Population structure, genetic diversity, and sexual state of the rice brown spot pathogen *Bipolaris oryzae* from three Asian countries

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*Bipolaris oryzae* causes brown spot in rice (*Oryza sativa*) inflicting substantial grain yield losses worldwide. Knowledge of the population structure, genetic diversity and sexual recombination of the fungal pathogen can help to implement effective disease management strategies. In this study, *B. oryzae* isolates sampled from Iran, the Philippines and Japan were analysed with 12 simple-sequence repeat (SSR) markers, newly developed from the genome sequence of the fungus. Among the 288 *B. oryzae* isolates genotyped, 278 unique haplotypes were identified. High genotype numbers (richness) with even distribution (evenness) were found within the collection sites. Both mating types, MAT1-1 and MAT1-2, were present in each collection area, and the sexual state was induced under controlled conditions with production of viable ascospores. However, the tests of linkage disequilibrium rejected the hypothesis of random mating. Discriminant analysis of principal components (DAPC) revealed that the *B. oryzae* collection formed three clusters, each consisting of isolates from different collection sites. Analysis of molecular variance (AMOVA) showed that genetic variation among clusters was 18.7%, with the rest of the variation distributed within clusters (*R_{ST} = 0.187, P < 0.001*). Statistically significant pairwise genetic differentiation was found between the clusters. These results show that Asian *B. oryzae* isolates are genetically diverse, and, overall, distributed in three groups. These findings will be helpful in managing the disease and guide the use of representative isolates needed for selection of resistant rice varieties.

**Keywords:** *Cochliobolus miyabeanus*, gene and genotypic diversity, microsatellites, population differentiation, random mating

Introduction

Rice brown spot (RBS), caused by *Bipolaris oryzae* (teleomorph: *Cochliobolus miyabeanus*), is an important disease for rice around the world (Ou, 1985; Webster & Gunnell, 1992; Barnwal \textit{et al.}, 2013). The disease was first described in 1900 in Indonesia where it is currently found in almost every rice-growing region (Ou, 1985). *Bipolaris oryzae* is frequently present on nonfungicide-treated rice seeds commercialized in southern and eastern parts of Asia (Mew & Gonzales, 2002), causing significant grain yield losses in these regions (Sunder \textit{et al.}, 2014). The fungus can also infect a wide range of grasses including switchgrass (*Panicum virgatum*; Krupinsky \textit{et al.}, 2004) and the American wild rice (*Zizania palustris*; Johnson & Percich, 1992).

The disease cycle and epidemiology of *B. oryzae* have been extensively studied on rice (Ou, 1985; Webster & Gunnell, 1992; Barnwal \textit{et al.}, 2013). Conidia and mycelia on seeds and in crop residues are thought to be the most common survival structures and sources of primary inoculum. The fungus infects leaves, panicles, glumes, stems, sheaths and grain hulls. The major damage in rice is due to fungal infections during the seedling stage that weaken plants and consequently reduce grain yield (Ou, 1985; Webster & Gunnell, 1992). Fungicide applications and proper plant nutrition are the major disease management strategies used by growers due to lack of high levels of genetic resistance in current commercial cultivars (Ou, 1985; Webster & Gunnell, 1992; Barnwal \textit{et al.}, 2013).

Within each agrosystem, knowledge of the population genetic structure of a pathogen, i.e. the magnitude and dispersion of the genetic variation among and within groups of individuals, is important in order to understand the factors that drive that variability, such as...
genetic mutation, mating system, migration, population size and natural selection (McDonald, 1997). Also, this information could have implications for effective disease management strategies and recommendations for breeding programmes. The genetic diversity of *B. oryzae* collections from diverse geographical origins and hosts has been analysed with a variety of molecular markers (Kamal & Mia, 2009; Castell-Miller & Samac, 2012; Burgos et al., 2013). For example, in a *B. oryzae* collection from the Philippines, population subdivision was found using variable number of tandem repeat (VNTR) markers, with the two major sources of variation being collection sites and rice varieties. The *Bipolaris* isolates in each collection site were genetically diverse, although clonality was present in some fields (Burgos et al., 2013). In another study, *B. oryzae* isolates collected from infested seeds from 16 rice-growing areas of India showed high diversity as measured by the polymorphism of inter-simple sequence repeat (ISSR) markers. There was no correlation between isolate groups as determined by a hierarchical clustering method and their collection sites (Archana et al., 2014). Genetic variability inferred by cluster analysis of molecular fingerprinting patterns was reported in additional studies of Asian *B. oryzae* strains from India (Kumar et al., 2011; Kandan et al., 2015), Bangladesh (Kamal & Mia, 2009) and Iran (Motlagh & Anvari, 2010). The latter study used random amplified polymorphic DNA (RAPD) markers with isolates belonging to only one province, Guillian, in the north of Iran. However, more comprehensive studies of Asian *B. oryzae* collections are lacking, although they would be relevant due to the international seed market among countries that could have implications for pathogen movement among those areas. Additionally, even though high genotypic diversity was found within Asian *B. oryzae* populations implying that sexual reproduction could potentially have occurred, the induction of the sexual state and fertility between field isolates from those countries was not demonstrated.

Sexual reproduction of this heterothallic fungus is controlled by the mating type locus *MAT1*, with a single isolate carrying either of the two alternative forms, *MAT1-1* or *MAT1-2*, which are located at the same chromosomal position and are essential for sexual reproduction. The DNA sequences of *MAT1-1* and *MAT1-2* are very dissimilar, and called idiomorphs, while outer DNA flanking areas are nearly identical between homologous chromosomes (Arie et al., 1997; Turgeon, 1998). The idiomorphs have been identified in field isolates pathogenic on switchgrass, wild rice and rice (Krupinsky et al., 2004; Castell-Miller & Samac, 2012; Condon et al., 2013). Frequency distribution of the two idiomorphs deviated from the 1:1 ratio in some field populations (Castell-Miller & Samac, 2012), while they were found equally distributed in other populations (Tsuda & Ueyama, 1976; Castell-Miller & Samac, 2012). The sexual state of *B. oryzae* has been induced under controlled conditions between Japanese strains, and between Japanese and USA isolates (Tsuda & Ueyama, 1976). Random mating between individuals affects structure of the pathogen populations through reshuffling alleles in multilocus individuals, and causes random association between unlinked alleles (Milgroom, 1996). Recombination of novel alleles could increase genotypic diversity and favour plant pathogens through acquisition of adaptive traits.

In this study, the population genetic structure and genetic diversity of *B. oryzae* isolates collected in Iran, the Philippines and Japan were investigated using single-sequence repeat (SSR) markers developed from the recently released genome sequence of a *B. oryzae* isolate originally collected from rice in the northeast region of Taiwan (http://genome.jgi.doe.gov/Cocmi1/Cocmi1.home.html; Condon et al., 2013). Additionally, the mating type genes were amplified, and the sexual state was induced between those Asian field isolates and a few from Bangladesh. The specific objectives of this study were to: (i) analyse the genetic diversity of Asian *B. oryzae* isolates based on geographic areas of collection, (ii) investigate whether isolates show signatures of random mating within their collection sites, (iii) demonstrate formation of the sexual state between field isolates, and (iv) determine the population genetic structure of the Asian *B. oryzae* collection.

**Materials and methods**

**Fungal isolates**

A total of 283 *B. oryzae* isolates was collected during 2011 and 2012 from naturally infected rice fields in the Iranian regions of Rasht (65 isolates), Amol (64 isolates), Bagh-e-Malek (59 isolates), Astara (64 isolates) and Gorgan (31 isolates) (Fig. 1; Table 1). These regions represent approximately 90% of the total rice production acreage of the country (ASID, 2011). Sixteen isolates were collected from other gramineous species: *Zea mays* (three isolates), *Echinocloa colona* (four isolates), *Paspalum scrobiculatum* (five isolates) and unidentified grasses (four isolates). Eight random sites, located 10 m apart, were sampled in each field. At each sampling site, four to eight leaves with RBS symptoms were collected. Thus, from each field, 32–64 leaves were obtained. The leaf samples were kept in paper envelopes, allowed to dry for 2–4 days at room temperature, and stored at 4 °C until use.

Additionally, 20, 8 and 6 isolates, collected from rice in the Philippines, Japan and Bangladesh, respectively, were included (Table 1). Those isolates were kindly provided by Drs C. M. Vera Cruz, T. Tsukiboshi and I. Hossain, respectively. In total, 317 *B. oryzae* isolates (Table 1) were analysed with SSR markers. Isolates from Bangladesh were only used in the estimation of the genetic variability of SSRs, and for crosses with the 16 isolates recovered from grasses other than rice to induce the sexual state.

**Fungal isolation, DNA extraction and ITS sequencing**

For isolation of the Iranian fungal isolates, leaf samples were cut into segments of approximately 1 cm², surface sterilized with 0.5% of sodium hypochlorite for 1 min, rinsed twice in sterile distilled water and briefly blotted on sterile filter paper to
eliminate excess water. The leaf pieces were then incubated at 25°C in Petri dishes lined with moist, sterile filter paper to allow fungal sporulation. Individual canoe-shaped conidia, typical of *B. oryzae*, were transferred onto water agar (2% WA) for germination, and single-spore cultures were maintained on potato dextrose agar (PDA) at 4°C until DNA extraction. All the cultures were stored on small pieces of sterile filter paper at 4°C.

For DNA extractions, each isolate was grown in 50 mL of potato dextrose broth (PDB) for 3–7 days with continuous shaking at 100 rpm. Mycelia were harvested by filtration and freeze-dried. DNA was extracted using a FastDNA kit in a FastPrep instrument (MP Biomedical) according to the manufacturer’s instructions. Quantity and quality of DNA were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies).

To verify species identity of some of the newly collected Iranian isolates, the internal transcribed spacers (ITSs) of the ribosomal DNA were amplified in 24 isolates, representative of collection areas and hosts, using the universal primers ITS1 and ITS4 (White et al., 1990) with PCR conditions as described by Berbee et al. (1999). Fourteen of those isolates were collected from rice in Bagh-e-Malek (five), Astara (three), Amol (four), Rasht (three), and the rest from *Z. mays* in Gorgan (two), *E. colona* from Rasht and Astara (two), *P. scrobiculatum* from Rasht (three), and unidentified grasses (two) located around rice fields in Rasht. The amplified products were cleaned and sequenced by Bioneer Inc. (Daejeon, South Korea).

Development of SSR primers

The genome of the *B. oryzae* strain WK-1C (ATCC 44560) was downloaded from JGI (http://genome.jgi.doe.gov/Cocmi1/Cocmi1.home.html; Condon et al., 2013) and used as a template to
develop SSR markers using the software MSATCOMMANDER v. 1.0.2 (Faircloth, 2008) with default settings. SSR loci containing more than eight dinucleotide repeats and located in the middle of scaffolds were selected for primer design using PRIMER3, which is integrated into MSATCOMMANDER. An M13 tag (5'-CAC-GAGGTTGAAAAAGAC-3') was added to the 5' end of each forward primer (Schuelke, 2000) to incorporate fluorescent-label. M13 primer (IRD700; LI-COR) for detection with a LI-COR 4300 DNA Sequencer. A total of 48 SSR markers with at least nine dinucleotide repeats were initially selected for primer design and tested on four B. oryzae isolates from four regions of Iran. All primers were synthesized by Eurofins Genomics.

For each SSR primer pair, PCR was performed in 10 μl total volume of a mixture containing 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl), 200 μM dNTP, 1.5 mM MgCl2, 1 pmol M13-labelled primer, 0.5 pmol of each 5'-tagged forward and reverse primer, 1 U Taq DNA polymerase (New England Biolabs) and 10–20 ng genomic DNA. PCR conditions were 95 °C for 2 min; 3 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s; 25 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s; and 1 cycle at 72 °C for 5 min. The PCR products were diluted 10-fold before loading onto 6% polyacrylamide gels. The sizes of DNA bands were determined by comparison to a 50–700 bp size standard (LI-COR). Two independent PCRs were amplified and run on polyacrylamide gels for all isolates. For each SSR primer pair, amplicons of the same size across different isolates were considered to be the same allele.

Genetic diversity analysis

Data were analysed using the R v. 1.1.4 package (Kamvar et al., 2014). The percentage of missing data (cut off = 0.05) per locus, and number of informative (polymorphic) loci were assessed with default settings. Genetic diversity analyses included number of alleles per locus (N_a); number of multilocus haplotypes or genotypic richness (MLG); expected genotypic richness with rarefaction correction based on the smallest sample size (eMLG, Hurlbert, 1971); genotypic evenness, which is the distribution of allele frequencies within a sample (E_d) resolved as (G – 1)(N_1 – 1), where G is the Stoddart & Taylor’s index and calculated as \( I_{SP} = \frac{N_1}{n} - \frac{p_1^2}{n} \); and N_a = e^d, with Shannon–Wiener’s H = –Σ(p_i × ln(p_i)) (Grünwald et al., 2003), where p_i is the frequency of the i_th genotype; and finally, unbiased expected heterozygosity (equivalent to unbiased gene diversity in haploids), defined as H_{SP} = [n / (n – 1)] × [1 – Σ(p_i^2)], where p is the allele frequency at a locus and n is the number of observed alleles in each locus (Nei, 1978; Grünwald et al., 2003). Private alleles (P) for each B. oryzae population were estimated with GENALEX v. 6.5.

Population structure analysis

The population genetic structure of the B. oryzae collection was identified using discriminant analysis of principal components (DAPC) (Jombart et al., 2010). First, the number of clusters that best fitted the original data was resolved using a K-means clustering of principal components (PC) by partitioning the total genetic variation between- and within-groups of isolates, maximizing the differences between clusters. Initially, successive increases in the number of clusters from 2 to 30 were used to find the best-supported model based on Bayesian information criteria (BIC). Then, DAPC was conducted, which first uses principal component analysis to transform the data into uncorrelated variables that are lower in number than the individuals analysed, followed by a discriminant analysis (Jombart et al., 2010) that maximizes the difference between clusters while minimizing the variability within them, and allows a probabilistic assignment of individuals to clusters. The number of principal components retained in the transformation step was 50. The analyses were performed with R v. 1.1.4.

Analysis of molecular variance, and pairwise-population differentiation

To further characterize the population genetic structure depicted by the DAPC analysis, a hierarchical analysis of molecular variance (AMOVA) and pairwise-cluster differentiation tests were performed in ARLEQUIN v. 3.5 software (Excoffier & Lischer, 2010). For the AMOVA test, the total genetic variation generated by the microsatellites was partitioned at two levels: among clusters and among haplotypes within clusters, and the significance of the covariance components at each level was tested by 1023 permutations (P = 0.001). Tests of pairwise-cluster differentiation (Skatkin, 1995) were conducted under the null hypothesis of nonpopulation (cluster) differentiation, by transforming RST distance values into an estimated RST, taking into account the microsatellite sizes under the assumption of a stepwise mutation model. The significance of each test was calculated using permutation tests (P = 0.001).

Multilocus gametic disequilibrium, and mating type ratio analyses of the Asian B. oryzae isolates

Linkage disequilibrium was tested for B. oryzae isolates within each collection site under the null hypothesis that alleles observed at different loci are unlinked in randomly mating populations (Agapow & Burt, 2001). The index of multilocus linkage disequilibrium, I_a, and the unbiased r^2 statistic that removes dependency on the number of loci, were calculated using R v. 1.1.4 permuting (default settings) alleles within each locus (P = 0.001).

The mating-type idiomorphs (MAT1-1 and MAT1-2) of B. oryzae were amplified in multiplex PCRs following the protocol of Arie et al. (1997). MAT1-1 was amplified with the primers CmMAT1F1 (5’-GACGCGTTCCTTCGGCATTA-3’) and CmMAT1R1 (5’-GGTCCGAAACCGAGGTAGT-3’), and MAT1-2 with CmMAT2F1 (5’-GGAGGTTGCTTCCTGTTGGCA-3’) and CmMAT2R1 (5’-GATGACTGGAATAACCGGCA-3’), which were kindly provided by Dr B. G. Turgeon, Cornell University, USA. PCR amplifications were performed in a 20 μl volume containing 10 ng genomic DNA, 1 x PCR buffer, 2 mM MgCl2, 200 μM dNTPs, 1 μM of each primer and 1 U Taq DNA polymerase in a PTC-100 thermal cycler (MJ Research, Inc.) programmed for 94 °C for 2 min; followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min; plus a final extension at 72 °C for 7 min. PCR-amplified products were run on a 2% agarose gel and amplicon sizes were determined based on a 100 bp DNA ladder.

Frequencies of MAT1-1 and MAT1-2 were tested for the 1:1 ratio with a chi-square for goodness-of-fit (χ^2) within collection sites.

Biological demonstration of the sexual state in Asian B. oryzae isolates

To test the fertility of B. oryzae isolates, the sexual state (C. miyabeana) between different mating types was induced with the methodology of Tsuda & Ueyama (1976). Briefly, each...
strain with the alternative mating type was grown on Sachs agar medium in a Petri dish separated by a piece of sterile rice straw. The dishes were placed in an incubator at 24 °C in the dark for 30 days. Initially, pairings in all pairwise combinations were done within each collection from Amol and Rasht to identify isolates with the best ability to produce fertile crosses. Two isolates, one from Amol (AA74, MAT1-1) and the other from Rasht (R44B, MAT1-2), collected in 2012 and 2011, respectively, yielded the highest number of fertile crosses (data not shown) and thus were used to pair in all combinations with isolates of alternative mating type from Iran (Astara, Bagh-e-Malek, Gorgan), Japan, the Philippines and Bangladesh. The occurrence of sexual reproduction was considered successful when ascospores from each cross were produced. Viability of ascospores was checked by germinating them on 1% WA. For visualization, ascosporas (pseudothecia) and ascospores were stained with lactophenol blue solution (Sigma-Aldrich) as directed by the manufacturer and observed in a BH2 microscope (Olympus) with ×40 and ×100 magnification.

Results

Fungal culture collections and analysis of ITS sequences

A total of 283 B. oryzae isolates were obtained from RBS affected leaves in rice fields from five Iranian regions (Fig. 1; Table 1). All B. oryzae isolates, derived from single spores, produced the typical canoe-shaped conidia characteristic of this species. Additionally, the ITS1 and ITS2 sequences of 24 B. oryzae isolates were amplified by PCR, sequenced and deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accessions KC315915–29, KC315935–39, and KC315941–44. They were 99–100% identical to each other and to those of reference B. oryzae isolates from rice of the Philippines (DQ300199), Thailand (JX256414), switchgrass (GU222690) and wild rice (HM147248) from USA.

SSR marker development and characterization

Of the 48 SSR primer pairs tested on four Iranian B. oryzae isolates, five failed to amplify DNA and six generated monomorphic bands, while the remaining 37 primer pairs amplified polymorphic amplicons. Of them, 12 primer pairs (Table 2) were chosen for genotyping the B. oryzae isolates used in this study due to their repeatability in PCR amplification and high polymorphism among the isolates. A total of 24 isolates with over 5% missing data (1 missing datum per genotype) were removed from further population genetic analyses. The average size of SSR loci used was 277 bp, varying from 201 bp (BOSSR_115) to 336 bp (BOSSR_102) (Table 2). All loci were informative. The number of alleles per locus ranged from five (BOSSR_355 locus) to 14 (BOSSR_430 and BOSSR_527 loci) with an average of nine alleles per locus. Unbiased expected heterozygosity (Nei, 1978) across loci varied from 0.59 (BOSSR_355) to 0.98 (BOSSR_115 and BOSSR_527), with an average of 0.90.

Genetic diversity, haplotypes and allele contents of B. oryzae isolates from different geographic origins

The genetic diversity of B. oryzae isolates was assessed within each collection site (Table 3). The estimated genotypic diversity measured through richness (eMLG) showed high genotype numbers (Table 3). The highest eMLG was found in the Philippine isolates and the lowest in the Iranian isolates from Gorgan (eMLG), even though the lowest numbers were close to the maximum expected value. Although Japanese isolates were not included and could not be directly compared to the rest of the populations, each individual was unique. Evenness (E3) indicated balanced distribution of MLG in the populations, approaching or reaching the maximum value of 1. Hexp was similar and high for all groups of isolates, with the lowest value for the Astara isolates and the highest for the Amol isolates. The highest number of private alleles was found in the isolates at Bagh-e-Malek (three), followed by isolates of Japan (two) and Amol (one).

Tests of linkage disequilibrium and analyses of mating type gene frequency

The multilocus index of association I A and the statistic r4d tests applied to clone-censored B. oryzae isolates within collection sites rejected the null hypothesis of gametic equilibrium, thus indicating that isolates in each collection site were not under random mating (Table 4). The amplification of mating type idiomorphs, MAT1-1 and MAT1-2, produced amplicon sizes of 556 and 523 bp, respectively. The idiomorph frequencies of Bagh-e-Malek and Astara isolates from Iran, as well as the Japanese and Philippine isolates, deviated from the ratio of 1:1, while the null hypothesis of equal ratios could not be rejected for the MAT1-1 and MAT1-2 of Amol, Rasht and Gorgan isolates (Table 4).

Induction of the sexual state of B. oryzae

The induction of the sexual state between Asian isolates was successful under controlled laboratory conditions. Spherical ascocarps with the typical elongated beak were observed over the rice straw (Fig. 2a,b) after 30 days' incubation. Crushed, opened ascocarps showed abundant ascii produced among paraphyses (Fig. 2c). Bitunicate ascii contained fully developed, typical helicoid-shaped ascospores (Fig. 2d,e,f). All ascospores tested were viable (data not shown).

The initial pairings in all possible combinations within isolates from Rasht or Amol produced the sexual state in less than 10% of the total number of crosses. The numbers of B. oryzae isolates from Iran, the Philippines, Japan and Bangladesh that yielded ascospores when paired with the alternative mating type strain, either AA74 (MAT1-1) from Amol or R44B (MAT1-2) from Rasht, are displayed in Table 5. Overall, the numbers of fertile crosses with the parent AA74 were higher than
with the parent R44B, except for the pairings involving the Japanese isolates. The highest number of fertile crosses was found between isolates of the testers and those from Rasht fields (Table 5).

Crosses between the tester isolates from rice fields of Rasht and Amol, and those collected from weed grasses were also successful. Five out of seven MAT1-2 isolates and six out of nine MAT1-1 isolates from weed grasses produced the sexual state when crossed to AA74 or R44B, respectively.

**Population structure analysis**

The population structure of the *B. oryzae* isolates was investigated using, first, a K-method to resolve the number of clusters, and, later, a DAPC analysis to maximize the difference between clusters while minimizing variability within them, and to assign individuals probabilistically into clusters.

As resolved by the K-means method, and based on the lowest associated likelihood (BIC), the number of groups that best represented the data was nine (Text S1; Fig. S1). However, the DAPC analysis of *B. oryzae* isolates with \( K = 9 \) indicated that these nine groups were gathered into three clusters (Fig. S2). Thus, \( K = 3 \) was selected to represent the data structure. Although substructuring was present within those three clusters, additional support for this subdivision came from preliminary analyses of pairwise population differentiation, and geographic barriers between collection areas, indicating that \( K = 3 \) was a meaningful number of clusters describing the data.

The DAPC scatter plot (Fig. 3a) showed that axis 1 (vertical) best summarized the differences of cluster 1 and cluster 3 from cluster 2, while axis 2 (horizontal) segregated cluster 2 and cluster 3 from cluster 1. The first two principal components contained enough information to resolve the genetic structure of the Asian *B. oryzae* isolates as indicated by the eigenvalues of the analysis (Fig. 3a inset), that is, those two vectors contained 88.1% of the cumulative variation. When plotted against the isolate densities, the first discriminant function differentiated the set of isolates in cluster 1 and cluster 3 from those in cluster 2 (Fig. 3b) where the frequencies of the two most contributing differential alleles corresponded to the SSR markers BOSSR_368 and BOSSR_519 (Fig. 3c), while the second discriminant function best segregated cluster 2 and cluster 3 from cluster 1 (Fig. 3d) and was due to the differential allele frequencies of markers BOSSR_102, BOSSR_150 and BOSSR_519 (Fig. 3e). Cluster 1 (\( n = 39 \)) contained many isolates from Bagh-e-Malek in Iran (36/51), one (out of six) isolate from Japan and a few (2/18) from the Philippines; cluster 2 (\( n = 104 \)) had more than two-thirds of isolates from Rasht (40/59) and three-quarters from Gorgan (21/28), over half of isolates from Amol (31/56), a

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**Table 2 Genetic characteristics of 12 simple-sequence repeat (SSR) loci of 293 Asian isolates of *Bipolaris oryzae***

<table>
<thead>
<tr>
<th>Locus name*</th>
<th>Primer sequence (5'-3')</th>
<th>SSR motif</th>
<th>SSR length (bp)</th>
<th>( N_a )</th>
<th>( H_{exp} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOSSR_102</td>
<td>F: TGTACCTATCCGCTTTCCCAG (AG) 9 336 9 0.93 R: CTCTTCAATCCGCACCACTCCACCC</td>
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<tr>
<td>BOSSR_115</td>
<td>F: TCTGACGCCCTATCAGCAAG (AT) 13 201 9 0.98 R: AGAAAGGGGAATGATCAGAGCACCC</td>
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<td></td>
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</tr>
<tr>
<td>BOSSR_150</td>
<td>F: CATGAGGGGCGTTGCAAAACTCT (AG) 15 324 10 0.96 R: AGCCAAAGGAGGGGCAAAGGACC</td>
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</tr>
<tr>
<td>BOSSR_223</td>
<td>F: AGTCACTGGTCTTTGAAAGGC (AC) 9 264 11 0.94 R: GATGAGGGCGATGGGATTTGAG</td>
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<td></td>
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</tr>
<tr>
<td>BOSSR_335</td>
<td>F: AGTGGGATCCAGAAATCCGACATG (AC) 8 323 11 0.94 R: CGAACTTGCTGTTGAGGATG</td>
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<td>BOSSR_355</td>
<td>F: CTTGGAATCTCCGCGACAATGAC (AC) 11 303 5 0.59 R: CATCTTGAACGCTTGTGAC</td>
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</tr>
<tr>
<td>BOSSR_368</td>
<td>F: GACCAGATACATCAGGACACCC (AC) 13 285 8 0.81 R: AGAAGAGGGTGGTGTGAACGGG</td>
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<tr>
<td>BOSSR_383</td>
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<td>F: GACGCCGCAAGGTAAGAAAG (AC) 14 291 14 0.96 R: GCAGAGGCTCCAGAAAGGCAATG</td>
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<tr>
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<td>F: CTCTTAAATGTCACCGGAGCTTC (AC) 9 298 9 0.93 R: GTTCTGAGCGGGAGGTCTTAC</td>
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<tr>
<td>BOSSR_519</td>
<td>F: TTTCTGACTGACAGATGACGC (AC) 15 203 6 0.77 R: GACAAAGCGATGCGTCTGAGTGCC</td>
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<tr>
<td>BOSSR_527</td>
<td>F: TGAGGGGATGAGGATAAGG (AC) 12 223 14 0.98 R: CGCCCCACATTCTCCCTCATTCC</td>
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</table>

*BOSSR: *B. oryzae* single sequence repeat (SSR).

*Number of alleles per locus.

few from Astara (4/60), one from Bagh-e-Malek, one from Japan and six from the Philippines, while cluster 3 (n = 135) was formed by most of the Astara isolates (56/60), less than half of Amol isolates (25/56), about one-third of Rasht (19/59) and Bagh-e-Malek (14/51) isolates, one quarter of Gorgan isolates (7/28), four from Japan and 10 from the Philippines (Fig. 3a; Table S1).

Analysis of molecular variance (AMOVA) and pairwise differentiation of Asian Bipolaris oryzae isolates

The three Asian Bipolaris oryzae clusters were further analysed by a hierarchical AMOVA and pairwise genetic differentiation. In the AMOVA test, total genetic variation was partitioned at two levels: among clusters and among Bipolaris oryzae haplotypes within clusters. This partition revealed that 18.7% of the total genetic variation was due to genetic differences among clusters, while the remainder and larger source of diversity (81.3%) was located among isolates within clusters (P < 0.001). All pairwise genetic comparisons between clusters showed that their corresponding RST values (Slatkin, 1995) were statistically significantly different (Table 6). The largest differences were found when comparing cluster 1, containing the majority of B. oryzae isolates from Bagh-e-Malek, a few isolates from the Philippines and one from Japan, with each of the other two clusters of B. oryzae isolates.

Discussion

In this study, the genetic structure of B. oryzae populations mainly collected from Iran was analysed using newly developed SSR markers. The 12 SSR markers used were polymorphic on the majority of the B. oryzae isolates. The number of alleles and polymorphisms detected were comparable to those reported on SSR markers developed for other plant pathogen ascomycetes (Yang & Zhong, 2008). These SSR markers should be useful in future studies on genetics and population biology of the fungus.

The main conclusions of this study are that: (i) B. oryzae isolates are genetically diverse within the geographic areas of collection, (ii) there was no evidence for random mating within collection sites, even though both mating types were present in all the areas examined and the formation of ascocarps with asci bearing viable ascospores was demonstrated between field isolates, and (iii) the Asian B. oryzae populations are structured into three clusters, each containing isolates from different geographic areas.

Within each geographic area of collection, the B. oryzae isolates were genetically diverse, with gene and genotypic diversity indices close to the maximum expected numbers. The high values of unbiased gene diversity could suggest that mutations have accumulated in the populations over a period of time, and/or that populations are of large size (McDonald, 1997). The genotypic diversity was due to both richness and
evenness. That is, most genotypes were unique and without predominance of any MLG within those populations. This high genetic diversity is in agreement with previous reports for other *B. oryzae* populations collected in Brazil, Bangladesh, India, the United States and the Philippines characterized by different molecular markers (Weikert-Oliveira *et al.*, 2002; Kamal & Mia, 2009; Kumar *et al.*, 2011; Castell-Miller & Samac, 2012; Burgos *et al.*, 2013).

Sexually reproducing heterothallic fungi are expected to have high genotypic diversity, low clonal fractions and alleles in linkage equilibrium (Milgroom, 1996). However, there was no evidence for random mating within the clone-censored *B. oryzae* populations, as measured by the multilocus linkage disequilibrium estimators $I_A$ and $r_d$, even though the $r_d$ estimator values were close to zero. Lack of support for linkage equilibrium was reported for *B. oryzae* populations collected from wild rice in the US (Castell-Miller & Samac, 2012). Linkage disequilibrium can be caused by linkage, migration, selection and genetic drift (Milgroom, 2015). Migration events can lead to admixture due to isolates carrying different alleles from those in the recipient population and it can take place during commercialization of hay and rice seeds among Asian farmers within neighbouring regions. Stochastic changes of allele frequencies (random genetic drift) can lead to a reduction of genetic diversity through generations in small populations.

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**Figure 2** Induction of the sexual state between Asian isolates of *Bipolaris oryzae* (*Cochliobolus miyabeanus*). (a) Spherical ascocarps emerging from a piece of sterile rice stem. (b) Mature and immature ascocarps with and without elongated beak, respectively, observed under dark conditions after 30 days of incubation. (c) Ascocarps with abundant asci produced among paraphyses. (d) Bitunicate ascus containing fully developed, typical helicoid-shaped ascospores. (e) Close-up of a bitunicate mature ascus containing ascospores. (f) Close-up of multicellular ascospores released from ascus. Fungal structures were stained with lactophenol blue solution. Bar in (b–d) = 100 μm, (e,f) = 10 μm.
(Milgroom, 1996, 2015). However, B. oryzae populations are large during the cropping season and thought not to have significant size reductions off-season because the fungus is believed to overwinter in grass residues. There is no directional selection on fungal populations from rice cultivars due to the lack of genetic resistance, although the effect of selection on fungal fitness due to other cultivar traits cannot be ruled out.

Both mating type idiomorphs, MAT1-1 and MAT1-2, were found in isolates at each location, with ratios differing from equality within each population of Bagh-e-Malek, Astara, the Philippines and Japan, and fitting a 1:1 segregation ratio in those of Rasht, Amol and Gorgan. Further, the sexual state producing viable ascospores was induced between some isolates of the alternative mating type within Rasht and Amol regions, and with isolates from other sites and hosts (i.e. weed grasses), indicating that under proper conditions random mating can be feasible. However, in field conditions, genotype adaptation to the environment (Schoustra et al., 2010), and other inherent biological factors of each genotype can influence the outcome of mating. For example, in Cochliobolus heterostrophus, mutations in genes involved in sexual development, such as the methyltransferase LAE1 and those of the velvet-complex Vel2 and Vos1, can negatively affect female fertility and significantly reduce the production of pseudothecia, asci and ascospores, as well as increase sensitivity to reactive oxygen species (Wang et al., 2014).

The sexual state has been previously demonstrated in isolates from Iran (Shamsi et al. (2010) and Japan (Tsuda & Ueyama, 1976). Bipolaris oryzae can survive on rice and wild grasses (Ou, 1985; Webster & Gunnell, 1992; Barnwal et al., 2013), where sexual recombination could take place, and/or in residues during the rice off-season period. If sexual recombination occurs sporadically, and even on a small scale, it still could have an important impact on the fungus population genetic structure by augmenting the genotypic diversity (Milgroom, 2015). However, more detailed analyses must be completed to confirm that random mating occurs in nature and contributes to genetic variability of the fungus. For example, genetically known (marked) isolates could be released at the beginning, and recaptured at the end, of the season to detect sexually recombined individuals and/or the reduction on linkage disequilibrium if sexual recombination occurs in the season (Milgroom, 2015). Indeed, other processes can account for high genetic variation, including DNA mutations and parasexual recombination. Hyphal anastomosis and heterokaryosis have been demonstrated for Bipolaris species including B. oryzae isolates collected from common rice (Lee et al., 1984). Further studies are required to understand better how diversity of B. oryzae genotypes is generated under field conditions.

The population genetic structure of the Asian B. oryzae collection was resolved overall within three clusters, based mostly on differential allelic information from four of the 12 SSR markers used. The isolate groups were not in complete agreement with the geographic collection sites. However, cluster 1 contained 70.6% of B. oryzae isolates collected at Bagh-e-Malek, which is located in the south of Iran and separated by the Zagros and Elburz Mountains from the northern areas. Private alleles were found in the Bagh-e-Malek population and could indicate some degree of isolation of B. oryzae isolates located in that area. Clusters 2 and 3 were genetically rich with B. oryzae isolates coming from all collection sites. However, cluster 2 has the majority of isolates from Gorgan and more than 50% of the isolates from Rasht and Amol, while cluster 3 harbours most of the isolates from Astara (93%) and more than 50% of the isolates from the Philippines and Japan. The Caspian Sea could limit isolate dispersal mainly between Astara and Gorgan areas. Isolates from Japan and the Philippines did not form a separate group from those from Iran, and, instead, the majority of them grouped with those of Astara located at the west of Gorgan. Movement of pathogen genotypes could explain the occurrence of B. oryzae isolates associated with different clusters within the same region. Bipolaris oryzae is a seedborne pathogen (Ou, 1985; Webster & Gunnell, 1992) and its movement between cultivation areas could be caused by the exchange of rice seeds, a practice commonly used among farmers in northern provinces of Iran. Additionally, pathogen dispersal could occur during the trade of rice straw for animal feed, and the sharing of

<table>
<thead>
<tr>
<th>Country</th>
<th>Location</th>
<th>No. of isolates paired</th>
<th>No. of fertile crosses with AA74 MAT1-1 (no. isolates with MAT1-2)</th>
<th>No. of fertile crosses with R44B MAT1-2 (no. isolates with MAT1-1)</th>
<th>No. of fertile crosses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iran</td>
<td>Rasht</td>
<td>65</td>
<td>21 (37)</td>
<td>9 (28)</td>
<td>30 (46)</td>
</tr>
<tr>
<td></td>
<td>Amol</td>
<td>64</td>
<td>16 (39)</td>
<td>5 (25)</td>
<td>21 (33)</td>
</tr>
<tr>
<td></td>
<td>Bagh-e-Malek</td>
<td>59</td>
<td>19 (49)</td>
<td>0 (10)</td>
<td>19 (32)</td>
</tr>
<tr>
<td></td>
<td>Astara</td>
<td>64</td>
<td>5 (17)</td>
<td>4 (47)</td>
<td>9 (14)</td>
</tr>
<tr>
<td></td>
<td>Gorgan</td>
<td>31</td>
<td>11 (17)</td>
<td>5 (14)</td>
<td>16 (52)</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>–</td>
<td>6</td>
<td>2 (5)</td>
<td>0 (1)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Japan</td>
<td>–</td>
<td>8</td>
<td>0 (1)</td>
<td>4 (7)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Philippines</td>
<td>–</td>
<td>20</td>
<td>10 (14)</td>
<td>2 (6)</td>
<td>12 (60)</td>
</tr>
<tr>
<td>Total</td>
<td>–</td>
<td>317</td>
<td>84 (179)</td>
<td>29 (138)</td>
<td>113 (36)</td>
</tr>
</tbody>
</table>
harvesting equipment on the coastal sites of rice agro-ecosystems on the Caspian Sea (ASID, 2011). Exchange of rice seeds among Asian countries is not uncommon (Mew & Gonzales, 2002). Incorporation of isolates from other south-central Asian countries could help to unravel the population structure of the \( \text{B. oryzae} \)–rice pathosystem in that region, and lead to a more efficient management system for RBS.

Preventive strategies, such as removal of infected residues from rice fields, proper fertilization, weed control and fungicide-treated seeds, could help reduce RBS severity and limit gene flow. Although host plant resistance is an effective and economical way to manage RBS (Barnwal et al., 2013), breeding efforts in Asian countries have put emphasis on more damaging diseases for rice, such as leaf blast and bacterial blight (Savary et al., 2011). The sources of resistance to RBS amongst \( O. \text{sativa} \) entries are scarce (Sato et al., 2008), thus other gene pools (e.g. \( Oryza nivara \)) have been explored to fill that gap (Barnwal et al., 2013). Considering the high genetic diversity in Asian \( \text{B. oryzae} \) populations, quantitative genetic resistance may offer an effective and long-term protection to rice when accompanied by proper crop management strategies. In addition, the use of genetically diverse isolates in breeding programmes for plant resistance screening needs to be emphasized, as

Table 6  Pairwise genetic differentiation (\( R_{ST} \)) between clusters of \( \text{Bipolaris oryzae} \) isolates resolved by discriminant analysis of principal components

<table>
<thead>
<tr>
<th>Cluster 2</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
<th>Cluster 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>–</td>
<td>0.315*</td>
<td>–</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>0.267*</td>
<td>0.190*</td>
<td>–</td>
</tr>
</tbody>
</table>

\( R_{ST} \) = fixation index (Slatkin, 1995); significance was determined by 1023 random permutations using ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010).

*Significant at \( \alpha = 0.01 \).

Figure 3  Discriminant analysis of principal components (DAPC) of the Asian \( \text{Bipolaris oryzae} \) isolates. (a) Scatterplot of three \( \text{B. oryzae} \) clusters resolved by DAPC. Pie charts show the \( \text{B. oryzae} \) isolate composition of each cluster: blue, Bagh-e-Malek, Iran; red, Japan; green, Philippines; violet, Rasht, Iran; grey, Amol, Iran; orange, Astara, Iran; yellow, Gorgan, Iran. Inset: The two DA eigenvalues (lower right) retained for cluster discrimination explained over 88% of the total variability of the data. Vertical line: axis1 and horizontal line: axis 2. (b) Density of \( \text{B. oryzae} \) isolates plotted against discriminant function 1 (DF1). (c) Loading plot of allele frequencies of markers BOSSR_368 and BOSSR_519 that most contributed to DF1. (d) Density of \( \text{B. oryzae} \) isolates plotted against discriminant function 2 (DF2). (e) Loading plot of allele frequencies of markers BOSSR_102, BOSSR_150 and BOSSR_519 that most contributed to DF2.
well as identification of the mechanistic pathway of \textit{B. oryzae} in causing disease on its hosts.

**Acknowledgments**

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**References**


Tsuda M, Ueyama A, 1976. Distribution of two mating types of \textit{Cochliobolus miyabeaenus} in field and a laboratory attempt to produce hybrids between isolates of \textit{Helminthosporium oryzae} from the USA and Japan. \textit{Annals of the Phytopathological Society of Japan} 42, 7–11.


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Supporting Information

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Figure S1 (a) Bayesian information criterion (BIC) values compared to the number of proposed clusters in the population genetic structure of the Bipolaris oryzae collection. (b) Cumulative total variance of the Asian B. oryzae isolates explained by the eigenvalues of the principal component (PC) analysis. Red line indicates the PC retained for implemented discriminant analysis of principal components (DAPC) analysis. (c) List of associated BIC values to the number of selected clusters (30) based on K-means clustering.

Figure S2 Discriminant analysis of principal components (DAPC) analysis of nine Asian Bipolaris oryzae clusters, based on the lowest associated likelihood (BIC) of K-means method.

Table S1 Asian Bipolaris oryzae isolates assigned to the three clusters based on discriminant analysis of principal components (DAPC).

Text S1. Selection of the number of clusters in the Bipolaris oryzae isolate collection by a K-means method.