Novel haplotypes and networks of AVR-Pik alleles in Magnaporthe oryzae

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Rice blast disease is one of the most destructive fungal diseases of rice world-wide. The avirulence (AVR) genes of Magnaporthe oryzae are recognized by the cognate resistance (R) genes of rice, and trigger race specific resistance. Here, we studied the possible evolutionary pathways in the evolution of AVR-Pik alleles by analyzing the DNA sequence variation and assayed for their avirulence function to the cognate Pik alleles resistance genes under field conditions in China. Results of PCR products showed that 278 isolates of M. oryzae carry AVR-Pik alleles among genomic DNA of 366 isolates of M. oryzae collected from Yunnan Province, China. Among of them, 66.7-90.3% of M. oryzae carry AVR-Pik alleles from six regions of Yunnan. Moreover, 10 AVR-Pik haplotypes encoding five novel AVR-Pik variants were identified among
201 isolates. The AVR-Pik alleles stepwise evolved to virulence from avirulent forms via base substitution. These findings demonstrate that AVR-Pik alleles are under positive selection and mutations are responsible for defeating race-specific resistance Pik alleles in nature.

Author summary
The interaction of resistance gene (R) of rice and avirulence (AVR) gene of rice blast fungi are belong to the gene-for-gene theory, the variation of AVR is one of the major reasons for generation new race. To detect the variation of AVR gene in isolates population of Magnaporthe oryzae collected from rice production fields, will helpful for evaluated the effectiveness of R gens in rice production areas. The Pik allele contained five R genes of Pik, Pikh, Pikp, Pikm and Piks, and the corresponding to the AVR genes of AVR-Pik/kh/kp/km/ks of M. oryzae. The Pik gene specifically recognizes and prevents infections by isolates of M. oryzae that contain AVR-Pik. The molecular variation of AVR-Pik alleles of M. oryzae and Pik alleles of rice remains unclear. Here we demonstrated that polymorphism and distribution of AVR-Pik alleles in Yunnan Province, China. By pathogenicity assays to detect function of the different haplotypes of AVR-Pik, for the first time we show detour and stepwise evolution of AVR-Pik alleles in rice production areas of Yunnan. The functional AVR-Pik possesses diversified sequence structures, and is under positive selection pressure in nature.

Introduction
In the long history of coexistence of parasitism and predation, the adaptive genetic mutation are including between hosts and pathogen. While the selective pressure was considered as the main force. So far, two hypotheses of arms race and trench warfare
evolution have been proposed between hosts resistance genes (R) and pathogens avirulence genes (AVR).[1] The arms race was received as principal hypothesis, in which both hosts R and pathogens AVR were under directional selection, and the alleles derived by mutation, in brief, pathogen evolved a virulence gene in order to overcome the host defense, on the other hand, the hosts evolved a new resistance allele to defeat the virulence genes of the pathogen. Where as, in trench warfare hypothesis, the evolution of both hosts R and pathogens AVR is non-directional.

Rice blast is one of the most destructive diseases of rice growing regions, caused by the filamentous ascomycetous fungi *Magnaporthe oryzae* (synonym of *Pyricularia oryzae*). Employing resistant rice varieties with major resistance (R) gene was considered of the most important strategy to control this disease, which is environmentally friendly and economical for decreasing crop loss by rice blast. So far, ≤26 R genes in rice have been cloned: *Pb1, Pia, Pib, Pid2, Pid3, Pik, Pikh/Pi54, Pikm, Pikp, Pish, Pit, Pita, Pizt, Pi1, Pi2, Pi5, Pi9, pi21, Pi25, Pi36, Pi37, Pi56, Pi63, PiCO39* (http://www.ricedata.cn/gene/gene_pi.htm), *Pi64*[2] and *Pigm*[3].

Rice resistance gene can recognize the corresponding AVR of *M. oryzae* and triggers the immunity reaction. So far, 12 AVR genes in *M. oryzae* have been cloned: *AVR-Pi54*[4], *AVR-Pi9*[5], *AVR-Pib*[6], *AVR-Pia*[7], *AVR-Pii*[7], *AVR-Pik/km/kp*[7], *AVR-Pizt*[8], *ACE1*[9], *AVR-Pita*[10], *AVR1-CO39*[11], *PWL1*[12], and *PWL2*[13].

The *AVR-Pik/km/kp* gene of *M. oryzae* inspects the effectiveness of *R* gene *Pik/km/kp*. *AVR-Pik/km/kp* encodes a putative secreted protein with 113 amino acids containing two conserved motifs: motif-1, [LI]xAR[SE][DSE]; and motif-2, [RK]CxxCxxxxxxxxxxxxxxH (similarity to the C2H2 zinc finger motif).[7] The *AVR-Pik/km/kp* gene was cloned from the isolate of Ina168 but absent in the assembled sequence of isolate 70-15, which is recognized by host resistance of Pik
protein and triggers the defense response.[7] Five AVR-Pik alleles (AVR-Pik-A, AVR-Pik-B, AVR-Pik-C, AVR-Pik-D, AVR-Pik-E) were found[7], and the AVR-Pik-D (20.5%) and AVR-Pik-E (1.4%) was detected among 77 isolates.[14] Four AVR-Pik alleles (AVR-Pik-A, AVR-Pik-C, AVR-Pik-D, AVR-Pik-E) were found among 39 isolates from world-wide (three isolates from Europe, six isolates from America, seven isolates from Africa and 23 isolates from Asia), and the AVR-Pik-D was the highest frequent allele (15 out 39), while the AVR-Pik-A, AVR-Pik-C, AVR-Pik-E alleles had similar frequencies (7-9 out of 39).[15] AVR-Pik/km/kp has evolved via gene gain/loss manners[7], while substitution mutations were observed in coding regions of the AVR-Pik/km/kp in M. oryzae populations and 16 SNPs were found in non-signal domain harbored regions in Chinese rice blast isolates.[16]

The Pik locus located on the long arm of chromosome 11, and the resistance function has been reported.[17-20] In the Pik locus, five rice blast R genes (Pik, Pik-m, Pik-p, Pik-h and Pik-s) involved, among of which four R genes (Pik, Pik-m, Pik-p and Pik-h) have been isolated,[18,21-24] and Pik was regard as a younger allele at the locus[22]. Pik, Pik-m, Pik-p and Pik-h were cloned and those were encode a putative CC-NBS-LRR protein.[18,23,25-26] The CC domain of Pik-1 physically binds the AVR-Pik effector of M. oryzae to trigger Pik-specific resistance.[15,23] The rice resistance gene Pik-s is still not cloned. The monogenic lines containing 24 rice blast resistance genes including Pik, Pik-m, Pik-p, Pik-h and Pik-s were developed, which will used to characterize the pathogenicity of rice blast fungus.[27]

Pikm and Pikp exhibited a high level of resistance to blast fungus from Fujian Province, and can used as resistance breeding parents in Fujian Province[28]. Pikm, Piks, and Pikp were moderate resistant in Sichuan and Guizhou Provinces, China.[29] Pikm, Piks, and Pik were moderate resistant, while Pikh exhibited high resistance in
Guangdong Province, China[30], and 35.4% of 82 rice germplasm resources carrying
Pikh detected by molecular.[31] While there were 80 carrying Pik locus in 229 rice
cultivars and breeding material from Fujian Province based on PCR detection.[32]
The different resistance spectrum of Pik, Pikm, Pikp, Pikh and Piks involved in the
Pik locus were detected by 282 blast isolates collected from Yunnan Province,
China.[33] The R genes of Pik locus exhibit high resistance to Chinese rice blast
fungus.

Further understanding the molecular evolution of AVR gene has potential
implications for the development of resistance breeding and the rational use of
resistance genes in production, and the deployment of more effective strategies to
control disease. Among long period interaction between the pathogen and its host, the
hosts apply the resistance genes to prevent infection by the pathogen, on the other
hand, the pathogen attempts to overcome them, and the co-evolution of pathogen and
its host was discernible at the genome level.[34-35] The pathogen via mutation to
adapt the host novel alleles and environment, while the pathogen genome structure is
strongly varied and impacted under host selection.[15,36-37]

The goal of the present study was to analyze DNA sequence variation of
AVR-Pik/km/kp alleles in field isolates of M. oryzae in order to understand the
variation and co-evolution mechanism of M. oryzae AVR-Pik/km/kp alleles and rice
Pik alleles in Yunnan Province.

Results
The Efficacy of Pik loci genes and detection frequency of AVR-Pik allele
Based on the disease reactions, the efficacy of Pik loci genes of Pik, Pikm, Pikp, Pikh
and Piks were examined. Some 223, 256, 154, 276 and 83 out of the 366 isolates
tested were avirulent to the Pik, Pikm, Pikp, Pikh and Piks gene containing rice monogenic line IRBLk-K, IRBLkm-Ts, IRBLkp-K60, IRBLkh-K3 and IRBLks-F5, respectively (Table 1), the avirulence frequency to Pik, Pikm, Pikp, Pikh and Piks were 60.9, 69.9, 42.1, 75.4 and 22.7%, respectively; while the remaining 143, 110, 212, 90 and 283 isolates were virulent to the corresponding R gene (Table 1). Out of 366 isolates, 278 AVR-Pik/km/kp alleles were amplified by AVR-Pik/km/kp (AVR-Pik allele) specific primers (pex31F/pex31R) (Table 1), the mean percentage of AVR-Pik/km/kp allele was 76.0%. The highest percentage of AVR-Pik/km/kp was 90.3% in M. oryzae population collected from north-eastern Yunnan, whereas the lowest was 66.7% from north-western Yunnan (Table 1). The percentages of AVR-Pik/km/kp were 77.8, 90.3, 66.7, 72.7, 89.3 and 68.3% in central, north-eastern, north-western, south-eastern, south-western and western Yunnan, respectively. Similarly, the percentages of AVR-Pik/km/kp were 74.5 and 77.0% in Xian/Indica (XI) and Geng/Japonica (GJ) rice growing regions in Yunnan. These findings suggest that Pik loci have different effective use in preventing infections by blast in most rice production areas in Yunnan.
Table 1. Distribution of AVR-Pik genes and avirulence isolates of M. oryzae collected from Yunnan, China to IRBLk-K, IRBLkm-Ts, IRBLkp-K60, IRBLkh-K3

| Locations   | No. of isolates | No. of isolates with AVR-Pik | PCR detection (%) | Pathogenicity assay* |
|-------------|-----------------|------------------------------|-------------------|----------------------|
|             |                 |                              |                   | No. of avirulence isolates and Frequency (%) |
|             |                 |                              |                   | IRBLk-K | IRBLkm-Ts | IRBLkp-K60 | IRBLkh-K3 | IRBLks-F5 |
| Central     | 54              | 42                            | 77.8              | 40(74.1) | 39(72.2) | 36(66.7) | 43(79.6) | 15(27.8) |
| North-eastern | 72          | 65                            | 90.3              | 62(86.1) | 64(88.9) | 52(72.2) | 68(94.4) | 15(20.8) |
| North-western | 15           | 10                            | 66.7              | 2(13.3)  | 4(26.7)  | 2(13.3)  | 5(33.3)  | 1(6.7)   |
| South-eastern | 33           | 24                            | 72.7              | 24(72.7) | 26(78.8) | 19(57.6) | 27(81.8) | 2(6.1)   |
| South-western | 28           | 25                            | 89.3              | 16(57.1) | 20(71.4) | 15(53.6) | 22(78.6) | 6(21.4)  |
| Western     | 164             | 112                           | 68.3              | 79(48.2) | 103(62.8) | 30(18.3) | 111(67.7) | 44(26.8) |
| Total       | 366             | 278                           | 76.0              | 223(60.9) | 256(69.9) | 154(42.1) | 276(75.4) | 83(22.7) |
| XI          | 149             | 111                           | 74.5              | 109(73.2) | 123(82.6) | 73(49.0) | 130(87.2) | 40(26.8) |
| GJ          | 217             | 167                           | 77.0              | 114(52.5) | 133(61.3) | 81(37.3) | 146(67.3) | 43(19.8) |
| Total       | 366             | 278                           | 76.0              | 223(60.9) | 256(69.9) | 154(42.1) | 276(75.4) | 83(22.7) |

* Indicates pathogenicity assay on monogenic line IRBLk-K, IRBLkm-Ts, IRBLkp-K60, IRBLkh-K3 and IRBLks-F5 containing Pik, Pikm, Pikp, Pikh and Piks, respectively.

The AVR-Pik/km/kp gene was identified association with AVR-Pik/km/kp alleles

The AVR-Pik/km/kp gene is an effector gene with 342 nucleotides encoding a putative secreted protein possessing one signal peptide of 57 first nucleotides in exon in the open reading frame (ORF)[7]. A total of 10 AVR-Pik haplotypes, including the five original AVR-Pik alleles of AVR-Pik_D (GenBank Accession No. AB498875) (H01), AVR-Pik_A (AB498876) (H02), AVR-Pik_B (AB498877) (H03), AVR-Pik_C (AB498878) (H04), and AVR-Pik_E (AB498879) (H05) were identified based on the DNA sequence assemblies of 201 isolates (Table 2). The remaining 77 isolates were sequenced, but they had double peaks, and were removed for further analysis. Five novel AVR-Pik/km/kp haplotyes of H06-H10 were identified. Alignment of DNA sequence assemblies of the AVR-Pik/km/kp gene from 201 isolates revealed that there were six polymorphic sites in the exon region, and none of them in the signal peptide...
region (Table 2). Six sites in the exon region resulted in amino acid substitutions (Table 3). Moreover, the AVR-Pik/km/kp allele sequence assemblies among the 201 isolates were predicted to produce 10 functional proteins (Table 3). Among these 10 proteins, amino acid variations were predicted to occur at five positions. All variations occurred throughout the protein, except of the putative secreted proteins possessing the [RK]CxxCxxxxxH motif (Table 3; S1 Fig). Amino acid variations at M78K were found in six isolates, all of which were virulent on monogenic lines IRBLk-K (with Pik), IRBLkm-Ts (with Pikm), IRBLkp-K60 (with Pikp), IRBLkh-K3 (with Pikh) and IRBLks-F5 (with Piks) (Table 3). This suggests that amino acid 78M is critical for avirulent function of AVR-Pik/km/kp/kh loci. The isolates of H01, H07 and H09 haplotypes held the avirulence genes of AVR-Pik/km/kp/kh, the isolates of H05 and H08 held AVR-Pik/km/kh, the isolates of H06 held AVR-Pikm/kh, the isolates of H02 and H03 haplotypes held AVR-Pikh, because those isolates were avirulent to corresponding R gene(s) (Table 3). While the isolates of H04 and H10 had overcome the resistance of all Pik alleles on the loci (Table 3). These findings suggest the novel avirulence gene AVR-Pikh was identified, and the evolution of AVR-Pik alleles of M. oryzae were involved. While the 10 haplotypes did not hold AVR-Piks, because of those isolates were virulent to monogenic line IRBLks-F5 (holding Pi-ks) (Table 3). Some 75 isolates contained AVR-Pik/km/kp/kh (frequency 36.4%), 55 isolates contained AVR-Pik/km/kh, (frequency 26.7%), four isolates contained AVR-Pikm/kh (frequency 1.9%), 50 isolates contained AVR-Pikh (frequency 24.9%). Some 17 isolates did not contain these avirulence gens (S1 Table). In summary, five novel AVR-Pik loci were identified, and 91.5% of total isolates contained AVR-Pikh, which is widely distributed in south-western China.
Table 2. Haplotypes of AVR-Pik loci in rice blast fungus of Yunnan, China

| Haplotype          | No. of isolates | % of total | Variant locus<sup>a</sup> |
|--------------------|-----------------|------------|---------------------------|
| AB498875 (AVR-Pik_D) | 45              | 22.4       | C            | C | G | C | T | G |
| AB498876 (AVR-Pik_A) | 46              | 22.9       | A            | G | A | . | . | . |
| AB498877 (AVR-Pik_B) | 4               | 2          | A            | G | A | . | . | A |
| AB498878 (AVR-Pik_C) | 11              | 5.5        | A            | . | . | A | . | . |
| AB498879 (AVR-Pik_E) | 51              | 25.4       | A            | . | . | . | . | . |
| H01                | 45              | 22.4       | .            | . | . | . | . | . |
| H02                | 46              | 22.9       | A            | G | A | . | . | . |
| H03                | 4               | 2          | A            | G | A | . | . | A |
| H04                | 11              | 5.5        | A            | . | . | A | . | . |
| H05                | 51              | 25.4       | A            | . | . | . | . | . |
| H06                | 4               | 2          | A            | . | . | A | . | . |
| H07                | 27              | 13.4       | .            | . | A | . | . | . |
| H08                | 4               | 2          | A            | . | A | A | . | . |
| H09                | 3               | 1.5        | .            | G | A | . | . | . |
| H10                | 6               | 3          | A            | G | A | . | A | . |

<sup>a</sup> . Indicates the same with AB498875 (GenBank Accession No.). The AB498875, AB498876, AB498877, AB498878 and AB498879 of AVR-Pik were obtained from GenBank, and which was the five different alleles of AVR-Pik_D, AVR-Pik_A, AVR-Pik_B, AVR-Pik_C, AVR-Pik_E, respectively.
The functional alleles from the reference of d indicate the corresponding functional alleles to responding R genes. The copyright holder for this preprint (which was posted December 17, 2018) has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC BY 4.0 International License.

| Pathogens | Functional alleles | Disease reaction | Variation locus | Total HP alleles |
|-----------|--------------------|-----------------|----------------|-----------------|
|          |        |                  |                |                 |
|          |        |                  |                |                 |

Table 3. Variation of the AVR-Pi loci in pathogenic fungi of Yunnan, China

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Stepwise evolution and haplotype diversity of AVR-Pik loci in M. oryzae

Among 10 AVR-Pik haplotypes, the haplotypes of H01, H02, H03, H04 and H05 were identical with the original AVR-Pik alleles of AVR-Pik_D (GenBank Accession No. AB498875), AVR-Pik_A (AB498876), AVR-Pik_B (AB498877), AVR-Pik_C (AB498878), and AVR-Pik_E (AB498879) (Table 2), respectively. Seven haplotypes were detected in 88, 37 and 39 M. oryzae from western, central and north-eastern Yunnan, respectively, six haplotypes were detected in 17 M. oryzae from south-eastern Yunnan, three haplotypes were detected in 10 M. oryzae from south-western Yunnan, and only one haplotype was detected in 10 M. oryzae from north-western Yunnan (Table 4). Ten and eight haplotypes were found in the GJ and XI rice growing regions, and the Diversity Index (DI) was 0.79 and 0.75 for those regions, respectively. Similarly, the DI was 0.78, 0.68, 0.65, 0.62, 0.54, and 0 for north-eastern, central, western, south-eastern, south-western, and north-western Yunnan, respectively (Table 4). In summary, the DI of AVR-Pik alleles was ordered in Yunnan Province as:

north-eastern>central>western>south-eastern>south-western>north-western. The DI of AVR-Pik alleles in GJ rice growing region was similar that of XI.
Table 4. Distribution of AVR-Pik haplotype in different rice-growing regions.

| Haplotype No. isolates (%) | Northeastern Central Southern East | Northwestern Southwestern Western | Total |
|----------------------------|-----------------------------------|----------------------------------|-------|
| H01                        | 3                                 | 15                               | 18    |
| H02                        | 4                                 | 27                               | 31    |
| H03                        | 4                                 | 1                                | 5     |
| H04                        | 4                                 | 4                                | 8     |
| H05                        | 4                                 | 22                               | 26    |
| H06                        | 4                                 | 9                                | 13    |
| H07                        | 4                                 | 27                               | 31    |
| H08                        | 4                                 | 5                                | 9     |
| H09                        | 4                                 | 3                                | 7     |
| H10                        | 4                                 | 3                                | 7     |

No. of haplotypes: 7

Index of diversity: 0.78

Index was calculated as the frequency of haplotypes types in the M. oryzae population following Fontaine's method.\[55]
Six nucleotide variations in the exons of AVR-Pik alleles was observed (S1 Fig and Table 2), and a haplotype network based on sequence variation was developed (Fig 1). Four micro-evolutionary clusters of AVR-Pik, AVR-Pikm, AVR-Pikp, and AVR-Pikh were observed among 201 field isolates (Fig 1). Five original AVR-Pik alleles of AVR-Pik_D (H01), AVR-Pik_A (H02), AVR-Pik_B (H03), AVR-Pik_C (H04), and AVR-Pik_E (H05) were involved in the networks. The isolates of H01, H05, H07, H08 and H09 were avirulent to IRBLk-K (with Pik), whereas the isolates of H02, H03, H04, H06, H07 and H10 were virulent to Pik (Table 3; Fig 1). The isolates of H01, H05, H06, H07, H08 and H09 were avirulent to IRBLkm-Ts (with Pikm), whereas the isolates of H02, H03, H04, and H10 were virulent to Pikm (Table 3; Fig 1). The isolates of H01, H07 and H09 were avirulent to IRBLkp-K60 (with Pikp), whereas the isolates of H02, H03, H04, H05, H06, H08 and H10 were virulent to Pikp (Table 3; Fig 1). The isolates of H01, H02, H03, H05, H06, H07, H08 and H09 were avirulent to IRBLkh-K3 (with Pikh), whereas the isolates of H04 and H10 were virulent to Pikh (Table 3; Fig 1). These findings suggest that there were four distinct stepwise evolved patterns of AVR-Pik, AVR-Pikm, AVR-Pikp, and AVR-Pikh in rice growing regions of Yunnan.

The possible scenario for M. oryzae AVR-Pik alleles-rice Pik alleles interactions and co-evolution were constructed (Fig 2). The AVR-Pik homolog H01 (AVR-Pik-D) were derived from an ancestral M. oryzae gene. The Pik allele, Piks, cannot recognize three alleles AVR-Pik-D (H01), H07 and H09, thus the other Pik allele, Pikp, evolved that can recognize three alleles AVR-Pik-D (H01), H07, H09, while the altered alleles H05 (AVR-Pik-E) and H08 were evolved to virulence from avirulent origins via nucleotide substitution to avoid recognition by Pikp (Table 2; Fig 2). For this situation, another Pik allele, Pik, evolved that can recognize five alleles AVR-Pik-D (H01), H07,
H09, \textit{AVR-Pik-E} (H05) and H08. Then, yet another \textit{AVR-Pik} allele, H06, was derived that cannot be recognized by \textit{Pikp} and \textit{Pik}. Next, the rice \textit{R} gene \textit{Pikm} was utilized that recognizes \textit{AVR-Pik-D} (H01), H07, H09, \textit{AVR-Pik-E} (H05), H08 and H06. Then, yet two \textit{AVR-Pik} alleles, \textit{AVR-Pik-A} (H02) and \textit{AVR-Pik-B} (H03), were derived that cannot be recognized by \textit{Pikp}, \textit{Pik} and \textit{Pikm}. Next, the rice \textit{R} gene \textit{Pikh} was utilized that recognizes \textit{AVR-Pik-D} (H01), H07, H09, \textit{AVR-Pik-E} (H05), H08, H06, \textit{AVR-Pik-A} (H02) and \textit{AVR-Pik-B} (H03). Then another two \textit{AVR-Pik} alleles, \textit{AVR-Pik-C} (H04) and H10, evolved that cannot be recognized by any of the five \textit{Pik} alleles (Table 2; Fig 2). Those showed stepwise evolution of \textit{AVR-Pik} and \textit{Pik} interaction and co-evolution. Interestingly, the \textit{AVR-Pik} allele H07 was derived from H01, that can be recognized by \textit{Pikp}, \textit{Pik} and \textit{Pikm}. Thus, the altered allele H06 from H07 can avoid recognition by \textit{Pikp} and \textit{Pik}, next the altered allele H08 from H06 can avoid recognition by \textit{Pikp}, while the altered allele H04 from H08 avoids recognition by any of the five \textit{Pik} alleles. Similarly, the H09 derived from H07, that can be recognized by \textit{Pikp}, \textit{Pik}, \textit{Pikm} and \textit{Pikh}, thus the altered allele H02 from H09 can avoid recognition by \textit{Pikp}, \textit{Pik}, and \textit{Pikm} (Table 2; Fig 2). The H05 allele can be recognized by \textit{Pik}, \textit{Pikm} and \textit{Pikh}, while the altered allele H04 from H05 can avoid recognition by \textit{Pikp}, \textit{Pik}, and \textit{Pikm} (Table 2; Fig 2). These results suggest that detour evolution of \textit{AVR-Pik} loci of \textit{M. oryzae} were involved during the interaction and co-evolution with the \textit{Pik} loci of \textit{Oryzae} in nature.

The virulent isolates of H04 and H10 were identified to \textit{Pik} loci (\textit{Pik}, \textit{Pikm}, \textit{Pikp}, \textit{Pikh}, \textit{Piks}) in most of regions, including north-eastern, south-eastern, south-western, and western Yunnan (Table 3-Table 4; Fig 1). These results suggest that the virulent evolution of \textit{AVR-Pik} loci occurred in most rice-producing regions of Yunnan.

The H01, H04, H05, H06 and H10 haplotypes were mainly distributed in \textit{XI} rice
Selection Pressure of AVR-Pik in M. oryzae

To determine the natural selection pressure of AVR-Pik in M. oryzae in Yunnan, the Tajima's Neutrality of AVR-Pik in M. oryzae was tested based on 201 AVR-Pik DNA sequences, and the Tajima's D was 1.19854 (Table supplement 2). The result suggests that AVR-Pik maybe under either strong population expansion or positive selection. The calculation results of three positive-selection models were highly consistent (Fig 3). The sliding window shows the distribution of the Ka/Ks values across all 113 amino acids under the M8, M8a, and M7 models (Fig 3). The results show that the Ka/Ks value of 46th, 47th, 48th, 67th and 78th sites was >1, suggesting that these sites were potentially subjected to purifying selection. The positive selection sites were only observed in the mature protein region among 201 M. oryzae isolates with AVR-Pik (Fig 3). These results showed that the amino acid sequence was conserved in the signal peptide compared with divergent mature protein region of AVR-Pik in M. oryzae.

To confirm the resistance performance of alleles of Pik in the field, we assayed the seedling and panicle blast disease with monogenic lines carrying the Pik, Pikm, Pikp, Pikh which were developed by JIRCAS and IRRI in fields in Mangshi, Lufeng and Yiliang Counties, respectively, in 2015 (Table supplement 3). The result show that IRBLkm-Ts (with Pikm), IRBLkp-K60 (with Pikp), and IRBLkh-K3 (with Pikh) were
resistant, while IRBLks-F5 (with Piks), IRBLk-Ka (with Pik) were susceptible in Mangshi County (Table supplement 3). These results suggest that *M. oryzae* of isolate population holding AVR-Pikm/kp/kh. IRBLkh-K3 (with Pikh) was resistant in Lufeng and Yiliang, and monogenic lines of IRBLks-F5 (with Piks), IRBLk-Ka (with Pik), IRBLkm-Ts (with Pikm) and IRBLkp-K60 (with Pikp) were susceptible in Lufeng and Yiliang Counties, suggesting that *M. oryzae* isolate population holding AVR-Pikh.

These results are consistent with PCR detection and pathogenicity assays.

**Discussion**

In this study, we found five new haplotypes in the *AVR-Pik* DNA sequences among field isolates of *M. oryzae* from various rice-producing regions in Yunnan. Numerous virulence isolates to the *Pik* gene containing rice varieties were identified in field isolates collected in Yunnan, suggesting that *Pik* was defeated in some rice production areas due to extensive development of *Pik* in China. The *Pik* alleles have been deployed and display high rice blast resistance in China.[20,22,32,38] Complete deletions have occurred in *AVR-Pik* sequences among field isolates of *M. oryzae* from various rice-producing countries[15-16], which accords with our results. Numerous isolates inspected from commercial rice fields, containing *AVR-Pik* suggest that *Pik* has effective in preventing rice blast disease. In Yunnan, rice cultivars with *Pikh*, *Pikp*, *Pikm*, *Piks*, *Pik* were resistant to 81.7, 62.8, 51.9, 43.4 and 39.4% of isolates (282 isolates), respectively[33]. Corresponding values from 146 isolates from Guandong Province were 88.4, 39.0, 0, 1.4 and 57.5%, respectively.[39] These results suggest that some *Pik* alleles has limited effects in these rice production areas. Continued analysis of *AVR-Pik* alleles in these isolates will help us understand the evolutionary mechanism of *AVR-Pik*, and to predict the stability and effectiveness of *Pik* alleles.
mediated resistance under natural conditions.

Effective variations of DNA sequence were observed in several AVR genes (AVR-Pita1, AVR-Pia, and AVR-Pii) in the telomere regions.[7,40-41] The transposable element insertion in the last exon of the ACE1 gene[9] and Pot3 inserted in AVR-Pizt and AVR-Pita1 all resulted in new virulent alleles. Based on the DNA sequence analysis,[8,42-43] four variations of point mutation, segmental deletion, complete absence (6.7%) and transposable element (TE) insertion were found in AVR-Pib, all of which result in losses of the avirulence function.[6] Three distinct expression profiles were found among seven functional of 16 nucleotide polymorphisms in the AVR-Pib genes.[6] These findings showed that M. oryzae uses the transposons to change the expression of AVR genes in defeating R genes. In the present study, the AVR-Pik gene was present in most blast populations (76.0%) in Yunnan (Table 1), which was similar to rice blast isolates in Hunan Province.[44] We found significantly more nucleotide variation in the protein coding region of AVR-Pik alleles, resulting in changes of amino acids suggesting that there exists intense selection pressure on AVR-Pik alleles in Yunnan.

DNA sequence variation was found in exon regions of AVR-Pik, and a total of 10 haplotypes were identified based on the six variant nucleotides among 201 isolates collected from Yunnan (Table 2). Five novel variant amino acids of the AVR-Pik loci variants in the 201 isolates were identified in the present study, leading to finding five new haplotypes. Based on the virulence analysis of the strains harboring this variation, haplotypes H01, H02, H05 and H07 are more frequent in the field isolates. This probably suggests that loss of these haplotypes may have a larger fitness penalty than others alleles in the M. oryzae population. These new alleles allow us to construct a more holonomic network among different alleles of AVR-Pik and some novel
haplotypes were found. We also identified the putative secreted proteins possessing the [LI]\xAR[SE][DSE] and [RK]CxxCxxxxxxxxxxxxH motif in 201 isolates with AVR-Pik alleles (Table 3) which was consistent with the results of Yoshida et al[7]. Some 126, 59, 94 and 15 isolates are variations at the amino acid position of H46N, P47A, G48D, A67D, respectively, and four and six isolates are variations in the amino acid position of M78I and M78K, respectively (Table 3). These results showed the amino acid position of 46th, 47th, 48th, 67th and 78th were the hot variation amino acid sites among proteins of AVR-Pik/km/kp/kh.

During the long co-evolution of plants and pathogens, the pathogen AVR genes are recognized by the cognate plant R genes in triggering effective defense responses. The divergences of AVR genes of the pathogen were shaped by host R genes and changing environmental conditions. We observed that the Diversity Index of AVR-Pik in XI and GJ regions were similar (Table 4) and variations of AVR-Pik were different between XI and GJ growing regions (Table 4). These results suggest that adaptive variations have occurred in commercial rice fields in Yunnan.

Yunnan is one of the diversification centers of the cultivated Asian rice Oryza sativa. Three wild species of O. rufipogon, O. officinalis and O. meyeriana existed in the area.[45] Over 5000 accessions of rice germplasms were collected from fields and preserved. Among them, 227 rice accessions were characterized by a set of differential rice blast isolates, and 38 and 25 of 227 rice accessions contained the rice blast resistance genes Pik and Pikm, respectively.[45] While the neutrality test of Tajima's D was 1.19854 (Table supplement 2), it suggests AVR-Pik/km/kp/kh loci may be under population expansion or purifying selection shaped by the cognate gene Pik loci in rice growing regions of Yunnan. Most of isolates carried AVR-Pikh and Pikh with high resistance in Yunnan and Guangdong Provinces. This may be due to Pikh...
being a widely distributed resistant gene in rice accessions. These results accord with
Zhai et al[22].

The AVR-Pik was recognition specificity by Pik of rice, and AVR-Pik directly
specific physically binds the N-terminal coiled-coil domain of Pik. These observations
were confirmed by yeast two-hybrid and co-immunoprecipitation in plant assays.[15]
Four alleles of AVR-Pik (AVR-Pik_D, AVR-Pik_E, AVR-Pik_A, AVR-Pik_C) in
Japanese isolate populations were in the manner of co-evolution with the rice Pik
alleles Pikp, Pik and Pikm.[15] Four alleles of AVR-Pik in Chinese M. oryzae
population stepwise evolution between rice Pik allele Pikp, Pik, Pikm, and Phk were
found.[16] Highly variable Pik alleles were observed, and both stepwise AVR-Pik of
M. oryzae and Pik of rice occurred in field conditions.[16] These observations
indicate that AVR-Pik has been strongly targeted by hosts.[16] In this present study,
we found both the detour and stepwise evolved AVR-Pik alleles-rice Pik alleles
interactions and co-evolution (Table 3; Fig 2), which implies high diversity of rice
varieties in Yunnan. The AVR-Pik alleles have been regularly under selection stress by
antagonistic alleles in host populations. Similarly, the wheat-infecting lineages from
Brazil and Bangladesh appeared genetically distinct and displayed reticulate evolution
on population genomic analyses of transcriptomic single nucleotide
polymorphisms.[46]

The stepwise mutation process has been demonstrated for virulence acquisition in
Fusarium oxysporum f. sp. Ciceris and Puccinia striiformis f. sp. Tritici.[47-49] In the
present study, we found one major mutation evolution of AVR-Pik allele and seven
minor mutation evolution patterns (Fig 2). The alternative mutation pattern can
seemingly convert from avirulence to virulence via seldom mutation, and showed
higher efficiency (Fig 2). These may be due to the strong positive selection pressure
by the corresponding Pik allele on the host and environment. Similarly, AVRL567 can
convert from avirulent to virulent by a set of stepwise mutants in amino acid
substitution.[50] Stepwise evolved procession has observed in AVR-Pik.[15-16] The
possible evolution of AVR-Pik found in the present study, showed more complex
evolution than expected in the rice growing regions of Yunnan.

**Conclusion**

We detected five novel haplotypes in the field population by using 201 isolates,
constructed a complex network of AVR-Pik alleles, and evaluated the effectiveness of
Pik alleles in rice production areas of Yunnan. Our findings support the premise that
functional AVR-Pik possesses diversified sequence structures and can avoid
recognition by host via multiple site variations. Haplotype H10 originates from
frequently distributed H2, H4 jump from H5 and/or H8, can overcome all detected Pik
alleles to date. Although H4 and H10 haplotypes have low frequency, but surveillance
of these two alleles in field populations is crucial because of its high risk of leading to
the breakout on the background of Pik rice variety. Management must retard the
selection stress on the allele, possibly by avoiding its proliferation in agricultural
practices. Prediction of blast occurring should be based on the frequency and
distribution of allele of multiple loci v.g. Pik and AVR-Pik in isolate populations in
field conditions.

**Materials and methods**

**Rice cultivars, fungal isolates, culture, and pathogenicity assays**

The Pik, Pikm, Pikp, Pikh, Piks gene containing rice monogenic line IRBLk-K,
IRBLkm-Ts, IRBLkp-K60, IRBLkh-K3 and IRBLks-F5, respectively, and the
susceptible backcrossing parent Lijiangxintuanheigu (LTH without *Pik*) were used for pathogenicity assays. A total of 366 isolates were collected, single spore purified, and examined. All isolates were stored at -20°C on filter paper and grown at room temperature under blue and white fluorescent lighting on petri dishes containing oatmeal agar for spore production. Disease reactions were determined using a modified standard pathogenicity assay, as described by Jia et al.[51] Specifically, rice seedlings at the 3- to 4- leaf stage were placed in a plastic bag and were spray inoculated with a spore suspension of 1-5×10⁵ spores/mL. After inoculation, the plastic bags were sealed to maintain high relative humidity (90-100%) for 24 h before removing the plants from bags. Subsequently, plants were maintained in a greenhouse for an additional six days, to allow the development of disease symptoms. The disease reactions were rated based on visual number and amount of lesions at the second youngest leaf using the 0-5 disease scale. A value of 0-1 is resistant, 2 is moderately resistant and 3-5 is susceptible. Five seedlings were used each time and the experiment was repeated one more time, and the mean disease scores were used to determine resistance versus susceptibility.

**DNA preparation, PCR amplification, and DNA sequencing**

Fungal isolates were grown in complete liquid media at 25°C for six-eight days to produce mycelium under dark conditions. DNA was then isolated from mycelia using the CTAB method.[52] Primers *pex31F* (5’-TCGCTTCCATTTTTA-3’) and *pex31R* (5’-GCCCATGCATTCTTAT-3’) were used to amplify the *AVR-Pik* allele and for sequencing using the methods of Yoshida et al.[7] Specifically, PCR reactions were performed using 2×Taq PCR MasterMix (Tiangen Biotech Co. Ltd., Beijing, China). Each PCR reaction consisted of the following components: 25 µl of Taq PCR
Master Mix (contains 25U of Taq DNA polymerase, 10X Tiangen PCR buffer, 15 mM MgCl₂, and 200 µM of each dNTP), 1 µl of each 10 µM primer, 2 µl of fungal genomic DNA, and 21 µl distilled water (provided by the Tiangen Kit). Reactions were performed in a BIO-RAD Thermal Cycler (C1000, Bio-Rad Laboratories, Life Science Research, Hercules, CA, USA) with the following PCR program: 1 cycle at 95°C for 3 min for initial denaturation, followed by 29 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final denaturation of 72°C for 7 min. All PCR reactions were repeated three times (20 µl for detection, 50 µl for sequencing). The size of the amplified fragment was estimated by DL2000 DNA Ladder (Tiangen Biotech Co. Ltd., Beijing, China). PCR products were sequenced using the same primers as mentioned above for PCR amplification. DNA was sequenced by Shanghai Life Technologies Biotechnology Co., Ltd. (Shanghai, China). The amplicon from each isolate was sequenced three times.

**Resistance evaluation of Pik alleles in field**

The monogenic lines of IRBLk-Ka, IRBLkm-Ts, IRBLks-F5, IRBLkp-K60, IRBLkh-K3 (carrying the Pik, Pikm, Piks, Pikp, Pikh, respectively.), which were planting in fields in Mangshi, Lufeng and Yiliang Counties of Yunnan Province, respectively, in 2015. The seedling and panicle blast disease were surveyed, and the resistance were evaluated.

**Data analysis**

DNA sequences of AVR-Pik were assembled by Vector NTI software Suite V.10 (Invitrogen, Carlsbad, California, USA) and aligned using DNASTAR V7.10 software (http://www.dnastar.com/). The number of DNA haplotypes, polymorphic sites (π),
and the sliding window were calculated using DnaSP v5.10.01 software.[53] Haplotype network analysis was performed using TCS1.21 (http://darwin.uvigo.es/).[54] Diversity Index was calculated as the frequency of haplotypes or protein types in the rice blast fungus population following the method of Fontaine et al.[55] Diversity Index = \( 1 - \sum_{i=1}^{n} p_i^2 \), where \( p_i \) is the frequency of the haplotype \( i \) in a population. Tajima's neutrality test was performed using MEGA V5.10.

**Figure captions**

**Fig 1. The haplotype network for the 10 AVR-Pik alleles.** The original AVR-Pik allele was designated as the H01 haplotype in the network. Each haplotype was separated by mutational events. All haplotypes were displayed as circles. The size of the circles corresponds to the haplotype frequency. The haplotype H01 to H05 were same with the AB498875, AB498876, AB498877, AB498878 and AB498879 (GenBank Accession No.) of AVR-Pik was obtained from GenBank. Green color indicates avirulent to the corresponding \( R \) gene, yellow color indicates virulent to the corresponding \( R \) gene.

**Fig 2. Possible scenario for M. oryzae AVR-Pik alleles-rice Pik alleles interactions and co-evolution.** Chronological order is given on the left (time order). AVR-Pik homolog H01 (AVR-Pik-D) were derived from an ancestral \( M. oryzae \) gene. AVR-Pik-D (H01), H07 and H09 are recognized by Pikp, thus the altered alleles AVR-Pik-E (H05) and H08 evolved. In response to this situation, another Pik allele, Pik, evolved that can recognize five alleles AVR-Pik-D (H01), H07, H09, AVR-Pik-E (H05) and H08. Then, yet another AVR-Pik allele, H06, was derived that cannot be recognized by Pikp and Pik. Next, the rice \( R \) gene Pikm was utilized that recognizes
AVR-Pik-D (H01), H07, H09, AVR-Pik-E (H05), H08 and H06. Then, yet two
AVR-Pik alleles, AVR-Pik-A (H02) and AVR-Pik-B (H03), were derived that cannot be
recognized by Pikp, Pik and Pikm. Next, the rice R gene Pikh was utilized that
recognizes AVR-Pik-D (H01), H07, H09, AVR-Pik-E (H05), H08, H06, AVR-Pik-A
(H02) and AVR-Pik-B (H03). Then other two AVR-Pik alleles, AVR-Pik-C (H04) and
H10, evolved that cannot be recognized by any of the five Pik alleles.

Fig 3. Sliding window of positive-selection sites of the AVR-Pik alleles under M8,
M8a, and M7 models. The Y-axis indicates the ratio of the rate of nonsynonymous
substitution (Ka) to the rate of synonymous substitution (Ks) (Ka/Ks); the X-axis
indicates the position of the AVR-Pik amino acids in the site. The signal region of the
variant structure is purple and the black area represents the mature protein region on
the label on top of the Figure.

Supporting information

S1 Fig. Diversification of AVR-Pik in avirulent isolates. Distribution of variation of
the AVR-Pik alleles was analyzed using sliding window. X-axis shows the distribution
of variation within the full region, including signal peptide and exon of AVR-Pik.
Lower pane indicates the corresponding schematic presentation of the signal peptide
and exon of AVR-Pik. Window length: 1; Step size: 1. π value corresponds with the
level of variation at each site because it is the sum of pair-wise differences divided by
the number of pairs within the population.

S1 Table. Distribution of AVR-Pik loci in rice blast fungus.

S2 Table. Tajima's Neutrality Test of AVR-Pik in M. oryzae. The analysis involved
201 nucleotide sequences of AVR-Pik. m indicates number of sequences, S indicates
number of segregating sites, Ps indicates S/n, Θ indicates p_s/a_1, π indicates nucleotide
diversity, and $D$ is the Tajima test statistic. Tajima's $D$: 1.19854, Statistical significance: Not significant, $P>0.10$.

**S3 Table.** Summary of disease reaction of monogenic lines with Pik alleles in fields.

The pathogenicity assay on the monogenic lines IRBLk-K, IRBLkm-Ts, IRBLkp-K60, IRBLkh-K3, and IRBLks-F5 containing the resistant genes of Pik, Pikm, Pikp, Pikh, and Piks, respectively. R and S indicate disease reaction was resistant and susceptible, respectively.

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