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Diversity and Evolution of the Avirulence Gene *AvrPi54* in Yunnan Rice Fields

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Abstract: Variance or complete loss of the avirulence gene (*Avr*) enables the pathogen to escape resistance protein (R) recognition. The field resistance effectiveness of the *R* gene is determined by its corresponding *Avr* gene in field isolates. To effectively deploy the rice blast *R* gene *Pi54*, the distribution, variation and evolution of the corresponding *Avr* gene, *AvrPi54*, were determined through PCR amplification, pathogenicity assay, gene sequences and evolutionary analysis. Among 451 *Pyricularia* isolates from rice and non-rice hosts, including *Oryza rufipogon*, *Digitaria sanguinalis*, *Eleusine coracana*, *E. indica* and *Musa* sp. in Yunnan province, the PCR amplification result showed that *AvrPi54* alleles existed among 218 (48.3%) isolates including rice isolates, *O. rufipogon* isolates and *E. coracana* isolates. Pathogenicity assay showed that 336 (74.5%) isolates were avirulent to Tetep (holding *Pi54*). Five *AvrPi54* haplotypes were identified among 142 isolates through the gene sequence. These haplotypes were determined to be avirulent to *Pi54* through pathogenicity assay. Four novel haplotypes (H2 to H5) of the *AvrPi54* gene would provide new target sites for rice blast control. Haplotype diversity analysis indicated that there existed a lower genetic diversity of *AvrPi54* for *P. oryzae* populations (five haplotypes, $H_d = 0.127$, $\pi = 2.9 \times 10^{-4}$) in this study. Neutrality tests showed that *AvrPi54*'s genetic variation was affected by purified selection. Haplotype network and phylogeny analysis showed that H1 was an ancestral haplotype and was widely distributed in rice isolates and *O. rufipogon* isolates, while H5 diverged early and evolved independently. These results indicate that the gene evolves slowly and stably and is a comparatively conserved *Avr* gene.

Keywords: *Pyricularia oryzae*; pathogenicity; *AvrPi54*; haplotype diversity; evolution

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1. Introduction

Rice is a staple cereal crop for more than one-third of people all over the world [1]. Nevertheless, rice production and global food security are threatened by catastrophic diseases engendered by pathogens and pests. *Pyricularia oryzae* (Syn. *Magnaporthe oryzae*), a filamentous ascomycete fungus, can cause rice blast disease and infect the leaves, nodes, collars, panicles and roots of rice at all growth periods [2]. More than 50 species of poaceous

plants, including important crops such as rice, wheat, barley, millet, oat and other species of the Poaceae, can be infected by the fungus [3–7]. The yield loss caused by rice blast disease is sufficient to nourish more than 60 million people each year [8,9]. The application of rice resources containing the major resistance gene (*R*) is the most economic, effective and ecological method to manage rice blasts. The *avirulence* gene (*Avr*) may encode pathogen molecules that are directly or indirectly perceived by the corresponding *R* gene product [10], and then host resistance responses are triggered by pathogen recognition and infection is stopped. Race-specific *R* genes of plants have evolved to detect the products of the corresponding *Avr* genes and trigger effective resistance to plant pathogens [11].

Currently, there are two principal hypotheses for the co-evolution of *R* and *Avr* genes in plants: "arms race" and "trench warfare". In an arms race, both *R* and *Avr* genes are under-diversified selections or positive selections, whereas in trench warfare, either *R* or *Avr* is an under-balanced selection or a purified selection, and the other is an under-diversified selection or a positive selection [11–13]. Based on diversity analysis, phylogeny and neutrality test of *Avr*/*R* pairs, an arms race or trench warfare could be derived between the gene pair interaction during the co-evolutionary process. It is valuable for the better application of the resistance gene. The most prominent example of an arms race in plant and pathogen interactions is the case study of flax and flax rust interactions. The *L* locus, harboring 12 allelic variants with extensive sequence diversity in flax, is responsible for the resistance to flax rust caused by *Melampsora. lini* [14]. *AvrL567* in *M. lini* is recognized by the *L5*, *L6* and *L7* alleles of the flax L gene. There are also 12 allelic variants in six isolates and a high diversity level in the non-synonymous DNA sequence polymorphisms in *AvrL567* [15]. Physical interactions were observed in yeast two-hybrid analysis between the L protein and AvrL567 protein. Interaction specificity was also observed in planta [16]. *AvrL567* interacts with *L* driven by arms race dynamics [16–18]. Another case of *Avr* gene and *R* gene interaction by an arms race is *AvrPik* of *P. oryzae* and the *Pik* locus of rice. There are high diversity levels in *AVR-Pik* and the cognate *Pik* with multiple alleles in which DNA replacements cause amino acid changes. *AVR-Pik* alleles are specially recognized by the various *Pik* alleles in a yeast two-hybrid assay and in an in planta co-immunoprecipitation assay. Thus, direct physical interactions between *AVR-Pik* and *Pik* are driven by arms races [18]. Some cases have indicated that the interaction between plants and the pathogen is driven by trench warfare. Under long-term balancing selection, the interaction between the protein RPM1 of *Arabidopsis* and two unrelated effector proteins of *Pseudomonas syringae* (AvrRpm1 and AvrB) was suggested to be caused by the trench warfare [19]. In trench warfare, the pathogen alternates with the host based on host resistance frequency, causing the pair-interaction organisms to advance and retreat. Co-evolution between *Avr-Pita* and *Pita* was suggested to be driven by trench warfare because of the diversified haplotypes and positive selection for *Avr-Pita* and the single haplotype and balanced selection for *Pita* [11,12]. The trench warfare interaction between *Avr-Pita* and *Pita* was also testified based on nucleotide diversity, neutrality statistics and phylogenetic analyses [13]. Disease epidemics take turns with periods of high host resistance, resulting in endless advances and retreats for the host and pathogen in the trench warfare model. Temporal variation in host and pathogen interactions comes up because disease spread is more likely when the frequency of resistance is low [19]. This frequency-dependent selection can result in the periodic recurrence of severe epidemics, causing the frequency of resistance to cycle. Transient benefits may be brought by arms races; durable resistance might be generated by a non-arms race in some molecular evolutionary examples [20].

The rice blast model is a classical gene-for-gene model [21] in which *Avr* genes in the pathogen have a functional relationship with specific *R* genes in rice [22,23]. Currently, there are more than 100 mapped blast *R* genes in rice and 26 mapped *Avr* genes in *P. oryzae*. Among the mapped *R* and *Avr* genes, 38 and 14 genes have been cloned, respectively [24]. Most cloned *R* genes encode NLR proteins, except for *Pid2*, *pi21*, and *Ptr*. While *Avr* genes in *P. oryzae* mostly encode small secreted proteins without distinct sequence homology. The cloned *Avr* genes play a critical role in revealing its variance and the interaction

with corresponding *R* genes in the Rice–*Pyricularia* pathosystem [25]. Among cloned R genes, nine corresponding *Avr* genes have also been cloned. These *R*/*Avr* pairs include *Pi-ta*/*AVR-Pita*, *Piz-t*/*AvrPiz-t*, *Pik*/*AVR-Pik*, *Pia*/*AVR-Pia*, *Pi-CO39*/*AVR1-CO39*, *Pi54*/*Avr-Pi54*, *Pii*/*AVR-Pii*, *Pi9*/*AvrPi9*, and *Pib*/*AvrPib*. Based on the interaction advances of seven *R*/*Avr* pairs, five of them interact directly, namely *Pi-ta*/*AVR-Pita*, *Pik*/*AVR-Pik*, *Pia*/*AVR-Pia*, *Pi-CO39*/*AVR1-CO39*, and *Pi54*/*AvrPi54*, whereas *Piz-t*/*AvrPiz-t* and *Pii*/*AVR-Pii* interact indirectly [24,25]. Except for the one-to-one interactions, another two interaction types were also found. One is two-to-one interactions, such as when Pik-1 and Pik-2 interact with AVR-Pik [26], the recently cloned two Avr proteins (MoHTR1 and MoHTR2) with the same target protein [27]. Another type is the interaction between two different Avr proteins and a single R protein complex, such as AVR1-CO39 or AVR-Pia, which could discern the heterodimer produced by RGA4 and RGA5.

Among the cloned *Avr* genes of *P. oryzae*, *AvrPi54* is the first one that has been used to apply genome-wide in silico analysis, protein modeling, and in silico docking to clone the candidate gene [28]. It was cloned from the avirulent isolate RML-29 (Mo-nwi-55) and is located at chromosome 4. A single exon of 462 bps encodes the AvrPi54 protein with 153 amino acids, and it is predicted to be a secreted protein with a 19-amino-acid signal peptide (SP) at the N-terminal region. There exist six β-sheets and no α-helices in the mature SP truncated protein. A low genetic diversity (three haplotypes, $\pi = 0.00048$, $H_d = 0.088$) at the *AvrPi54* locus of 45 isolates was found in India [13]. It activates immunity to rice mediated by the *Pi54* gene. *Pi54*, which was formerly named *Pikh*, a counterpart resistance gene of *AvrPi54*, is a durable and broad-spectrum resistance gene [29,30]. It is cloned from an *indica* rice variety, Tetep. A functional complementation assay showed that *Pi54* conferred resistance to diversified *P. oryzae* isolates [30–32]. The transgenic TP309 lines with *Pi54* were verified to stimulate the rice defense system including the activation of defense response genes and transcription factors, resulting in hypersensitive response and disease resistance [33]. The sub-cellular localization of Pi54 with Green Fluorescent Protein (GFP) showed that the GFP signal was found in some leaf tissue structures, such as the stomata, upper epidermal and mesophyll cells, vascular bundle, and walls of bundle sheath and bulliform cells [34]. The results were further verified by immunocytochemical studies. *Pi54rh* and *Pi54of*, which belong to two orthology genes of *Pi54*, have also been cloned and functionally validated from wild rice *O. rhzomatis* and *O. officinalis* [35,36].

Because of its durable and broad-spectrum resistance to *P. oryzae* [30], the variance of *Pi54* has been widely studied. The abundant sequence variation in the *Pi54* gene is found to be responsible for its broad spectrum resistance among different rice landraces and wild relatives [37–41]. Allele mining of *Pi54* among eight wild rice species and six cultivated rice lines revealed its structural variation and impact on the phenotypes [37]. In total, 50 new haplotypes of the *Pi54* gene were identified among 92 rice lines, and the sequence variation in two consensus regions (163 and 144 bps) was used to design the specific DNA markers of the allele [38]. The allelic diversity of the *Pi54* gene among 885 Indian rice genotypes was explored. Nine new alleles were identified based on the DNA sequences [39]. Sixteen *Pi54* alleles were sequenced from landraces and wild *Oryza* species which have genomes ranging from AA to EE [40]. Among the 16 *Pi54* alleles, $Pi54^{ab}$ and $Pi54^{btj}$ have potential application value in a breeding program of rice blast resistance and identified SNPs and Indel can be used to develop allele-specific DNA markers. Two divergent structures of the *Pi54* gene and its locus in two *O. sativa* ssp. japonica cultivars Nipponbare and Sasanishiki were reported [41]. And the *Pi54* gene was also widely applied to rice breeding and production for blast control. The gene was introduced into the rice cultivar 'Improved Pusa Basmati 1' [42], an Indian rice cultivar MRU1010 [43], four sterile lines for improving blast resistance [44], an India popular susceptible variety 'Tellahamsa' [45], and two elite restorer lines (CB 174 R and CB 87 R) in *India* [46]. A relatively higher distribution frequency (>15%) of the *Pi54* gene was detected among 277 parental lines in China [47]. In total, 26 out of 48 rice varieties in the Huang-huai japonica rice region of China were detected to hold the

Pi54 gene through the molecular marker [48]. In total, 41 of 80 key parent resources of rice were identified to hold the *Pi54* gene [49].

Variance or complete loss of the *Avr* genes enables the pathogen to escape R protein recognition for its growth and reproduction [50,51]. So, field efficacy of the *R* gene is determined by its corresponding *Avr* gene of field isolates. Further study of the *Avr* gene of the pathogen isolates in the natural field may provide beneficial knowledge on how to apply the *R* gene to control rice blast disease in field crops [52]. Yunnan province of South China is famous on a mountainous region with an extremely diverse topography and climate, and is extremely rich in species and vegetation types, and the landscape varies [53]. Due to its abundant biodiversity, Yunnan is listed as one of the top conservation priorities in the world [54]. Using a large panel of accessions containing 446 *O. rufipogon* accessions and 1083 *O. sativa* varieties, the domestication sweeps and GWAS analyses indicated that *O. sativa* japonica rice was first domesticated from a specific *O. rufipogon* population around the middle area of the Pearl River in southern China [55]. And that *O. sativa* indica rice was subsequently derived from hybrids between japonica rice and local wild rice when the initial japonica rice cultivars spread into South East and South Asia [55]. And Yunnan is one of three genetic diversity centers of cultivated rice in China [56]. South China (Yunnan)–Laos–North Thailand region was revealed as the diversity center and the putative origin center of *P. oryzae* populations [57]. It is of great scientific value to study the variation and evolution of *Avr* genes in Yunnan, the diversity center of rice and *P. oryzae*. Compared to *Pi54*, little is known about the variance, distribution and evolution of its corresponding gene *AvrPi54*. In order to make better use of the *Pi54* gene, here, we report the molecular mechanisms of diversification, evolution and selection pressure of *AvrPi54* among the field isolates in Yunnan province, China. The study would provide useful information on resistance breeding and varieties layout for rice blast control.

2. Materials and Methods

2.1. Fungal Isolates, Rice Cultivars, Culture, and Pathogenicity Assay

Blast isolates were collected from rice and non-rice host in rice-growing regions of Yunnan Province, China. A paper bag was used to seal five samples from each block. In the previous study, it was described how to store isolates, produce spores, culture rice seedlings, and conduct pathogenicity assay [58]. Each sample was moistly incubated on a sterile filter paper in a 9 cm Petri dish at 25 °C for 24 h. Through the tip of a glass pin, single spores were isolated under a stereomicroscope and cultured in PDA medium (potato at 200 g/L, dextrose at 20 g/L, and agar at 15 g/L). Single-spored isolates were grown on filter papers in OMA medium (oatmeal at 40 g/L, dextrose at 20 g/L, and agar at 15 g/L) for long-term storage at −20 °C. The isolate was cultured in OMA medium (20 g/L oatmeal, 15 g/L agar, 10 g/L sucrose) for 7 days in a dark incubator at 25 °C, and then aerial mycelia were gently washed off with a paintbrush and distilled water. Then, the colonies were light up at 25 °C for 3 days under fluorescent light to produce sporulation. Conidia suspension was gained by softly brushing and flooding the medium surface with distilled water containing 0.01% Tween 20 detergent. The concentration of the suspension was checked to 4×10^4 conidia/mL for inoculation. In this study, 424 rice blast isolates were obtained from 14 prefectures of 7 regions in Yunnan province (Table 1). The seven regions included Central (Kunming, KM; Yuxi, YX; Chuxiong, CX and Qujing, QJ), West (Dali, DL; Baoshan, BS and Dehong, DH), Northwest (Lijiang, LJ), Northeast (Zhaotong, ZT), South (Honghe, HH and Xishuangbanna, BN), Southwest (Lincang, LC and Puer, PE) and Southeast (Wenshan, WS). According to the production area, these isolates of rice group were divided into a *GJ* group from *Geng/Japonica* rice area or *XI* group from *Xian/Indica* rice area. In order to understand the pathogenicity and distribution of the *AvrPi54* gene among isolates from other hosts, 27 isolates from non-rice host were included in this study. Isolates of the non-rice group were, respectively, collected from *O. rufipogon* (18 isolates), *Digitaria sanguinalis* (1 isolates), *Eleusine coracana* (1 isolate), *E. indica* (1 isolate), and *Musa* sp.

(6 isolates) in Yunnan province. Methods of non-rice isolate storage, spore production, the pathogenicity assay were same as above.

Table 1. *AvrPi54* gene amplification and pathogenicity assay to Tetep among *Pyricularia* isolates in Yunnan province of China.

Isolates Groups	No.	PCR Amplification of *AvrPi54*		No. Seq	Pathogenicity Assay to Tetep	
		+ (%)	- (%)		A (%)	V (%)
Central	62	41 (66.1)	21 (33.9)	22	50 (80.6)	12 (19.4)
KM	28	20 (71.4)	8 (28.6)	10	25 (89.3)	3 (11.7)
YX	5	4 (80.0)	1 (20.0)	4	4 (80)	1 (20)
CX	16	8 (50.0)	8 (50.0)	3	11 (68.8)	5 (31.2)
QJ	13	9 (69.2)	4 (30.8)	5	10 (76.9)	3 (23.1)
West	191	93 (48.7)	98 (51.3)	51	135 (70.7)	56 (29.3)
BS	102	59 (57.8)	43 (42.2)	29	70 (68.6)	32 (31.4)
DL	34	8 (23.5)	26 (76.5)	7	19 (55.9)	15 (44.1)
DH	55	26 (47.3)	29 (52.7)	15	46 (83.6)	9 (16.4)
Northwest	26	11 (42.3)	15 (57.7)	10	15 (57.7)	11 (42.3)
LJ	26	11 (42.3)	15 (57.7)	10	15 (57.7)	11 (42.3)
Southwest	35	19 (54.3)	16 (45.7)	18	27 (77.1)	8 (22.9)
LC	22	13 (59.1)	9 (40.9)	12	19 (86.4)	3 (13.6)
PE	13	6 (46.2)	7 (53.8)	6	8 (61.5)	5 (38.5)
Southeast	16	6 (37.5)	10 (62.5)	4	12 (75)	4 (25)
WS	16	6 (37.5)	10 (62.5)	4	12 (75)	4 (25)
South	42	19 (45.2)	23 (54.8)	14	25 (59.5)	17 (40.5)
BN	21	9 (42.9)	12 (57.1)	5	12 (57.1)	9 (42.9)
HH	21	10 (47.6)	11 (52.4)	9	13 (61.9)	8 (38.1)
Northeast	52	21 (40.4)	31 (59.6)	17	45 (86.5)	7 (13.5)
ZT	52	21 (40.4)	31 (59.6)	17	45 (86.5)	7 (13.5)
Rice host	424	210 (49.5)	214 (50.5)	136	309 (72.9)	115 (27.1)
GJ	276	140 (50.7)	136 (49.3)	85	200 (72.5)	76 (27.5)
XI	148	70 (47.3)	78 (52.7)	51	109 (74.3)	39 (25.7)
Non-rice host	27	8 (29.6)	19 (70.4)	6	27 (100)	0 (0)
Oryza rufipogon	18	7 (38.9)	11 (61.1)	5	18 (100)	0 (0)
Eleusine coracana	1	1 (100.0)	0 (0.0)	1	1 (100)	0 (0)
E. indica	1	0 (0.0)	1 (100.0)	0	1 (100)	0 (0)
Digitaria sanguinalis	1	0 (0.0)	1 (100.0)	0	1 (100)	0 (0)
Musa sp.	6	0 (0.0)	6 (100.0)	0	6 (100)	0 (0)
Total	451	218 (48.3)	233 (51.7)	142	336 (74.5)	115 (25.5)

No.: isolate numbers in pathogenicity assay and PCR detection of *Avr54* gene; +: numbers of isolates with PCR product of *AvrPi54* gene, percentage in parentheses; -: numbers of isolates without PCR product of *AvrPi54* gene, percentage in parentheses; No. seq: numbers of sequenced isolates for *AvrPi54* gene; A: avirulent isolate numbers in pathogenicity assay, percentage in parentheses; V: virulent isolate numbers in pathogenicity assay, percentage in parentheses; *GJ* and *XI* indicate isolates from Geng/Japonica and Xian/Indica rice production area, respectively.

Rice resistance cultivar Tetep containing *Pi54* (friendly shared by Professor Yang Sihai in Nanjing University) and susceptible control variety LTH without *Pi54* were used for pathogenicity assay. Ten rice seedlings per cultivar were planted in a plastic tray for spray inoculation. At leaf stage 3.5, 20 mL conidial suspension was sprayed and inoculated for rice seedlings. The inoculated rice plants were placed for 24 h in a controlled dark incubator at 25 °C with 95% relative humidity. Finally, these plants moved back to the greenhouse. Seven days after inoculation, the disease reactions were evaluated using a modified standard pathogenicity assay [59]. Based on the visual number and size of lesions, the disease reactions were determined at the second youngest leaf using a 5-scale rating system (0–2: resistance; 3–5: susceptible). Each experiment was repeated threefold. The pathogenicity of an isolate was evaluated by comparing the disease reaction to the resistant and susceptible varieties.

2.2. DNA Preparation, PCR Amplification, and DNA Sequencing

Fungal mycelia were quietly grown in a complete liquid medium (LB; NaCl at 10 g/L, yeast extract at 5 g/L, and tryptone at 10 g/L.) for 6 to 8 days at 25 °C under dark conditions. DNA was extracted from mycelia using the CTAB method [60]. Forward primer AvrPi54-F (5′-GGTGATAGGCTGACCAACACAAC-3′) and reverse primer AvrPi54-R (5′-GCATGGCGTGGCATGTATAGC-3′) were designed in this study according to sequences of gene accession number HF545677 (isolate Mo-nwi-55 *AvrPi54* gene) and CP050923 (*Pyricularia oryzae* strain LpKY97 chromosome 4) and specifically applied to amplify complete gene including non-CDS and CDS. PCR reaction consisted of the following components: 1 µL of fungal genomic DNA, 0.5 µL of each 10 µM primer, 12.5 µL of 2 × Pfu PCR Master Mix (Tiangen Biotech Co. LTD, Beijing, China), and 10.5 µL sterile water. PCR program was performed in a BIO-RAD Thermal Cycler (C1000, Bio-Rad Laboratories, Life Science Research, Hercules, CA, USA) with 1 cycle at 95 °C for 3 min for initial denaturation, followed by 35 cycles at 95 °C for 30 s, 65 °C for 45 s, 72 °C for 45 s, and a final extension of 72 °C for 7 min. All PCR reactions were amplified thrice. The size of the amplified fragment was estimated by DL2000 DNA Ladder (Tiangen Biotech Co. LTD, Beijing, China). AvrPi54-F, AvrPi54midF (5′-CATCGTCGGCACCGTCAC-3′), AvrPi54midF2 (5′-GATCCTCACCTATGGCTG-3′) and AvrPi54midR (5′-ACATTAGCCATTGCGTGC-3′) were used as sequencing primers. PCR products were sequenced three times by Shanghai Life Technologies Biotechnology Co., Ltd. (Shanghai, China).

The criteria for selecting sequencing isolates were as follows: (1) randomly selected isolates may cover the sampled region as far as possible in order to know the genetic variation of the gene as a whole; (2) it was ensured that no less than 3 isolates were sequenced in every sampled prefecture; (3) if isolates with the *AvrPi54* gene were very few in a prefecture, all would be sequenced.

2.3. Data Analysis

Sequences were assembled with Seqman software in lasergen7.0 [61]. Assembled sequences were aligned with ClustalW 1.81 [62]. The number of DNA haplotypes and polymorphic sites and the amount of nucleotide diversity (π) and haplotype diversity (H_d) [63,64] were calculated with the program DnaSP 5.10.01 [65]. Haplotype was determined by variable sites. Neutrality tests of Tajima's D [66] were also conducted using this program. The haplotype distribution map was constructed using ArcGIS 10.2 (Redlands, CA, USA), in which prefecture coordinates were the same as in our previous study represented by the capital coordinates of the prefecture [67]. Haplotype network analysis was performed using TCS 1.21 [68]. A phylogenetic tree was constructed using the Neighbor-Joining method in Mega 7.0 software [69]. The evolutionary distances were computed using the Maximum Composite Likelihood method. The bootstrap test (1000 replicates) was performed to obtain confidence estimates.

3. Results

3.1. The Distribution and Pathogenicity Assay of Avirulence Gene AvrPi54 in Yunnan Province

By *AvrPi54*-specific amplification, 218 (48.3%) out of 451 isolates were found to hold the *AvrPi54* alleles (Table 1). Among the 27 non-rice isolates, 7 *Ofound rufipogon* isolates and 1 *E. coracana* isolate were found to hold the *AvrPi54* alleles. *Musa* isolates, *D. sanguinalis* isolates, and *E.indica* isolates were found not to hold the *AvrPi54* alleles. Among the 424 rice isolates, 210 (49.5%) were detected to hold *AvrPi54* alleles. The detection frequency of *AvrPi54* was, respectively, 66.1, 54.3, 48.7, 42.3, 40.4, 37.5, and 28.1% in central, southeast, west, northwest, northeast, southwest and south. The frequency of *AvrPi54* was varied from 23.5 to 80% among the fourteen prefectures, and, respectively, 50.7 and 47.3% in *GJ* and *XI* regions.

According to the pathogenicity assay, 336 (74.5%) out of the 451 tested isolates were avirulent to Tetep (containing *Pi54*), while the 115 isolates (25.5%) were virulent (Table 1). All the non-rice isolates were avirulent to Tetep. Among the rice isolates, the frequency

of avirulence isolates to *Pi54* was, respectively, 86.5, 80.6, 77.1, 75.0, 70.7, 59.5, and 57.7% in northeast, central, southwest, southeast, west, south, northwest. The frequency of avirulence isolates was varied from 57.1 to 89.3% among the fourteen prefectures, and, respectively, 72.5 and 74.3% in the *GJ* and *XI* regions.

3.2. Variance, Distribution of AvrPi54 Haplotypes

Amplification products of 142 isolates, including 136 rice isolates and six non-rice isolates, were sequenced. Published *AvrPi54* gene sequence HF545677 acted as reference sequence which is 1258 bp in length. After assembled and aligned analysis, 13 variable sites were detected (Table 2), of which 11 sites were located in non-coding regions (non-CDS) and 2 sites were located in the coding region (CDS). These variable sites included one indel, nine transitions and three transversions. In total, five haplotypes (H1 to H5) based on 142 *AvrPi54* sequences were identified (Genbank accession No. OR545387-545391). Compared to 93 published *AvrPi54* gene sequences from GenBank, four novel haplotypes were identified, including H2, H3, H4 and H5.

Table 2. Frequency and variable sites of *AvrPi54* haplotypes in this study.

Haplotype	Frequency	Non-CDS			CDS					Non-CDS				
		140	141	223	619	683	732	795	841	918–935	1151	1159	1171	1180
HF545677		C	C	G	G	T	T	G	C	TGTAGATGGAGTGGTTGA	G	G	G	A
H1	132	C	C	G	G	T	T	G	C	TGTAGATGGAGTGGTTGA	G	G	G	A
H2	5	C	C	G	G	T	T	G	C	TGTAGATGGAGTGGTTGA	G	G	A	A
H3	1	C	C	G	G	T	T	G	C	TGTAGATGGAGTGGTTGA	G	C	G	A
H4	1	C	C	G	A	T	T	G	C	TGTAGATGGAGTGGTTGA	G	G	G	A
H5	3	T	T	A	G	C	C	A	A	----------	A	G	G	C

Amino acid sequences were translated according to the *AvrPi54* coding region of five haplotypes. CCN97897 as the referring protein, variable sites and three AvrPi54 protein types (P1 to P3) were gained (Table 3). P1, the same as CCN97897, was encoded and shared by H1, H2 and H3. P2 and P3 were encoded by H4 and H5, respectively. Compared to CCN97897, the 137th amino acid Valine was changed into Isoleucine in P2, and the 5th amino acid Alanine was changed into Threonine in P3. Compared to 93 published CDS sequences of AvrPi54 in GenBank, P2 was a novel encoded protein type. The five haplotype isolates were pathogenic to LTH, but not to Tetep (Figure 1). The result indicated that these haplotypes in Yunnan province still hold avirulance function.

Table 3. Protein type encoded by *AvrPi54* gene CDS in Yunnan Province.

Protein Type	Frequency	Amino Acid Sites	
		5	137
CCN97897	-	A	V
P1	140	A	V
P2	1	A	I
P3	1	T	V

H1 was identical to HF545677 and widely distributed in 14 prefectures in Yunnnan province including 127 rice isolates and 5 *O. rufipogon* isolates (Table 4, Figure 2). H2 was distributed in LJ and YX prefectures including five rice isolates. H3 was only distributed in the BN prefecture including one rice isolate. H4 was only distributed in the PE prefecture including one rice isolate. H5, including three isolates, was only distributed in the BS prefecture and *E. coracana* isolates. And H1 was a shared haplotype in the *GJ* and *XI* regions, H2 and H5 only in the *GJ* region, H3 and H4 only in the *XI* region.

Table 4. Distribution of Pyricularia oryzae *AvrPi54* haplotype.

Groups	H1	H2	H3	H4	H5	No. Samples	No. Variable Sites
Central	21	1	0	0	0	22	1
KM	10	0	0	0	0	10	0
YX	3	1	0	0	0	4	1
QJ	5	0	0	0	0	5	0
CX	3	0	0	0	0	3	0
West	49	0	0	0	2	51	9
DL	7	0	0	0	0	7	0
BS	27	0	0	0	2	29	9
DH	15	0	0	0	0	15	0
Northwest	6	4	0	0	0	10	1
LJ	6	4	0	0	0	10	1
Northeast	17	0	0	0	0	17	0
ZT	17	0	0	0	0	17	0
South	13	0	1	0	0	14	1
HH	9	0	0	0	0	9	0
BN	4	0	1	0	0	5	1
Southwest	17	0	0	1	0	18	1
LC	12	0	0	0	0	12	0
PE	5	0	0	1	0	6	1
Southeast	4	0	0	0	0	4	0
WS	4	0	0	0	0	4	0
Rice host	127	5	1	1	2	136	12
GJ	78	5	0	0	2	85	10
XI	49	0	1	1	0	51	2
Non-rice host	5	0	0	0	1	6	9
Oryza rufipogon	5	0	0	0	0	5	0
Eleusine coracana	0	0	0	0	1	1	0
Total	132	5	1	1	3	142	12

No. samples: isolate numbers in haplotype analysis; No. variable sites: mumbers of variable sites.

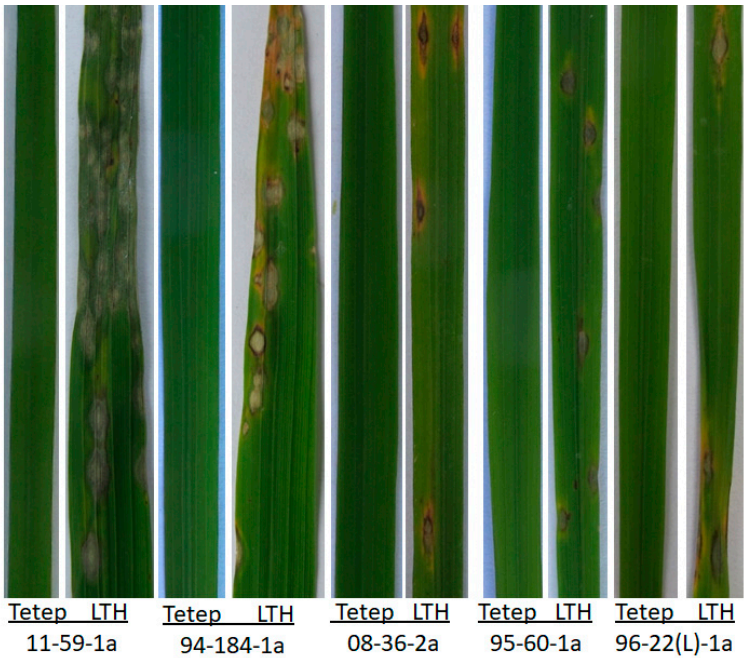

Figure 1. Disease reaction of representative isolates among 5 haplotypes to Tetep and LTH. Note: Tetep, a resistant variety to *Pi54* gene; LTH, a susceptible variety to *Pi54* gene; 11-59-1a, 94-184-1a, 08-36-2a, 95-60-1a and 96-22(L)-1a were, respectively, representative isolates of haplotypes H1 to H5.

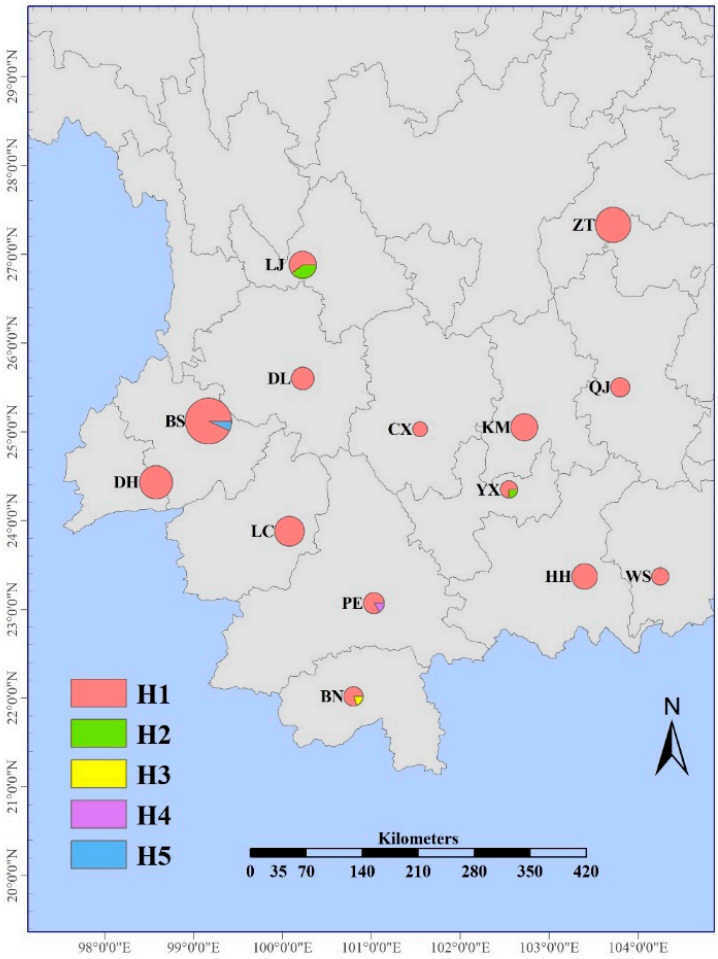

Figure 2. Distribution of *AvrPi54* haplotypes in Yunnan Province.

3.3. AvrPi54 Haplotypes Diversity and Network

AvrPi54 genetic diversity of *Pyricularia* isolates in Yunnan province was estimated in the program DnaSP 5.10.01 (Table 4). Due to the limited number of non-rice isolates, their diversity analysis was not completed. The *AvrPi54* gene diversity was low in Yunnan province (five haplotypes, $H_d = 0.127$, $\pi = 2.9 \times 10^{-4}$). The diversity of the *GJ* region (three haplotypes, $H_d = 0.156$, $\pi = 4.3 \times 10^{-4}$) was higher than that of the *XI* region (three haplotypes, $H_d = 0.078$, $\pi = 0.6 \times 10^{-4}$). There were only five regions with *AvrPi54* haplotype diversity in Yunnan rice regions, with the exception of the northeast and southeast. The value of H_d was ranked from high to low in the northwest, south, southwest, central and west of Yunnan. The value of π was arranged from high to low in the west, northwest, south, southwest, and central of Yunnan. These results indicated that there existed lower genetic diversity for *AvrPi54* in Yunnan *P. oryzae* populations.

Using DnaSP 5.10.01 software, neutrality tests were performed to determine if the genetic variation of *AvrPi54* was affected by natural selection. A negative and significant value (Tajiama's D = −2.1254 *, $p < 0.05$) was detected in the neutrality test (Table 5). The test suggested that *AvrPi54* genetic variation was mainly affected by purified selection and did not follow the neutral theory. Maybe the gene experienced the events of population expansion after the bottleneck effect.

To understand the evolutionary relationship of *AvrPi54* haplotypes in *P. oryzae*, the network of the haplotypes was constructed (Figure 3). Five haplotypes were divided into two clades. One clade consisted of haplotypes H1, H2, H3, and H4. Another clade only had one haplotype H5. H1, with a high frequency, was the ancestral haplotype, from which

H2, H3 and H4 were directly derived. Ten sites including nine substitutions and one indel varied between H1 and H5. The network also indicated that H1 and H5 separated earlier.

Table 5. Diversity and the neutrality test of Tajima's D of *Pyricularia oryzae* isolates in Yunnan rice region.

Groups	Total	No. Hap	H_d	$\pi \times 10^{-4}$	Tajima's D
Rice host	136	5	0.127	2.9	−2.1254 *
GJ	85	3	0.156	4.3	−1.92196 *
XI	51	3	0.078	0.6	−1.46227
Central	22	2	0.091	0.7	−1.1624
West	51	2	0.077	5.6	−1.8244 *
Northwest	10	2	0.533	4.2	1.30268
Northeast	17	1	0	0	-
South	14	2	0.143	1.1	−1.1552
Southwest	18	2	0.111	0.9	−1.1647
Southeast	4	1	0	0	-

No. hap: numbers of haplotypes; H_d: haplotype diversity; π: Nucleotide diversity; -: not analyzed. *: significant, $p < 0.05$.

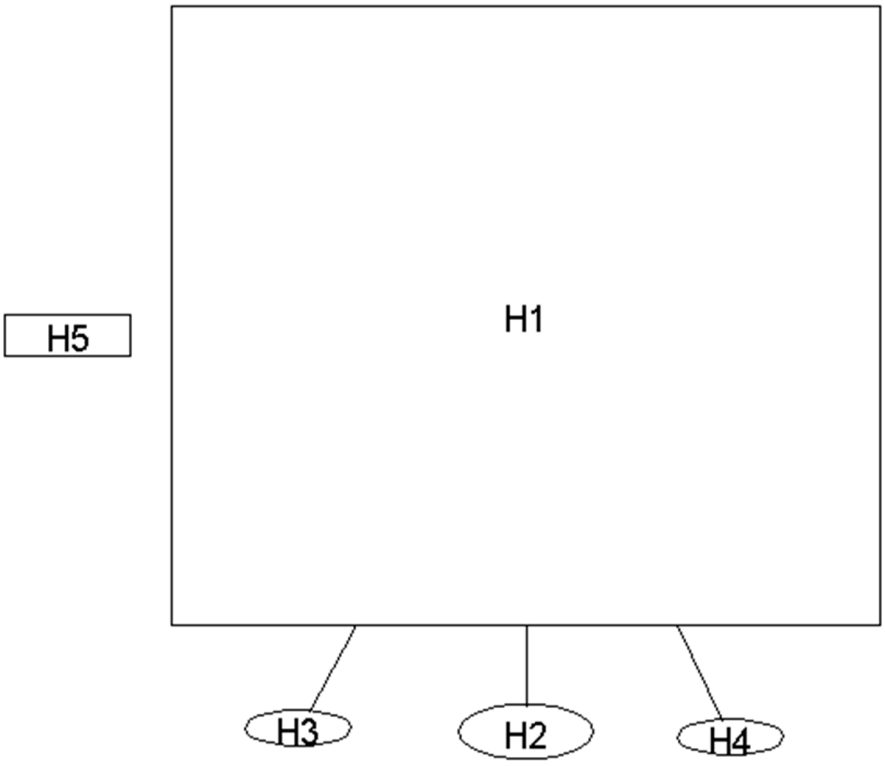

Figure 3. Network of 5 *AvrPi54* haplotypes based on 142 *Pyricularia* isolates in Yunnan province. Note: Every haplotype was separated by mutational events. Alphabet and numbers in circles indicate haplotype identity. All haplotypes are displayed as rectangles or circles. The size of the rectangles or circles corresponds to the haplotype frequency. Rectangle indicated ancestral haplotype. H1 haplotype was identical to *AvrPi54* obtained from GenBank (accession no. HF545677).

3.4. Phylogenetic Analysis of AvrPi54

Based on the *AvrPi54* gene sequences of 142 *P. oryzae* isolates, the evolutionary history was inferred using the Neighbor-Joining method in Mega 7.0 software. NJ tree was constructed under 1000 bootstrap replications (Figure 4). Isolates marked in a red circle belonged to haplotype H1 and gathered in a clade. Isolates marked in a yellow circle (belonging to H3) and marked purple circle (belonging to H4) were mixed in the clade. Isolates marked in a green circle were clustered into a clade belonging to haplotype H2.

And isolates marked in a blue circle were formed into another clade belonging to haplotype H5. According to the NJ tree, H1 was more closely related to H2, H3 and H4 than to H5. The results were consistent with the haplotype network.

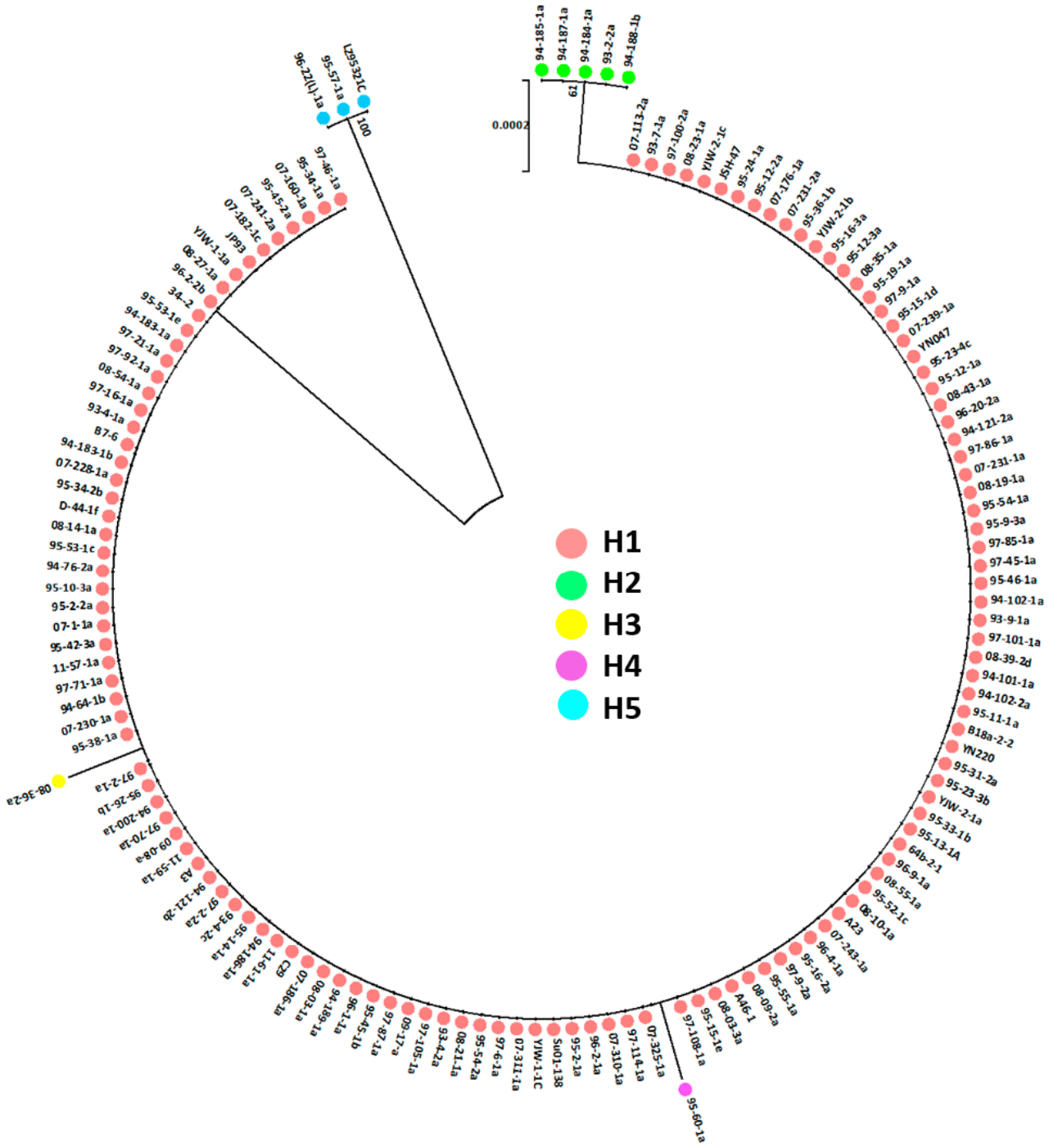

Figure 4. NJ tree for *AvrPi54* gene based on 142 *Pyricularia oryzae* isolates. Note: Numbers above branches indicate bootstrap support above 50%; Different color circles indicate different haplotypes.

4. Discussion

Understanding the distribution and pathogenicity of the *Avr* gene is useful for the efficient application of the corresponding *R* gene. In this study, the distribution of *AvrPi54* was identified through specific primer amplification. The results indicated that 48.3% of isolates of Yunnan province hold the *AvrPi54* alleles. Except for rice isolates, non-rice

isolates including *O. rufipogon* and *E. coracana* also hold the *AvrPi54* alleles. It is well known that *O. rufipogon* is the ancestral progenitor of rice (*O. sativa*). The fact that the *AvrPi54* gene was detected among isolates from *O. rufipogon* indicated that it has existed during the co-evolutionary history between *O. rufipogon* and *P. oryzae*, and it originated earlier. In the previous studies [7,70,71], *P. oryzae* is divided into several subgroups according to its restricted range of host species. Major subgroups included the *Oryza* pathotype, *Setaria* pathotype, *Panicum* pathotype, *Eleusine* pathotype and *Digitaria* pathotype. According to our result, the *AvrPi54* gene was shared between the *Oryza* pathotype and *Eleusine* pathotype. It indicated there existed some gene flow between the *Oryza* pathotype and *Eleusine* pathotype. *E. coracana* would be a potential host resource for rice blasts.

In this study, the *Pi54* gene still has some applicable value in central, southeast, west, and northeast of Yunnan province according to the distribution of *AvrPi54* and the pathogenicity assay (Table 1). According to the pathogenicity assay, 74.5% of isolates were avirulent to the resistant variety Tetep. There were two reasons for the difference between the frequency of *AvrPi54* PCR amplification and that of avirulent isolates by pathogenicity assay. One reason was that other *Avr*/*R* pairs played a role in resistance, and the other was that the primer binding site variation lead to amplification failure.

Mutations (point mutation, deletion, TE and frameshift) of the *Avr* gene may result in the loss of its avirulent functionality. These cases in *P. oryzae* have been reviewed in our previous study [67]. The sequence analysis is beneficial to understand *Avr* gene distribution, the characteristics of variation and the mining of new alleles. In this study, five haplotypes were obtained by sequencing analysis of the *AvrPi54* gene among 136 rice isolates and 6 non-rice isolates. And they were avirulent to Tetep through pathogenicity assay. The results suggested that the variance of *AvrPi54* in Yunnan did not alter its virulence. The existence of the avirulent function of *AvrPi54* is one of the causes that *Pi54* plays the role of a durable broad-spectrum resistance gene. Among the five *AvrPi54* haplotypes, H1, identical to HF545677, was not only detected among 5 *O. rufipogon* isolates but also widely distributed in 14 prefectures of seven regions in Yunnan Province including 127 rice isolates (*GJ* and *XI* rice isolates). The network of *AvrPi54* haplotypes also indicated that H2, H3 and H4 were originated from H1 (Figure 3). A similar result was found in the NJ tree (Figure 4). These results indicated that H1 was an ancestral haplotype. Four novel haplotypes (H2 to H5) of the *AvrPi54* gene detected in this study would provide some new target sites for rice blast control.

In a previous study [13], balancing selection at the *AvrPi54* locus and positive selection at *Pi54* locus were, respectively, detected in *P. oryzae* and *O. sativa* populations. *Pi54* is diversified with higher nucleotide diversity and haplotype diversity (π = 0.03164, H_d = 0.929, 40 haplotypes) among 72 rice landraces, while their counterparts, *AvrPi54,* are conserved with lower nucleotide diversity and haplotype diversity ($\pi = 4.8 \times 10^{-4}$, H_d = 0.088, three haplotypes) among 45 avirulent isolates. Trench warfare co-evolution between *Pi54* and *AvrPi54* was speculated [13]. Even though more than three times the isolates from the previous paper [13] were used in this study, a low diversity level of nucleotides and haplotypes for *AvrPi54* was still detected ($\pi = 2.9 \times 10^{-4}$, H_d = 0.127, five haplotypes). These results mean that *AvrPi54* evolves slowly and stably, and is a comparatively conserved *Avr* gene, similar to the previous study [13]. The neutrality test indicated that *AvrPi54* was affected by purified selection. Maybe *P. oryzae* population in Yunnan experienced different evolutionary events, compared to the balanced selection in a previous paper [13]. It perhaps experienced population expansion after the bottleneck effect in this study. In the network of *AvrPi54* haplotypes (Figure 3), H5 is a separated haplotype without a link to other haplotypes. A similar result was found in the NJ tree (Figure 4). The independent position of the H5 haplotype may be a relic of the bottleneck effect. Whether trench warfare is suited for co-evolution between *Pi54* and *AvrPi54* in Yunnan would be further to study the variation and evolutionary of *Pi54*.

5. Conclusions

Through our study, the distribution, variation, pathogenicity and evolution of the *AvrPi54* gene were well understood among *P. oryzae* populations in Yunnan province. In summary, *AvrPi54* is a conserved avirulent gene with a low diversity level of haplotypes in the Yunnan rice production regions. The gene's variance did not alter its avirulent function and was affected by purified selection. Four novel haplotypes (H2 to H5) of the *AvrPi54* gene detected in this study would offer new target sites for disease monitoring. These results provide useful information on *Pi54* resistance gene breeding and variety layout for rice blast control.

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