of different specific resistance genes at different times and in different areas may be preferable to accumulating multiple resistance genes in a single variety (pyramiding). Varieties with nonspecific resistance could also be used, either as an alternative strategy or in combination with pyramiding, although the gradual breakdown of this type of resistance is also possible (McDonald & Linde 2002). Integrated management, based on various control methods (agronomic, genetic, chemical), could also be useful, to decrease selection pressure and slow adaptation.

This study provides new information about the centre of origin of *M. oryzae*. Asian cultivated rice, *Oryza sativa* (Londo *et al.* 2006), diversified and was domesticated in two centres, in southern China and northern India. Previous studies have identified the Himalayan foothills as a centre of genetic diversity for the *M. oryzae* strains pathogenic on rice (Zeigler 1998; Kumar *et al.* 1999; Tharreau *et al.* 2009). Populations from the centre of origin of a species are generally expected to be more diverse than populations from invaded areas, because migration from the origin is often accompanied by bottlenecks or founder effects, leading to a loss of diversity (Sakai *et al.* 2001; Dlugosch & Parker 2008). Our findings confirm that gene diversity and allelic richness are greater in populations from Asia than in populations from elsewhere. The occurrence of sexual reproduction exclusively in this region also provides additional evidence that Asia is the centre of origin for this species. The USA and CH4 populations seem to constitute exceptions with particular demographic histories. Gene diversity was high in the USA population, whereas genotypic diversity was low, consistent with the presence of a mixture of clones, probably due to multiple introductions (Tharreau *et al.* 2009). By contrast, the gene diversity of the CH4 population was lower than that of other Asian populations, probably due to a bottleneck resulting from selection by the host. However, further population genetics studies on worldwide collections are required to confirm that Asia is the centre of origin of rice blast disease.

Our results raise questions about the evolution of sexual reproduction during the spread of pathogens outside their centre of origin. According to the general theory of sex evolution, sexual reproduction is likely to be selected in heterogeneous environments (Lenormand & Otto 2000; Otto & Lenormand 2002). In the context of host-pathogen coevolution, sexual reproduction of the host is thought to be favoured by strong parasitic pressure exerted by a diverse parasite population, as this reproductive system allows the recombination of defence systems to combat parasites (Hamilton *et al.* 1990; Otto & Michalakis 1998; Lively 2010). Conversely, pathogens are also likely to maintain sexual reproduction in areas of high host genetic diversity (Stukenbrock & McDonald 2008). In particular, for fungi making extensive use of sexual reproduction, theoretical models predict that, regardless of mating types ratios, female fertility may be more frequent in native (diverse) than in agricultural (more uniform) environments (Leslie & Klein 1996). Our study provides experimental support for these predictions, because female-fertile strains and sexual reproduction were maintained in areas in which diverse traditional varieties are grown. By contrast, asexual reproduction was observed in the fields of highly uniform rice cultivars. These findings are consistent with the hypothesis that sexual reproduction is maintained only when the pathogen is confronted with diverse host populations. A loss of sexual reproduction in homogeneous environments has been documented in various other species, including aphids (Gilabert *et al.* 2009; Simon *et al.* 2010) and rotifers (Becks & Agrawal 2010). *Phytophthora infestans* (Gómez-Alpizar *et al.* 2007) and *Puccinia striiformis f.sp. tritici* (Bahri *et al.* 2009; Mboup *et al.* 2009; Ali *et al.* 2010) provide additional examples of Oomycete or fungal pathogens that have lost the ability to reproduce sexually in invaded agrosystems. By contrast, other well-studied fungal pathogens, such as *Mycosphaerella graminicola* (Linde *et al.* 2002), *Mycosphaerella fijiensis* (Rivas *et al.* 2004), *Phaeosphaeria nodorum* (Stukenbrock *et al.* 2006), *Ustilago scitaminea* (Raboin *et al.* 2007) and *Venturia inaequalis* (Gladieux *et al.* 2008), have retained both sexual and asexual reproduction systems, even in uniform agrosystems. In these species, the sexual cycle is essential for overwintering (*M. graminicola*, *P. nodorum* and *V. inaequalis*), dissemination (*M. fijiensis*) or infection

(*U. scitaminea*). The requirement of sexual reproduction for these processes may account for the conservation of sexual reproduction by these species outside of their native area, despite the relative uniformity of the invaded environments. These fungal pathogens demonstrate that sexual reproduction may be retained in individual species owing to various short-term constraints (Gouyon 1999).

The evidence of sexual reproduction in Asia also raises questions about the maintenance of sexual reproduction in centres of origin. It remains unclear whether sexual reproduction confers a short-term ecological advantage on *M. oryzae* in native areas. It may provide the pathogen with a means of overwintering. The asexual spores of *M. oryzae* are not very resistant, and perithecia are probably more resistant to adverse conditions. Fruiting bodies are known to serve as overwintering structures in some fungi (Agrios 1997). In *M. oryzae*, sexual reproduction would be maintained in traditional agro-ecosystems, to enable the pathogen to survive the winter, when there is no rice in the field, and to produce a source of primary inoculum for the following season. In more intensive agro-ecosystems, infected seeds harvested and stored by humans may allow the pathogen to overwinter and serve as a primary source of inoculum. Whatever the selection pressure, our results provide experimental evidence that changes in agro-ecosystems can have a major impact on the evolution of the reproduction system of associated plant pathogens.

Fungal pathogens of cultivated plants provide us with a unique opportunity to study the evolution of sex because (i) a single species may use several reproductive strategies and (ii) evolutionary changes may occur faster in agro-ecosystems than in natural ecosystems, owing to high reciprocal selection pressures (McDonald & Linde 2002; Stukenbrock & McDonald 2008). *M. oryzae*, with its coexistence of sexual and asexual populations, thus joins other well-studied species, including aphids (Simon *et al.* 2010) and freshwater snails (Lively & Jokela 2002; Lively 2010), as an excellent model for this field of research.

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This study is part of the PhD thesis of D.S. on the rice blast fungus, *Magnaporthe oryzae*. She has studied the population structure and the modification of the reproduction following worldwide dissemination of the pathogen under the supervision of E.F. and D.T.. J.M. and H.A. are technicians working

with E.F. and D.T.. V.R. develops theoretical approaches on the adaptive potential and applies them to plant pathogens. E.B. develops models in populations genetics to understand the forces shaping diversity. E.F. and D.T. study, through biological, genetic and genomic approaches, how *Magnaporthe oryzae* adapts to its hosts. P.X. is a rice breeder in Yunnan Province of China. Y.S., C.Y.L. and J.L.N. are plant pathologists with long and varied experience on rice blast.

Data accessibility

Sample locations and microsatellite data: DRYAD entry doi: 10.5061/dryad.67891086.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Microsatellite primers.

Table S2 Genetic diversity and multilocus linkage disequilibrium in the simulated populations.

Fig. S1 Worldwide distribution of mating types and female fertility in a non-populational collection of *M. oryzae* strains from rice (samples collected at the continental scale).

Fig. S2 Relationship between *G:N* and the number of scored loci in the different populations.

Fig. S3 Pairwise linkage disequilibrium in five populations.

Fig. S4 Pairwise linkage disequilibrium between two consecutive years in the CH1 population.

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