DEVELOPMENT OF MOLECULAR TOOLS FOR SIMULTANEOUS ANALYSES OF MULTIPLE KEY TRAITS IN THE CULTIVATED AND WEEDY RICE (ORYZA SATIVA L.)

Universiti Malaya

NORAIKIM BINTI MOHD HANAFIAH

FACULTY OF SCIENCE
UIVERSITI MALAYA
KUALA LUMPUR

2022

DEVELOPMENT OF MOLECULAR TOOLS FOR SIMULTANEOUS ANALYSES OF MULTIPLE KEY TRAITS IN THE CULTIVATED AND WEEDY RICE (*Oryza sativa* L.)

NORAIKIM BINTI MOHD HANAFIAH

THESIS SUBMITTED IN THE FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY / SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2022

Universiti Malaya

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION FORM

Name of Candidate: **NORAIKIM BINTI MOHD HANAFIAH**

Matric No.: **17029031/3 / SVA180026**

Name of Degree: **DOCTOR OF PHILOSOPHY**

Title of Thesis ('this Work")

DEVELOPMENT OF MOLECULAR TOOLS FOR SIMULTANEOUS ANALYSES OF MULTIPLE KEY TRAITS IN THE CULTIVATED AND WEEDY RICE (*Oryza sativa* L.)

Field of Study:

BIOTECHNOLOGY

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exits was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this work to the Universiti Malaya ('UM"), who henceforth shall be owner of copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date: 23/12/2022

Subscribed and solemnly declared before,

Witness's Signature Date: 23/12/2022

Name:

Designation:

Universiti Malaya

DEVELOPMENT OF MOLECULAR TOOLS FOR SIMULTANEOUS ANALYSES OF MULTIPLE KEY TRAITS IN THE CULTIVATED AND WEEDY RICE (*Oryza sativa* L.)

ABSTRACT

In the twenty-first century, a new paradigm of agriculture that utilises fewer resources than it did during the green revolution is required to feed the constantly expanding population while combating climate change, particularly for global major crops such as rice (*Oryza sativa* L.), which feeds more than half of the world's population. More improved rice varieties must be developed to meet changing demands, such as fragrant rice with desirable amylose content, as well as to combat the negative effects of global warming, which increases major biotic stressors such as bacterial leaf blight (BLB), sheath blight (SB), and blast. One viable option is to use weedy germplasms that have been reported to contain many valuable genes; however, the challenge is that available literature on the potential use of the genetic resources of these species in the development of climate-resilient varieties is sparse. It is also critical to develop reliable yet affordable methods to assist rice breeding programmes in resource-constrained settings, particularly in developing countries like Malaysia. Although numerous important genes have been reported for major rice diseases and quality, it remains unclear whether these genes work for local cultivated and weedy rice. The aims of this study were to synthesise previous studies on rice biology and genetics, highlighting key research gaps and future directions in rice improvement using weedy rice, and to identify reported genes and molecular markers suitable for simultaneously screening of important biotic stresses and quality traits in rice. This study used the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) protocol to conduct the systematic review, and involved the development of multiplex polymerase chain reaction (PCR)-assays to identify BLB, SB,

Universiti Malaya

and blast with fragrance and amylose content in both local cultivated and weedy rice. The amplification products were analysed using high-resolution gel and capillary electrophoresis, and the results were validated using sequencing. A total of 30 primer sets were selected from the literature to be tested, with 12 found to be functional polymorphic via uniplex PCR. Of these, six polymorphic primer sets (pTA248, RM6836, RM8225, RM202, *fgr*- SNP, and *Wx*-SSR) were chosen for the development of multiplex assays due to their clear polymorphisms and non-overlapping band sizes. After validation through sequencing, eight multiplex assays that can analyse up to four primer sets were developed in this study. High-resolution gel agarose electrophoresis was found to be less expensive (USD 0.30/sample) and more practical for screening disease resistance and quality genes in rice, particularly in laboratories with limited resources. One of the developed multiplex assays (Multiplex-7: RM8225, RM202, pTA248) was then used to screen 100 weedy rice biotypes, with 12%, 19%, and 89% of the biotypes showing resistance to SB, blast and BLB, respectively. A total of fifteen weedy rice biotypes used in molecular screening were selected for greenhouse phenotyping, and the molecular and phenotyping results were compatible for all three biotic stresses studied. This demonstrates that the multiplex assay developed in this study can be used to effectively screen all the major rice diseases in weedy rice biotypes in a single PCR reaction, which can aid in the exploration of the use of weedy rice. Overall, the findings of this project can assist rice breeding programmes in Malaysia and other rice producing countries.

Keywords: rice, disease resistance, grain quality, multiplex PCR, systematic review

Universiti Malaya

PEMBANGUNAN *MOLECULAR TOOL* UNTUK ANALISIS SERENTAK PELBAGAI CIRI UTAMA DALAM KULTIVAR PADI DAN PADI ANGIN (*Oryza sativa* L.)

Abstrak

Pada abad kedua puluh satu, paradigma baharu pertanian yang menggunakan sumber yang lebih sedikit daripada yang digunakan semasa revolusi hijau adalah diperlukan bagi memenuhi keperluan makanan populasi yang terus berkembang disamping mengatasi perubahan iklim, terutamanya untuk tanaman utama global seperti padi (*Oryza sativa* L.), yang membekalkan sumber makanan kepada lebih separuh daripada penduduk dunia. Lebih banyak varieti padi yang lebih baik mesti dibangunkan untuk memenuhi perubahan permintaan, seperti beras wangi dengan kandungan amilosa yang disukai, di samping bagi mengatasi kesan negatif pemanasan global, yang meningkatkan tekanan biotik utama seperti hawar daun bakteria (BLB), hawar pelepah (SB), dan penyakit blasta. Salah satu cara yang berdaya maju ialah dengan menggunakan germplasma padi angin yang telah dilaporkan mengandungi banyak gen berharga; namun, cabarannya ialah kajian yang ada mengenai potensi penggunaan sumber genetik spesies ini dalam pembangunan varieti tahan iklim adalah terhad. Ia juga penting untuk membangunkan kaedah yang boleh dipercayai dan berpatutan untuk membantu program pembiakan padi dalam persekitaran yang mempunyai sumber terhad, terutamanya di negara sedang membangun seperti Malaysia.Walaupun banyak gen penting telah dilaporkan untuk penyakit utama dan kualiti padi, akan tetapi ia masih tidak jelas sama ada gen ini berfungsi untuk kultivar padi tempatan dan padi angin Malaysia. Matlamat kajian ini adalah untuk mensintesis kajian terdahulu tentang biologi dan genetik padi, memfokuskan jurang penyelidikan utama dan hala tuju masa depan untuk menggunakan padi angin dalam penambahbaikan padi kultivar, dan untuk mengenal pasti gen yang dilaporkan dan penanda molekul yang sesuai untuk pemeriksaan serentak tekanan biotik dan ciri kualiti dalam nasi. Kajian ini

menggunakan protokol *Preferred Reporting Items for Systematic Reviews and Meta-Analysis* (PRISMA) untuk menjalankan kajian sistematik, dan melibatkan pembangunan ujian tindak balas berantai polimerase multipleks untuk mengenal pasti BLB, SB, dan penyakit blasta dengan aroma dan kandungan amilosa dalam kedua-dua kultivar tempatan dan padi angin. Produk amplifikasi dianalisis menggunakan gel resolusi tinggi dan elektroforesis kapilari, dapatan kajian disahkan menggunakan penjujukan. Sebanyak 30 set primer telah dipilih daripada kajian lepas untuk diuji, dengan 12 didapati polimorfik fungsian melalui PCR unipleks, dan enam set primer polimorfik ini (pTA248, RM6836, RM8225, RM202, *fgr*-SNP dan *Wx*-SSR) telah dipilih untuk pembangunan ujian multipleks kerana polimorfisme yang jelas dan saiz jalur yang tidak bertindih. Selepas pengesahan melalui penjujukan, lapan ujian multipleks yang boleh menganalisis sehingga empat set primer telah dibangunkan dalam kajian ini. Elektroforesis gel agarosa resolusi tinggi didapati lebih murah (USD0.30/sampel) dan lebih praktikal untuk menyaring rintangan penyakit dan gen kualiti dalam padi, terutamanya di makmal dengan sumber terhad. Satu daripada ujian multipleks yang dibangunkan (Multiplex-7: RM8225, RM202, pTA248) kemudiannya digunakan untuk menyaring 100 biotaip padi angin, dengan 12%, 19%, dan 89% daripada biotaip menunjukkan rintangan kepada SB, penyakit blasta dan BLB, masing-masing. Sebanyak lima belas biotaip padi angin yang digunakan dalam pemeriksaan molekul telah dipilih untuk fenotaip rumah tanaman, dan keputusan molekul dan fenotaip adalah serasi untuk ketiga-tiga tekanan biotik yang dikaji. Ini menunjukkan bahawa ujian multipleks yang dibangunkan dalam kajian ini boleh digunakan untuk menyaring dengan berkesan semua penyakit padi utama dalam biotaip padi angin dengan berkesan dalam satu tindak balas PCR,yang boleh membantu dalam penerokaan manfaat padi angin. Secara keseluruhannya, dapatan projek ini dapat membantu program pembiakan padi di Malaysia dan negara pengeluar padi yang lain.

Kata kunci: padi, rintangan penyakit, kualiti beras, PCR multipleks, kajian sistematik

Universiti Malaya

ACKNOWLEDGEMENTS

Firstly, I would like to thank my God, for letting me through all the difficulties. I have experienced Your guidance day by day. You are the one who let me finish my study. I will keep trusting and worshipping you for my entire life.

Next, I would like to express my sincere gratitude to my supervisors, Dr. Muhamad Shakirin bin Mispan, Dr. Acga Cheng and Prof. Dr. Lim Phaik Eem for their continuous guidance and advice. Their patience, motivation, enthusiasm and immerse knowledge carried me through all the stages of my research. I could not have imagined having a better mentor for my PhD study.

Besides, I would like to thank all staffs and laboratory members in Institute of Biological Sciences especially in Functional Omics and Bioprocess Development, Biotechnology laboratory as well as the Ecology and Biodiversity Laboratory.

A special thanks to my family. Words cannot express how grateful I am to my mother, father, my mother-in law, father-in-law, and my beautiful and kind sisters for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me this far. I would also like to thank my beloved husband, Muhammad Shazlan Shah. Thank you for supporting me for everything, and especially I cannot thank you enough for encouraging me throughout this experience.

Universiti Malaya

TABLE OF CONTENTS

ABSTRACT .... **iii**

ABSTRAK .... **v**

ACKNOWLEDGEMENTS .... **vii**

TABLE OF CONTENTS .... **viii**

LIST OF FIGURES .... **xii**

LIST OF TABLES .... **xix**

LIST OF SYMBOLS AND ABBREVIATIONS .... **xxi**

CHAPTER 1: INTRODUCTION **1**

1.1 Research Background 1

1.2 Problem Statement 4

1.3 Research Questions 6

1.4 Research Objectives 6

CHAPTER 2: LITERATURE REVIEW **7**

2.1 Global Food Security under a Changing Climate 7

2.2 Rice (Oryza sativa L.) 9

2.2.1 The Fundamentals of Rice 9

2.2.2 Rice Cultivation and Utilisation 11

2.2.3 Weedy Rice 13

2.3 Highlights of Rice Research since the Green Revolution 18

2.3.1 Early Green Revolution 18

2.3.2 Late Green Revolution 19

2.3.3 The 21st Century 20

2.4 Major Diseases in Rice 22

2.4.1 Bacterial Leaf Blight (BLB) 22

Universiti Malaya

2.4.2 Sheath Blight (SB) ... 24
2.4.3 Blast (B) ... 27
2.5 Major Grain Quality Traits in Rice ... 29
2.5.1 Fragrance ... 29
2.5.2 Amylose Content ... 32
2.6 Molecular Approaches for Rice Improvement ... 35
2.7 Sustainable Rice Production ... 40
CHAPTER 3: METHODOLOGY ... 42
3.1 Systematic Review ... 42
3.2 Plant Materials ... 44
3.3 Identification and Validation of Molecular Markers ... 44
3.4 Genomic DNA Extraction and Quality Check ... 45
3.5 Amplification of Targeted Genes ... 50
3.5.1 Uniplex PCR ... 50
3.5.2 Development and Optimization of Multiplex PCR ... 50
3.6 Analysis of Polymorphisms ... 51
3.6.1 Gel Electrophoresis ... 51
3.6.2 Capillary Electrophoresis and Sequencing ... 53
3.7 Genotyping of Weedy Rice Biotypes ... 53
3.8 Phenotyping for Biotic Stress ... 54
3.8.1 Blast Resistance ... 54
3.8.1.1 Inoculum Preparation of *Magnaporthe oryzae* ... 54
3.8.1.2 Inoculation of *Magnaporthe oryzae* ... 54
3.8.2 Sheath Blight (SB) Resistance ... 55
3.8.2.1 Inoculum Preparation of *Rhizoctonia solani* ... 55
3.8.2.2 Inoculation of *Rhizoctonia solani* ... 56

Universiti Malaya

3.8.3 Bacterial Leaf Blight (BLB) Resistance 57

3.8.3.1 Inoculum Preparation of *Xanthomonas oryzae* 57

3.8.3.2 Inoculation of *Xanthomonas oryzae* 58

3.9 Data Analysis 59

CHAPTER 4: RESULTS 60

4.1 Systematic Review 60

4.1.1 Initial Identification and Screening of Records 60

4.1.2 Eligibility Assessment of Selected Documents 61

4.1.3 Quantitative and Qualitative Analyses 62

4.2 Molecular Analyses 64

4.2.1 DNA Quality Check and Quantification 64

4.2.2 Uniplex PCR Analysis 65

4.2.2.1 Uniplex Genotyping for Blast Resistance 66

4.2.2.2 Uniplex Genotyping for Sheath Blight (SB) Resistance 66

4.2.2.3 Uniplex Genotyping for Bacterial Leaf Blight (BLB) Resistance……………………………………………..69

4.2.2.4 Uniplex Genotyping for Grain Quality 70

4.2.3 Multiplex PCR Analysis 71

4.2.3.1 High-resolution Agarose Multiplex Genotyping 71

4.2.3.2 Capillary Electrophoresis and Sequencing 76

4.3 Multiplex Screening of Weedy Rice Biotypes for Biotic Stresses 78

Universiti Malaya

CHAPTER 5: DISCUSSION.. 90

5.1 Rice Research Development for Food Security in the Face of Climate Change...90

5.2 Development of Multiplex PCR Assays for Screening of Important Traits in Rice…………………………………………………………………………92

5.3 Weedy Rice as Potential Genetic Resources for Rice Breeding Programme..….95

CHAPTER 6: CONCLUSION 100

6.1 Conclusion 100

6.2 Recommendations for Future Research 101

REFERENCES 102

LIST OF PUBLICATIONS AND PAPERS PRESENTED 128

APPENDICES 129

Universiti Malaya

LIST OF FIGURES

Figure 2.1 : Image of weedy rice (red circle) and cultivated rice (black circle) in field at Tanjung Karang, Selangor………………......... 17

Figure 2.2 : Lesion caused by bacterial blight (Source:https:// http://www.knowledgebank.irri.org/decision-tools/rice-doctor/rice-doctor-fact-sheets/item/bacterial-blight)…............. 23

Figure 2.3 : Lesion caused by sheath blight (Source: https:// http://www.knowledgebank.irri.org/training/fact-sheets/pest-management/diseases/item/sheath-blight)……......................... 25

Figure 2.4 : Lesion caused by blast disease (Source: https:// http://www.knowledgebank.irri.org/training/fact-sheets/pest-management/diseases/item/blast-leaf-collar)........................... 27

Figure 2.5 : The location of four primers, EAP, ESP, IFAP and INSP used to identify fragrance characteristics in rice………..................... 31

Figure 2.6 : A) Amylose structure, (B) amylopectin structure. Source: Martin & Smith, 1995 ………………………………………… 33

Figure 3.1 : General flow of systematic review of literature search from identification, screening, eligibility and included based on PRISMA flow diagram. The flow was adapted from https://www.prisma-statement.org accessed on February 2020………………………………………………………… 43

Figure 3.2 : Overview of gel electrophoresis-based using three different primers. Individual DNA sample were screened with single primer set per reaction as Uniplex (A) and multiplex (B) PCR………………………………….............................. 52

Figure 3.3 : Inoculum preparation of *Magnaporthe oryzae* for rice blast pathogenicity screening. A) Washing off mycelia in sterile water for spore suspension and B) using a haemocytometer to adjust to a concentration of 1 X 10^5 spores per ml under light microscope before inoculation…………………..…………… 55

Figure 3.4 : Bottle microchambers used after inoculation of *Rhizoctonia solani* for sheath blight pathogenicity screening……………… 57

Figure 3.5 : Inoculation of *Xanthomonas oryzae* v. *oryzae* (Xoo) to weedy rice plants to screen bacterial leaf blight pathogenicity. A) Dipping scissor tips into the Xoo suspension; B) Cutting the leaf tip away from the leaf……………………………………. 58

Figure 4.1 : Number of articles (n) at each stage of PRISMA analysis……. 61

Figure 4.2 : The major focus themes of research articles obtained………… 63

Universiti Malaya

Figure 4.3 : Major milestones in rice research since green revolution. Source: Mohd Hanafiah et al. (2020)……………………....... 63

Figure 4.4 : Plausible strategies to achieve sustainable rice production. Source: Mohd Hanafiah et al. (2020)….…………………… 64

Figure 4.5 : Genomic DNA samples separated using 1% agarose gel electrophoresis at 100 V for 1 h. Lane 1: 1 kb DNA ladder, Lanes 2 -15: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3………………............. 65

Figure 4.6 : Amplified RM6836 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: Mahsuri Mutant, MR219, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………………………………….. 67

Figure 4.7 : Amplified RM8225 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: Mahsuri Mutant, MR219, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………………………………….. 67

Figure 4.8 : Amplified RM202 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: Pulut Hitam 9, MR219, Mahsuri Mutant, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………………………………….. 67

Figure 4.9 : Amplified pTA248 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: MR219, Ria, Mahsuri Mutant, Pulut Hitam 9, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………………………………….. 70

Figure 4.10 : Amplified *fgr*-SNP products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: MRQ74, MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3……………………………………………………………….. 70

Figure 4.11 : Amplified *Wx*-SSR products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106,

Universiti Malaya

MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3……………………………………………………….. 71

Figure 4.12 : (Multiplex-1): Amplified multiplex *Wx*-SSR and *fgr*-SNP products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with amylose content [~110 bp (>25% amylose); ~130 bp (<25% amylose)] and fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MRQ74, MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3……………….............. 73

Figure 4.13 : (Multiplex-2): Amplified multiplex *Wx*-SSR and pTA248 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with amylose content [~110 bp (>25% amylose); ~130 bp (<25% amylose)] and bacterial leaf blight resistance [~600 bp (susceptible); ~700 bp (resistant)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…..……………………………………………………… 73

Figure 4.14 : (Mutiplex-3): Amplified multiplex *fgr*-SNP and pTA248 products separated using 4% high-resolution agarose gel electrophoresis at 120 V fr 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and bacterial leaf blight resistance [~600 bp (susceptible); ~700 bp (resistant)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Ria, MRQ74, Mahsuri Mutant, Pulut Hitam 9, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3……………………….......... 74

Figure 4.15 : (Multiplex-4): Amplified multiplex *fgr*-SNP and RM8225 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and blast resistance [~200 bp (resistant); ~220 bp (susceptible)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MRQ74, MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………………… 74

Figure 4.16 : (Multiplex-5): Amplified multiplex *fgr*-SNP and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and sheath blight resistance [~160 bp (resistant); ~180 bp (susceptible)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Pulut Hitam 9, MRQ74, Mahsuri Mutant, Ria,

Universiti Malaya

MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3………………..................... 75

Figure 4.17 : (Multiplex-6): Amplified multiplex pTA248, RM6836 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~250 bp (susceptible); ~230 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………….. 75

Figure 4.18 : (Multiplex-7): Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3…………………………….. 76

Figure 4.19 : (Multiplex-8): Amplified multiplex *Wx*-SSR, RM202, RM8225 and *fgr*-SNP products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and sheath blight resistance [~160 bp (resistant); ~180 bp (susceptible)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MRQ74, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3……………………………………………………………….. 76

Figure 4.20 : Electrophenogram results from capillary electrophoresis for local checks (A) MR219, (B) Mahsuri Mutant and (C) Pulut Hitam 9. The primer peaks of RM6836 and RM202 were presented as HEX (green) and NED (black), respectively. The red peaks represent internal size standard 500-ROX. The x- and y-axis represent size (bp) of the PCR products in bases and relative fluorescence intensity, respectively………………….... 77

Figure 4.21 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR1, WR03,

Universiti Malaya

WR05, WR06, WR09, WR10, WR11, WR12, WR13, WR15, WR17, WR19, WR02, WR04…………………………………. 80

Figure 4.22 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR20, WR21, WR22, WR23, WR24, WR25, WR26, WR27, WR28, WR29, WR30, WR31, WR07, WR16………………………… 81

Figure 4.23 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR33, WR34, WR36, WR37, WR38, WR39, WR40, WR41, WR42, WR43, WR44, WR45, WR32, WR35………………………… 81

Figure 4.24 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 19: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR47, WR50, WR51, WR52, WR53, WR54, WR55, WR56, WR57, WR58, WR48, WR49, WR59…………………………………. 82

Figure 4.25 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR60, WR61, WR62, WR63, WR64, WR65, WR67, WR68, WR69, WR70, WR72, WR73, WR66, WR71……………………….. 82

Figure 4.26 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp

Universiti Malaya

(susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR74, WR75, WR76, WR77, WR78, WR79, WR80, WR81, WR83, WR85, WR86, WR84, WR87………………………………… 83

Figure 4.27 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 19: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR88, WR92, WR93, WR94, WR96, WR97, WR98, WR99, WR100, WR89, WR90, WR91, WR95………………………………… 83

Figure 4.28 : Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 11: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR8, WR14, WR18, WR46, WR82……………………………………… 84

Figure 4.29 : Frequency distribution of weedy rice biotypes associated with major rice diseases in Malaysia. Resistant and susceptible genotypes were determined based on molecular screening…… 85

Figure 4.30 : Allelic frequency of weedy rice samples for markers associated with major rice diseases in Malaysia………………………….. 86

Figure 4.31 : Symptoms after phenotyping for biotic stresses and disease scoring scale on selected weedy rice samples for (A) sheath blight, (B) blast, and (C) bacterial leaf blight…...................... 88

Figure 4.32 : Disease score for selected weedy rice samples for sheath blight (blue), rice blast (orange), and bacterial leaf blight (grey) diseases. Predetermined susceptible (S) and resistant (R) weedy rice based on genotype scoring were indicated above the bar…………………………………………………………... 88

Figure 4.33 : Principal coordinate analysis (PcoA) showing the genetic distances between weedy rice samples from different locations and selected cultivated rice. Weedy rice biotypes from Pekan (Pahang), Sungai Burung (Selangor), Sungai Leman (Selangor), Sungai Besar (Selangor), and Seberang Perak (Perak) are represented by fonts in red, blue, purple, green, and black, while cultivated rice are represented by font in yellow.

Universiti Malaya

Weedy rice lines marked with an asterisk have heterozygosity in at least one of the loci………………………………………. 89

Figure 5.1 : Observations in field showed weedy rice(s) in bacterial leaf blight (BLB) infected fields possessed various degrees of infection from A) highly susceptible to B) strong resistance to BLB. This indicates rich genetic diversity in weedy rice as a potential to provide useful genetic resources for rice breeding programme……………………... 96

Universiti Malaya

LIST OF TABLES

Table 2.1 : Global rice production environments, climate and major producing areas……………………………………………...... 12

Table 2.2 : Examples of important genes linked to biotic and abiotic stresses in weedy rice……………………………………….. 17

Table 2.3 : Examples of linked marker for BLB resistance in rice…........ 24

Table 2.4 : Examples of reported QTLs for sheath blight resistance in rice…………………………………………………….…...... 26

Table 2.5 : Examples of reported blast resistance genes in rice………… 28

Table 2.6 : Comparative characteristics of various widely used molecular marker systems for plant genome analyses………………….. 39

Table 3.1 Details of the selected genes and primers…………….......... 46

Table 3.2 Local checks and expected size of PCR products…………… 50

Table 3.3 : Details on multiplex assays developed……………………....... 51

Table 4.1 : Criteria list for inclusion and exclusion of selected articles in eligibility assessment. Source: Salleh et al. (2020)………….. 62

Table 4.2 : Purity readings of extracted DNA samples of selected rice varieties…………………………………………………….... 65

Table 4.3 : Uniplex PCR product sizes for molecular markers linked to blast resistance based on agarose gel image.………………... 66

Table 4.4 : Uniplex PCR product sizes for molecular markers linked to sheath blight (SB) resistance……………………………........... 68

Table 4.5 : Uniplex PCR product sizes for molecular markers linked to bacterial leaf blight (BLB) resistance……………………….. 69

Table 4.6 : Description of multiplex results of fourteen selected local rice varieties…………………………………………………..... 72

Table 4.7 : Comparison of capillary electrophoresis and sequencing results to determine resistant (R) or susceptible (S) response of RM6836 and RM202 towards blast and sheath blight (SB), respectively………………………………………………….. 78

Table 4.8 : Description of multiplex results of 100 weedy rice biotypes (ID) and its association with sheath blight (SB), blast and bacterial leaf blight (BLB). Molecular size representing susceptible, resistant and heterozygous was labelled as S, R and H, respectively……………………………………......... 79

Universiti Malaya

Table 4.9 : Genetic diversity of weedy rice samples based on three (3) loci associated with sheath blight (SB), rice blast, and bacterial leaf blight (BLB) diseases at five (5) different rice fields………………………………………………………… 87

Universiti Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

bp	:	base pair
CO^2	:	carbon dioxide
^oC	:	degree Celsius
µl	:	micro litre
min	:	minute
KOH	:	potassium hydroxide
s	:	second
AI	:	artificial intelligence
ANOVA	:	analysis of variance
ASP	:	allele-specific primer
BLB	:	bacterial leaf blight
CRISPR	:	clustered regularly interspaced short palindromic repeats
DNA	:	deoxyribonucleic acid
DSI	:	Disease Scoring Index
EAP	:	external antisense primer
ESP	:	external sense primer
GBSS	:	granule - bound starch synthase
GFSI	:	Global Food Security Index
GWAS	:	genome-wide association studies
IDT	:	Integrated DNA Technologies
IFAP	:	internal fragrant antisense primer
INSP	:	internal nonfragrant sense primer
IoT	:	Internet of Things

Universiti Malaya

IRRI	:	International Rice Research Institute
MAS	:	marker-assisted selection
NA	:	nutrient agar
NGS	:	next-generation sequencing
MARDI	:	Malaysian Agricultural Research and Development Institute
mRNA	:	messenger ribonucleic acid
NCBI	:	National Center for Biotechnology Information
NPK	:	nitrogen, phosphorus and potassium
PCoA	:	principal coordinate analysis
PCR	:	polymerase chain reaction
PDA	:	potato dextrose agar
PRISMA	:	Preferred Reporting Items for Systematic Reviews and Meta-analysis
QTL	:	quantitative trait loci
RBI	:	Rice Bowl Index
SB	:	sheath blight
SDGs	:	Sustainable Development Goals
SES	:	Standard Evaluation System
SNP	:	single nucleotide polymorphisms
SRI	:	system of rice intensification
SSL	:	self-sufficiency level
SSR	:	simple sequence repeat
TALEN	:	transcription activator-like effector nuclease
UAVs	:	unmanned aerial vehicles
ZFN	:	zinc finger nuclease

Universiti Malaya

LIST OF APPENDICES

Appendix A : Uniplex gel images for molecular markers linked to blast resistance.. 129

Appendix B : Uniplex gel images for molecular markers linked to sheath blight resistance... 131

Appendix C : Uniplex gel images for molecular markers linked to bacterial leaf blight.. 134

Appendix D : Sequencing results.. 137

Universiti Malaya

CHAPTER 1: INTRODUCTION

1.1 Research Background

The true grass family Poaceae (or Gramineae) has long been considered as the most economically important plant family for food production. It comprises of more than 10,000 species, including the global big three cereals: wheat (*Triticum aestivum*), maize (*Zea mays*), and rice (*Oryza sativa*) (Gibson, 2009). For hundreds of years, subsequent polyploidy events have driven the survival of many major cereal crops, including rice, which played a significant historical role in its development. However, rapid climate change poses an urgent threat to the production of these crops worldwide (Cheng et al., 2022). Rice, with over 40,000 distinct varieties grown on every continent except Antarctica (Abraham et al., 2016; Khush, 1997), is a dependable staple for more than half of the entire world's population. This includes about 550 million undernourished people living in Asia (Food and Agriculture Organization [FAO], 2018). Presently, approximately 90% of the global rice is both produced and consumed in Asia, predominantly in China and India (Bandumula, 2017; Schneider & Asch, 2020). This important crop is a critical source of income for more than 200 million households in developing countries, being the central theme of many local economic development programmes (Khazanah Research Institute, 2019; Muthayya et al., 2014).

Rice is the main primary source of nutrition, particularly carbohydrates, for most of the Asian countries, including Malaysia (Firdaus et al., 2020). It has been reported that the average annual rice consumption per capita in Malaysia was 80 kg in 2016, which recorded about 26% of daily total calorie intake (Khazanah Research Institute, 2019). However, local rice production and harvested areas have remained relatively constant since the 1990s compared to other Asian countries such as Indonesia, Vietnam, Thailand, and the Philippines (Khazanah Research Institute, 2019). Besides, Malaysia has not

Universiti Malaya

achieved a 100% self-sufficiency level (Arshad et al., 2011; Rahim et al., 2017) and imported approximately 30% of rice mostly from Thailand, Vietnam, and Pakistan (Adnan & Nordin, 2020; Khazanah Research Institute, 2019). Decreased water resources and the emergence of new diseases are among the major challenges to increasing rice production globally (Hasan, 2015; Mohd Hanafiah et al., 2020; Sethuraman et al., 2021).

Generally, rice can be grown in a wide range of agro-climatic conditions, and its production is influenced largely by many biotic (such as blast disease) and abiotic stresses (such as salinity) (Miah et al., 2017; Sahila et al., 2022). The significant changes in climatic conditions, which are the main cause of biotic and abiotic stresses pose a severe threat to sustainable crop production (Raza et al., 2019; Shahzad et al., 2021). According to Fukuoka et al. (2015), stable food production can be achieved by enhancing disease resistance in crops. The fact that the global demand for rice is increasing while its production per capita is decreasing makes it crucial for researchers to look further for critical ways to improve the crop (Mohd Hanafiah et al., 2020).

Additionally, the current rice industry emphasises on the development of high-quality rice as it greatly affects palatability and consumer acceptability. One of the major rice quality characteristics is fragrance (or aroma) (Cheng et al., 2017; Hu et al., 2020). Special, pleasant fragrant rice such as the Basmati rice is preferred by many consumers and possesses great market in Asia for decades, although it often costs higher than the non-fragrant rice (Hu et al., 2020). Amylose content, which has a significant influence on the cooking and eating quality of rice, is another major grain quality feature that has been consistently improved to meet changing customer demand (Cheng et al., 2012; Hu et al., 2021).

Globally, one notable challenge in rice production is the presence of weedy rice (Mahmod et al., 2021; Sun et al., 2013). However, several previous studies showed that weedy rice has novel sources of resistance gene to common rice diseases such as bacterial

Universiti Malaya

leaf blight (BLB) (Nadir et al., 2017), sheath blight (SB) (Jia et al., 2022), and blast (Jia & Gealy, 2018) caused by the pathogens *Xanthomonas oryzae*, *Rhizoctonia solani,* and *Magnaporthe oryzae*, respectively. The biodiversity of weedy rice poses a challenge for rice production, but it also provides an opportunity for comprehensive biological and genetic studies of adaptive traits which have been lost due to rice cultivation (Jia & Gealy, 2018; Zhu et al., 2021). Evolved as an intermediate between wild and rice cultivars, weedy rice typically exhibits high resource competitiveness against cultivated rice. Thus, its competitive ability and adaptive evolutionary traits such as stress tolerance, increased seed dispersal, and dormancy may be useful in optimising resource utilisation and rice yield in the current climate instability (Mohd Hanafiah et al., 2020). One of the viable ways of incorporating resilience into modern rice with narrow genetic backgrounds is by introgressing alleles of its weedy and wild germplasms that harbor novel genes responsive biotic and abiotic stresses (Mohd Hanafiah et al., 2020).

Over the past few decades, molecular techniques are among the advanced approaches used to improve crop yields and other important traits such as disease resistance (Bigini et al., 2021; Das et al., 2017). The completion of the rice genome sequence has contributed to significant advancements in its genetics and breeding and paved the way for the sequencing of more complicated crop genomes such as wheat and maize (Jackson, 2016). One of the rapid yet reliable techniques to improve crops is multiplex polymerase chain reaction (PCR), a variant of PCR which utilises two or more pairs of specific primers in a single PCR system to amplify multiple target genes/loci simultaneously (Cheng et al., 2015; Mohd Hanafiah et al., 2021). Many past studies have demonstrated that multiplex PCR could be a cost-effective and efficient assay for pyramiding genes in rice breeding programmes. Examples include the detection of BLB resistance genes (Hajira et al., 2016; Meenakshi & Salgotra, 2020; Salgotra et al., 2011; Yap et al., 2016), BLB resistance and fragrance genes (Salgotra et al., 2011), and bacterial pathogens that

Universiti Malaya

cause important diseases on rice (Bangratz et al., 2020; Cui et al., 2016). Nonetheless, this molecular tool has not been applied to screen weedy rice biotypes, especially those in Malaysia.

To aid rice breeding programmes in resource-limited settings, particularly in developing countries like Malaysia, it is vital to establish the means to effectively screen the targeted traits at the lowest possible cost (Cobb et al., 2019; Delannay et al., 2012). Although many important genes and markers have been reported for major rice quality and diseases, it remains unclear whether these genes and markers work for Malaysian cultivated and weedy rice. Additionally, the available literature on the potential utilisation of weedy rice in the development of climate-resilient varieties are relatively scarce (Mohd Hanafiah et al., 2020). The present study sought to synthesise the past research on rice biology and genetics, highlighting the main research gaps and future directions in rice improvement, including the utilisation of weedy rice. Additionally, this study also aimed to identify reported genes and molecular markers that are suitable for simultaneously screening of BLB, SB, and blast with fragrance and amylose content in local cultivated and weedy rice through multiplex PCR assays. The results obtained from this project can assist the rice breeding programmes in Malaysia, and possibly beyond.

1.2 Problem Statement

To meet the ever-increasing and changing demand for rice, many researchers worldwide have been working on developing more resilient and palatable varieties. Although cultivated rice can be grown in a wide range of agro-climatic conditions, climate change can increase stressors such as biotic stresses, posing a serious threat to its production (Bokor et al., 2021; Raza et al., 2019). As a result, researchers must develop innovative yet dependable strategies to improve rice, particularly as global demand rises

Universiti Malaya

while production per capita falls (Mohd Hanafiah et al., 2020, 2021). The current rice industry also prioritises high-quality rice production, including rice with desirable fragrance and amylose content, which has a significant impact on palatability and consumer acceptance. At present, Malaysia has not accomplished 100% self-sufficiency level and imported about 30% of rice (Adnan & Nordin, 2020; Khazanah Research Institute, 2019). Local rice production has been impacted by several major biotic stresses, including blast, SB, and BLB diseases. These diseases have been reported to significantly reduce rice yield and quality (Bao et al., 2021; Chukwu et al., 2019a; NurulNahar et al., 2020).

Weedy rice (*O. sativa* f. *spontanea*) is one of major weeds affecting many major rice growing areas in the world (Bzour et al., 2018; Mahmod et al., 2021). Interestingly, several recent studies suggested that weedy rice could have novel genetic mechanisms that can prevent diseases, and their genetic information could be useful for the improvement of cultivated rice (Jia et al., 2018; Nadir et al., 2017). Nonetheless, the potential benefits and utilistaion of weedy rice, particularly the biotypes in Malaysia, remain unclear (Mohd Hanafiah et al., 2020). Hence, it is vital to identify the knowledge gaps and future directions of rice research, especially in adopting potential weedy rice biotypes in breeding programmes. It is worth noting that rice researchers these days often aim to improve multiple key traits (such as agronomic, quality, and disease resistance) within a single breeding programme that involves a large number of samples (Ashkani et al., 2015; Mohd Hanafiah et al., 2021). Therefore, it is critical to develop reliable and cost-effective screening tools capable of screening multiple targeted traits in both cultivated and weedy rice, particularly for breeders in developing or less-developed countries with limited resources.

Universiti Malaya

1.3 Research Questions

1. What are the current gaps and future directions in rice research, and how can weedy rice be used in modern rice breeding programmes?
2. Are the reported molecular markers associated with disease resistance (bacterial leaf blight, sheath blight, and blast) genes in the public domain suitable to be utilised in local rice?
3. Which reported molecular markers underpinning the important disease resistance and grain quality (fragrance and amylose content) traits in rice can be multiplexed and analysed simultaneously in a single PCR reaction?
4. What are the promising traits in weedy rice biotypes that can be used to develop commercial rice with better resistance to biotic stresses?

1.4 Research Objectives

1. To conduct a systematic review on the critical research gaps and future directions in rice research, as well as the potential utilisation of weedy rice in modern rice breeding programmes.
2. To identify and validate available molecular markers closely linked to genes that bestow resistance to major biotic stresses, inclusive of bacterial leaf blight, sheath blight, and blast in local rice.
3. To develop reliable and cost-effective multiplex PCR assays to simultaneously analyse major disease resistance and grain quality traits, including fragrance and amylose content, in rice.
4. To screen putative genes associated with biotic stresses in local weedy rice biotypes using multiplex PCR assays.

Universiti Malaya

CHAPTER 2: LITERATURE REVIEW

2.1 Global Food Security under a Changing Climate

Food security is defined as a condition in which people continuously have sufficient physical and economic access to safe and nutritious food to meet their dietary needs and preferences for a healthy life (Cheng et al., 2019; World Food Summit, 1996). One of the major challenges in the 21st century is to sustain global food and nutrition security (Mohd Hanafiah et al., 2020). The primary effect of food insecurity is malnourishment, which leads to shortages, excesses or imbalances in the energy and/or nutritional intake of an individual (Mbow et al., 2019; Sabi, 2021). By 2050, the world's population is expected to reach 9.8 billion people, with approximately 27% (2.1 billion) more than the current population (Marzi et al., 2021; Searchinger et al., 2019; United Nations Department of Economic and Social Affairs, 2017). As the global population continues to grow with rising incomes, the demand for quality food is increasing and consumer preferences are rapidly changing (Christiaensen et al., 2021; Ortega & Tschirley, 2017). In Malaysia, rice is considered as the most important crop under the food subsector as it is the staple food for majority of the nation and also the primary source of income and livelihood for about 40% of farmers (Firdaus et al., 2020).

Climate change, which can affect both temperature and precipitation (Fedoroff et al., 2010), will bring a variety of negative impacts on all four pillars of food security that include availability, access, utilisation, and stability (Clapp et al., 2021; Mbow et al., 2019). Global warming is putting more pressure on some already daunting challenges in food production (Nelson et al., 2010), mainly through its effect on annual crop yields (Poudel & Shaw, 2016). For example, every one-degree rise in temperature will contribute to approximately 10% reduction in rice yields in Indochinese Peninsula (J. Z. Li et al., 2017). The average global temperatures has risen 1°C above pre-industrial levels

Universiti Malaya

and, at current rates of warming, are projected to reach 1.5°C within two decades (Intergovernmental Panel on Climate Change [IPCC], 2018).

Apart from population increase and climate change, arable land and water shortages also have significant consequences for agriculture's capacity to satisfy the demands for grain, feed, fiber, and fuel while reducing the environmental effects of its cultivation (Fedoroff et al., 2010; Ghosh et al., 2020; Popp et al., 2014). With a growing global population, global demand for food, feed, fibre, and fuel is expected to rise steadily for the next three to four decades, forcing farmers to significantly increase food grain production (Ghosh et al., 2020). The strategy to reach a more sustainable future for all or better known collectively as Sustainable Development Goals (SDGs) was created by the United Nations in 2015 as an overarching call for action to protect the planet and put an end to poverty and ensure that people live in peace and prosperity. It is important to point out that agriculture, which is the world's largest consumer of natural resources (such as water and land), plays a direct role in reaching some of the 17 established SDGs, particularly with respect to water, biodiversity, climate change, poverty, sustainable energy and urban areas (Keesstra et al., 2016; Sethuraman et al., 2021).

Besides food availability that determines the self-sufficiency level (SSL), other aspects that are equally valuable to measure national food security include sustainability of the environment, food safety, and affordability (Harun et al., 2021; Khazanah Research Institute, 2019). Take rice, for example, Malaysia has recorded a higher Global Food Security Index (GFSI) and Rice Bowl Index (RBI) compared to some rice exporting countries such as Thailand and Vietnam, indicating that the country has better addressed other food security pillars, including access, utilisation and stability (Cheng, 2018; FAO et al., 2017; Khazanah Research Institute, 2019).

Since the 1970s, the multiple components of the green revolution, including the cultivation of high-yielding crop varieties with the increased use of fertiliser and

Universiti Malaya

irrigation, have been contributing to increased cereal output, mainly wheat, maize and rice (Qaim, 2020; Sheehy & Mitchell, 2011). While the production of major cereals has steadily increased since the green revolution, the yields of many developed varieties have been stagnant, largely due to biotic and abiotic stresses (Ladha et al., 2021). In the current era, a new model of green agriculture, where less resources are used for growing crops, is needed to feed the rising population in the midst of climate change (Mohd Hanafiah et al., 2020).

2.2 Rice (*Oryza sativa* L.)

2.2.1 The Fundamentals of Rice

Rice, a species belonging to the family Poaceae (or Gramineae), is one of the most important cereal crops, being the staple food for more than 50% of the world's 7.8 billion inhabitants (Kumagai et al., 2010; Nisar et al., 2018). It is considered one of the big three global cereals, along with wheat (*Triticum aestivum*) and maize (*Zea mays*) (Ajala & Gana, 2015; Mohd Hanafiah et al., 2020). Grown in every region worldwide except Antarctica (Fukagawa & Ziska, 2019; Khush, 1997; Ziska et al., 2012), rice has been first domesticated in China since 10,000 years ago (Awika, 2011). Two cultivated rice species include *O. sativa* and *O. glaberrima*, which are the most cultivated and African cultivated species, respectively (Roma-Burgos et al., 2021). Ecologically, rice can be categorised into three varieties: long-series *indica* (grown in Tropical and Subtropical Asia); short or medium-grained *japonica* (grown in temperate regions such as Japan and northern China); and medium-grained *javanica* rice (grown in the Philippines, Madagascar, and Indonesia mountainous areas) (Muthayya et al., 2014). To meet the future demand, rice production is required to increase by 0.6-0.9% annually by 2050 (Biswas et al., 2021; Jagadish et al., 2010; Mohapatra et al., 2017).

Universiti Malaya

With a genome size of approximately 430 Mb, rice is the first crop to be fully sequenced in the mid-2000s. It is an important biological model for crop research due to its small genome size (Y. Li et al., 2018; H. Yu et al., 2021). The genus *Oryza* is classified into five genetical groups, including *O. sativa* complex (AA), *O. officianalis* complex (BB, CC, EE, BBCC and CCDD), *O. ridleyi* complex (HHJJ) and *O. granulata* complex (FF) (Kumagai et al., 2010). Rice is a diploid species with 12 chromosomes (2n=24) (Barbhuiya, 2017). Asian rice has a diploid AA genome that has a relatively high sexual compatibility and perfect chromosome pairing in meiosis. Two progenitors of *O. sativa*, which include *O. rufipogon* and *O. nivara*, are perennial and annual, respectively. Rice gene pool can be divided into three classes: primary, secondary, and tertiary. The primary gene pool consists of Asian cultivated rice, weedy rice (*O. sativa* f. *spontanea*), ancestor wild species (*O. rufipogon* and *O. nivara*), and other AA-genome variant species. On the other hand, the secondary gene pool encompasses other non-AA-genome species, and the tertiary gene pool consists of species from other genera in the tribe Oryzeae (Lu & Snow, 2005).

The nutrient composition of rice depends on several factors, including the nature of the soil and the environments in which it grows, agricultural practices, and processing practices such as parboiling and milling, post-harvest conditions, and handling (Kennedy et al., 2003; Rohman et al., 2014). Rice is a great source of thiamine (vitamin B1), riboflavine (vitamin B2), and niacin (vitamin B3) (Rohman et al., 2014). However, there are little to no vitamin C, D, and A in rice (Kennedy et al., 2003). Biofortification is one of the strategies used to alleviate micronutrients deficiency in rice (Kiran et al., 2022; Rhowell Jr. et al., 2021). One of the most remarkable examples is the development of beta-carotene-producing golden rice that provides good source of vitamin A (Tu et al., 2000). It was only after about two decades that this developed transgenic biofortified crop received approval of food safety from several countries (such as Australia, New Zealand,

Universiti Malaya

and the United States) for human consumption and animal feed (Cornell Alliance for Science, 2018; International Service for the Acquisition of Agri-biotech Applications [ISAAA], 2019).

2.2.2 Rice Cultivation and Utilisation

It is estimated that more than 90% of rice is produced and consumed in Asia, although nearly half a billion people living in this region are still undernourished (FAO et al., 2019; Muthayya et al., 2014). Asian rice production will most likely decide the future trend of global rice production, with India and China being the world's largest rice growing area and producer, respectively (Gadal et al., 2019). These two countries account for approximately 50% of the rice produced and consumed globally (Muthayya et al., 2014). In some Asian countries, for example Cambodia, up to 90% of agricultural land is used for rice production (Stuart et al., 2020). A total of 17 Asian and Pacific nations, nine North and South America and eight countries in Africa depend predominantly on rice as staple food (Gadal et al., 2019).

Rice cultivation dominates the regions of Asia due mainly to their suitable tropical climate and hydrological regimes. Rice is vulnerable to water deficits because it is commonly cultivated under flood conditions where large amounts of fresh water or sufficient rainfall must be available (Awika, 2011). Rice can be grown in a wide range of solar radiation, from a potential of 25% in the main rice season in Myanmar, Thailand, and India up to a potential of 95% in southern Egypt and Sudan (Gadal et al., 2019; Global Rice Science Partnership [GRISP], 2003). A conference conducted by the International Rice Research (IRRI) in 1982 had developed a global classification system for rice growing environments to avoid confusion and uncertainty in identifying different rice environments, as shown in Table 2.1 (Gadal et al., 2019; Khush, 1984).

Universiti Malaya

Table 2.1: Global rice production environments, climate and major producing areas

Main categories	Subcategories	Climate	Major producing areas
Irrigated	▪ Irrigated with favourable temperature ▪ Irrigated, low temperature, tropical zone ▪ Irrigated, low-temperature, temperate zone	Tropics, subtropics, and temperate	Malaysia, Indonesia, Sri Lanka, Vietnam, the Philippines, south-eastern India, Southern China, Bangladesh, South Asia hills, Indo-Gangetic Plain, central China, Japan, Korean peninsula, north-eastern China, southern Brazil, southern USA, Egypt, Iran, Italy, Spain, California (USA), Peru, and South-eastern Australia
Rainfed lowland	▪ Rainfed shallow, favourable ▪ Rainfed shallow, drought-prone ▪ Rainfed shallow, drought- and submergence-prone ▪ Rainfed shallow, submergence-prone ▪ Rainfed medium deep, waterlogged	Tropics	Cambodia, North-East Thailand, eastern India, Indonesia, Myanmar, Nigeria
Deep water	▪ Deep water ▪ Very deep water	Tropics	River deltas of South Asia and South-East Asia, Mali
Upland	▪ Favourable upland with long growing season ▪ Favourable upland with short growing season ▪ Unfavourable upland with long growing season ▪ Unfavourable upland with short growing season	Tropics	South Asia, South-East Asia, Brazilian Cerrado, and Western Africa
Tidal wetlands	▪ Tidal wetlands with perennially fresh water ▪ Tidal wetlands with seasonally or perennially saline water ▪ Tidal wetlands with acid sulfate soils ▪ Tidal wetlands with peat soils	Tropics, subtropics, and temperate	Sumatra, Kalimantan, Papua, Sulawesi, and Midwestern Korea, Japan

Universiti Malaya

Among cereal crops, rice is mainly grown for direct human consumption, with very little used for other purposes (Awika, 2011). It is the staple food for Malaysians, being the primary source of carbohydrates (Shakri et al., 2021). In 2016, one Malaysian was reported to consume averagely 80 kg of rice, which is about 26% of daily total calorie intake (Khazanah Research Institute, 2019). Nevertheless, local rice production has remained relatively constant since the early 1990s compared to other Asian countries (such as Thailand and Vietnam) which has been showing an increasing trend. Additionally, it is worthwhile to point out that the local rice self-sufficiency level has remained at the lowest compared with other Southeast Asian countries with more than 30% of rice imported mostly from Thailand, Vietnam, and Pakistan (Khazanah Research Institute, 2019).

2.2.3 Weedy Rice

Weedy rice (*O. sativa* f. *spontanea*.), also known as "padi angin" in Malaysia (due to its easily shattering seeds) is known to cause unpleasant agronomic traits and serious threats to the global rice production (Baki & Mispan, 2010; He et al., 2017; Nadir et al., 2017; Sudianto et al., 2016). In Malaysia, weedy rice was first being reported in the Northwest Selangor Project rice fields in 1988 since the introduction of direct-seeding method replacing transplanting method in the late 1980s (Wahab & Suhaimi, 1991) followed by the Muda area in Kedah in 1990 (Mohammed Zuki & Kamarudin, 1994), Besut, Terengganu in 1995 and later in 1996 reported to be found in Seberang Perai, Penang and Kerian, Perak(Azmi et al., 2000). Wide-scale adoption of the direct-seeding method has impacted the increased infestation of weedy rice across Malaysian rice granaries. The spread has generally been favored by use of commercial rice seeds contaminated by weeds. Therefore, various measures have been taken in order to minimise weedy rice infestations and consequential production losses (Nadir et al., 2017).

As a conspecific weed for rice, weedy rice refers to various forms of unwanted plants belonging to the *Oryza* genus and is basically intermediates between cultivated and wild rice (*Oryza* spp.) for many adaptive traits (Mispan et al., 2013; Oka, 1988). Although, many reports suggest that weedy rice may include other wild *Oryza* species including *O. barthii*, *O. glaberrima*, *O. longistaminata*, *O. nivara*, *O. punctata*, and *O. latifolia* (Holm, 1997), this study focuses only on the conspecific to the rice *O. sativa* which is the most widespread in the fields thus the most similar to the rice cultivars (Ruzmi et al., 2021).

Weedy rice has become one of the millennial notorious weed species in rice granaries and ecosystems all over the world (Mispan et al., 2019). Weed infestations, if not effectively controlled, can result in significant economic losses by reducing rice quality and yield (Nadir et al., 2017). Under direct-seeded rice conditions, weed-induced yield losses can reach 100%. Weedy rice infestation rate was reported for 30 and 70% of the rice-grown area in European and US countries, respectively (Gealy, 2005), 80% in Cuba, 50% in Senegal, and 60% in Costa Rica (Nadir et al., 2017). Similarly, in Malaysia, weedy rice infestation significantly reduces yield of up to 74% (Azmi & Karim, 2008). Weedy rice with densities of 35 to 40 plants m^{-2} can reduce tall rice cultivar yields by 60% and yield of short cultivar by 90%, indicating greater losses than grass weeds (Ziska et al., 2015).

Typically, weedy rice can be distinguished from cultivated rice only after weedy traits become more apparent at tillering and post-tillering stages. The weedy traits including leaves with more hispid on both surfaces, taller plant stature, easily shattering grains, pigmentation of the pericarp, and the presence of awns of variable lengths (Baki & Mispan, 2010) differentiating weedy rice from cultivated rice varieties. Additionally, other common weedy traits enabling weedy rice to become a weed are seed shattering, seed dormancy and high competitiveness with cultivated rice (Roma-Burgos et al., 2021).

Besides, weedy rice is also likely to have vigorous vegetative growth and long awns (Kanapeckas et al., 2016; Olajumoke, et al., 2016).

Types of weedy rice can be distinguished by their spikelet characteristics and the colour of seed coat (hull). Malaysian weedy rice spikelets can be awned or awnless, and their hulls can be strawhull, intermediate strawhull, brownhull, or blackhull (Sudianto et al., 2016). Parallel trichomes on the weedy rice hull offer a firmer anchoring of seeds on the soil surface, facilitating seed burial and preventing them from being washed away by heavy rain (Abraham & Jose, 2015). Malaysian weedy rice populations can be divided into four morphological groups (Sudianto et al., 2016). The first group consists primarily of awned, blackhull, and brownhull varieties derived from the wild *Oryza* populations supported by population genetics data. The majority of strawhull awnless types belong to Group 2 derived from Malaysian elite *indica* rice cultivars with high shattering. The third cluster consists predominantly of brownhull morphotypes. The fourth cluster contains a mixture of other weedy morphotypes, lending credence to the natural outcrossing between crop and weed and among weedy populations of various types, resulting in an admixture of plant traits (Sudianto et al., 2016). The complexity of the origin of weedy rice in Malaysia is not yet fully understood (Ruzmi et al., 2021).

A population genetics study of Malaysian weedy rice using simple sequence repeat (SSR) markers revealed three possible origins: (1) traditional Malaysian rice cultivars or landraces (a mixture of *japonica* and *indica* varieties), (2) Malaysian elite rice cultivars that are genetically different from the traditional landraces, and (3) the wild rice *O. rufipogon* (Song et al., 2014). The first group consists of populations that are genetically similar to the companion rice cultivars, while the second group comprises populations that are admixtures of the rice cultivars and *O. rufipogon*. Both cultivated varieties and *O. rufipogon* contribute to the high genetic diversity of the South Asian weedy rice

(Huang et al., 2017). The contribution of crop–weed gene flow to the evolution of weedy rice is also demonstrated in other Southeast Asian regions (Huang et al., 2017).

In recent years, unique research patterns have been observed in many global rice improvement schemes, including uncovering the useful traits of weedy rice such as disease resistance (Mohd Hanafiah et al., 2020). While its presence may reduce both the quantity and quality of the cultivated grains, weedy rice has recently been documented to have novel sources of stress tolerance or resistance (Jia & Gealy, 2018). Ironically, the competitive ability and adaptive evolutionary traits of weedy rice such as stress tolerance, increased seed dispersal, and dormancy (Kanapeckas et al., 2016; Oka, 1988; Suh, 2003; Vidotto & Ferrero, 2000) could be useful to maximizing resource use efficiency and yield of rice amidst the current rapid climate uncertainties (Mohd Hanafiah et al., 2020). In the study conducted by Ziska et al. (2012), weedy rice responded positively to higher carbon dioxide (CO_2) concentration and temperature levels, and showing a greater height with greater formation of panicle and tiller.

Exploiting the full potential of weedy rice, particularly its gene pools, can be advantageous for modern rice breeding and evolutionary studies (Mohd Hanafiah et al., 2021; Nadir et al., 2017). Figure 2.1 shows co-occurence of weedy rice among cultivated rice in a Malaysian rice field. Table 2.2 summarizes some important studies linked to biotic and abiotic stresses in weedy rice. The virtue of weedy rice is eventually deliberated, influenced most likely by increased knowledge and awareness of the adverse effects of climate change.

Universiti Malaya

Figure 2.1: Image of weedy rice (red circle) and cultivated rice (black circle) in field at Tanjung Karang, Selangor

Table 2.2: Examples of important genes linked to biotic and abiotic stresses in weedy rice

Gene (s)	Biotic or abiotic stress	Reference
Asr1	Salinity stress	Chen et al. (2004)
Bar	Glufosinate-ammonium (Basta) herbicide	He et al. (2017)
EXPA3	Salinity tolerance	Qiu et al. (2017)
HKT, *NHX1* and *SOS1*	Salinity stress	Zhang et al. (2018)
OVP1	Cold stress	Bevilacqua et al. (2015)
PDR8	Non-host resistance	Chen et al. (2004)
Pi-ta and *Ptr(t)*	Blast	Lee et al. (2011)
Rc, *Bh4* ad *Phr1*	Aging	Sun et al. (2013)
Sn16	Bacterial blight	Chen et al. (2004)
PAPH1	Drought tolerance	Han et al. (2022)
OsAPX2, OsHSP24.15, OsHSP71.10 and *OsHSP85.88*	Drought tolerance	Agostinetto et al. (2022)

Sources: Agostinetto et al. (2022); Han et al. (2022); Mohd Hanafiah et al. (2020)

2.3 Highlights of Rice Research since The Green Revolution

The green revolution established in the 1960s by the IRRI was aimed to develop high-yielding rice varieties. The first semi-dwarf, high-yielding rice variety IR8, popularly known as the "miracle seeds", was developed in 1966, saving millions of lives in a number of famine-prone countries, particularly those in Asia such as India and China (Hargrove & Cabanilla, 1979). However, its dependency on massive quantities of fertiliser and irrigation to optimise production has triggered controversy over several decades (Massawe et al., 2016).

2.3.1 Early Green Revolution

The discovery of the semi-dwarfing (*sd-1*) gene by late Dr. Norman E. Borlaug, a Nobel Peace Prize Laureate known as the Father of Green Revolution, has significantly improved the production of high-yielding varieties worldwide, notably for the big three cereals (Mohd Hanafiah et al., 2020). The semi-dwarf trait became important in avoiding plants from lodging. Between 1966 and 1986, short-statured rice varieties took up roughly 60% of the global rice land (Khush, 1987). The first high-yielding rice variety IR8 was derived from the cross between Dee-geo-woo-gen (DGWG) - a Chinese dwarf variety with *Sd-1* gene, and Indonesian Peta, which is a tall and robust variety with pleasant taste (Hargrove & Cabanilla, 1979). Released in 1966, the IR8 soon became the most planted rice variety in some areas of Asia. Nonetheless, it has several disadvantages, with the major ones being its long growth period (i.e. 130 days to maturity) and vulnerability to several diseases and insects (Khush, 2001).

The success of the development of IR8 was acknowledged by rice breeders worldwide (Mohd Hanafiah et al., 2020; Peng et al., 2021). Many breeding projects extensively used semi-dwarf varieties as donor parent for other major food crops including wheat (Syme,

Universiti Malaya

1970) and maize (Welch, 1983). However, the major issues of growing modern varieties include the excessive utilisation of chemical fertilisers and pesticides with the need to adopt efficient irrigation systems (Mohd Hanafiah et al., 2020). Additionally, an increase in monoculture whereby a single crop is continuously being cultivated in a field causes modern varieties to be more prone to changing environment (Cheng et al., 2022). Monoculture also leads to a decrease in crop genetic diversity, triggering the susceptibility of crops to agricultural threats, in particular disease and pest infestation (Cheng, 2018; Massawe et al., 2016).

2.3.2 Late Green Revolution

The global production of rice, wheat, and maize in many parts of the world increased regularly since the 1960s, and it was nearly doubled within a mere two decades that consequently reduced famine and hunger crises (Ameen & Raza, 2017). After the IR8 variety was released in 1966, roughly 70% of the global rice land in the mid-1990s was planted with high-yielding rice varieties with improved grain quality, shorter growth duration, and resistance to multiple diseases and insects (Khush, 1995).

In the late green revolution, the effective use of molecular and cellular approaches in rice study was greatly improved. Genetic rice engineering began in the 1980s, and the first transgenic rice was reported in the late 1980s (Toriyama et al., 1988; Zhang et al., 1988). Much focus was given to developing rice with resistance toward insects (Duan et al., 1996), pests (Pinto et al., 1999), viruses (Uchimiya et al., 1993), and diseases such as SB (Uchimiya et al., 1993) and BLB (Song et al., 1995). One renowned example is the genetically engineered, insect-resistant Bt rice which was developed by introducing the insecticidal genes from *Bacillus thuringiensis* Berliner (Bt) into rice (Tu et al., 2000). Although Bt rice showed good resistance to yellow and striped stem borer, both in

Universiti Malaya

laboratory and in field conditions, its commercial planting was long delayed due to regulatory restrictions for food safety concerns (Yang et al., 2014).

With the development of linkage and quantitative trait loci (QTL) maps, marker-assisted selection (MAS) is the most common method used internationally. This is particularly applicable for developing high-yielding rice with improved resistance to biotic and tolerance to abiotic stress, which was one of the primary goals of improving global rice production in the late green revolution period (Babujee & Gnanamanickam, 2000; Khush, 1997). For example, the development of rice introgressed with *Xa* genes for bacterial blight resistance (Pradhan et al., 2019). Genome-wide association studies (GWAS) are another important tools used to dissect the genetics and identify markers associated with complex traits in rice, including disease resistance such as BLB, stress tolerance, flowering time, plant height, grain yield, and grain shape for use in MAS (Chukwu et al., 2019a; Das & Rao, 2015; Huang et al., 2017; Yano et al., 2016).

2.3.3 The 21st Century

The completion of the rice genome in the mid-2000s marked a significant milestone in rice research. Although rice is one of the most distinctive models for plant biology research (Izawa & Shimamoto, 1996; Somerville & Koornneef, 2002), it is a C3 crop with significantly lower photosynthetic efficiency than some C4 crops such as maize and sorghum (*Sorghum bicolor*) (Wang et al., 2012). Much research was devoted to engineering C4 photosynthetic traits into rice, which could increase its yield up to 50% while using half the water (Lin et al., 2019; Wang et al., 2016).

Currently, there are four major tools for genome editing in rice, namely zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), meganuclease, and clustered regularly interspaced short palindromic repeats (CRISPR / Cas method).

Universiti Malaya

The CRISPR / Cas system, which uses the adaptive mechanism of prokaryotes to foreign deoxyribonucleic acid (DNA) fragments, has successfully produced mutagenesis in transgenic rice (Miao et al., 2013). There has been a significant increase in the use of CRISPR / Cas genome editing in plant study, particularly after several successful attempts in monocots (such as rice and sorghum) and dicots (such as thale cress, *Arabidopsis thaliana* and tobacco, *Nicotiana tabacum*) (Jiang et al., 2013). Presently, the CRISPR / Cas9 method is frequently used to edit genes associated with yield, quality, and disease resistance in rice (Mohd Hanafiah et al., 2020).

The advancement of omics technologies during the past decades has greatly enhanced the capability for rice gene discovery and functional genomics, resulting in a significant improvement in the performance of molecular methods used in rice research (Collard & Mackill, 2008; Mohd Hanafiah et al., 2020). Developing host resistance is an efficient technique to decrease the yield losses caused by biotic stresses in crops (Molla et al., 2019). In addition, the incorporation of molecular methods into conventional rice breeding programmes has significantly increased the opportunities of breaching the yield barrier (Biswal et al., 2017). For example, DNA marker technology, derived from molecular genetics and genomics offers great promise for plant breeding to develop durable disease resistance, abiotic stress tolerance as well as nutrient and water-use efficiency.

Many highly specialised equipments have also been developed based on sophisticated molecular techniques used for genotyping (Collard & Mackill, 2008). In recent years, the rapid growth of genomics, next-generation sequencing (NGS), and related DNA innovations have resulted in a quantum leap in molecular understanding of critical plant breeding traits (Biswal et al., 2017). Robust and low-cost molecular screening of resistant genotypes is critical for breeding disease-resistant and high-quality rice varieties, especially in resource-constrained developing nations such as Malaysia (Ashkani et al.,

Universiti Malaya

2015).

2.4 Major Diseases in Rice

In general, plants need to develop a variety of responses to acclimate to environmental stresses in order to tolerate adverse environmental conditions due to its sessile nature (Gull et al., 2019; Miryeganeh, 2021). With the increase in adverse weather conditions due to climate change and the constant threat of pathogens and pests, there is an immediate need to grow crop varieties that can survive multiple stresses (Cohen & Leach, 2019). Both biotic and abiotic stresses are reported to be causing significant agricultural problems by reducing crop growth and productivity (Majeed & Muhammad, 2019; Sombunjitt et al., 2017). Several environmental factors have affected sustainable farming in developing countries, with higher incidences of extreme weather and a number of environmental issues influencing the sustainability of agricultural development (Das et al., 2017). Globally, diseases are one of the main causes of loss in rice production (Acharya et al., 2018). Major rice diseases across the world include bacterial leaf blight (BLB), sheath blight (SB), and blast caused by the pathogens *X. oryzae* pv. *oryzae* (Hajira et al., 2016), *R. solani* (Y. -Y Yu et al., 2017), and *M. oryzae* (Wang et al., 2014), respectively.

Universiti Malaya

2.4.1 Bacterial Leaf Blight (BLB)

The BLB or leaf blight caused by *Xoo* - a Gram-negative bacteria - is one of the most damaging rice diseases. Generally, *Xanthomonas* is a widespread bacterial genus that comprises approximately 30 pathogenic species known to cause disease in over 300 plant hosts (Alvarez-Martinez et al., 2021; Meenakshi & Salgotra, 2020). First discovered in 1884 in the province of Fukuoka, Japan (Ou, 1985; Sombunjitt et al., 2017), BLB has

been reported in various parts of Asia, North Australia, Africa, and the USA (Sabar et al., 2016). It is particularly endemic to many parts of Asia and West Africa (Mazid et al., 2013).

The severity of the BLB depends on many factors, including host susceptibility, growth stages, and environmental conditions (Sabar et al., 2016; Sombunjitt et al., 2017). BLB causes wilting of seedlings, yellowing and drying of leaves which can damage 20-30% of rice yield (Figure 2.2) (Sombunjitt et al., 2017). In severe cases, it can result up to 80% of yield losses (Pustika & Yolanda, 2021; Sombunjitt et al., 2017; Weerasinghe et al., 2017). Plants that are infected during the booting stage will result in poor quality grains with a high proportion of broken kernels (Weerasinghe et al., 2017). The chemical control practice for this disease has yet to be developed (Weerasinghe et al., 2017).

Figure 2.2: Lesion caused by bacterial blight (Source: http://www.knowledgebank.irri.org/decision-tools/rice-doctor/rice-doctor-fact-sheets/item/bacterial-blight)

University of Malaya

To date, more than 42 BLB resistance genes have been reported, and some of these genes have been utilised in MAS either individually or in combination (Meenakshi & Salgotra, 2020; Shu et al., 2021). Examples of linked marker for the disease is presented in Table 2.3. The greatest number of resistance genes against BLB have been identified in *O. sativa* L. ssp. *indica* rice and wild rice (*O. longistaminata*, *O. rufipogon*, *O. minuta* and *O. officinalis*). Some resistance genes have also been identified from *O. sativa* L. ssp. *japonica* rice (Sombunjitt et al., 2017). Several studies have developed multiplex assays for simultaneous detection of multiple resistance genes against BLB, including *Xa21*, *xa5* and *Xa13* (Hajira et al., 2016), *Xa4*, *xa5* (RM604), *Xa7* (Xa7F/7-1R/7-2R), *xa13* and *Xa21* (Yap et al., 2016), and *Xa13* (Xa13 prom) and *Xa21* (pTA248) (Meenakshi & Salgotra, 2020).

Table 2.3: Examples of linked marker for BLB resistance in rice

Chr. No.	Resistance gene	Linked marker	Rice variety
3	*xa42*	KGC3_16.1 and RM15189	XM14, a mutant of IR24
4	*Xa-1*	XNpb235, XNpb264, and C600	Temperate japonica
4	*Xa2*	HZR950-5 and HZR970-4	Tetep
6	*Xa7*	ID7	DV85
11	*Xa21*	pTA248	*O. longistaminata*
11	*Xa4*	RM224	TKM6
11	*Xa39*	RM21 and RM206	FF329 (Huang-Hua-Zhan (HHZ) × PSBRC66 (P66))
11	*Xa40(t)*	RM27320 and ID55	11325/ Anmi'×'11325/Ilpum

Source: Chukwu et al. (2019b)

2.4.2 Sheath blight (SB)

Similar to BLB, SB is a rice disease that reduces the quantity and quality of rice. It is the most detrimental rice disease globally after rice blast (Dey et al., 2019), being the most common disease in nearly all rice-growing countries in the developing world, including Malaysia (Joshi et al., 2016). Amid favourable conditions, SB may cause up to

Universiti Malaya

50% in rice production loss (Vân Bạch, 2021; Zheng et al., 2013). Yield losses depend mainly on the plant growth phases where infection occurs, resistance degree of a particular genotype as well as the prevailing environmental conditions (Joshi et al., 2016).

The SB is caused by a semi-saprophytic soil-borne contagious pathogen, *R. solani* Kuhn that thrives on a wide range of hosts. This pathogen can survive either as sclerotia or mycelia in host plants' debris (Dey et al., 2019). Sclerotia floats to the surface of flooded water in rice fields and germinates on plant sheaths, creating an infection appressoria. Initial signs of infection include approximately 1 cm oval, oblong or ellipsoid, greenish-grey, water-soaked spots that occur on leaf sheaths near the water line. The pathogen spreads through hyphae surface and creates new infection mechanisms in the plant, causing severe necrotic damage (Figure 2.3) (Joshi et al., 2016). It is important to note that morphological traits like plant height, heading date, and stem thickness positively correlated with sheath blight resistance (Dey et al., 2019). The short stature of the *sd-1* semi-dwarf locus was closely linked to higher sheath blight infection (Dey et al., 2019; Sharma et al., 2009).

Figure 2.3: Lesion caused by sheath blight (Source: http://www.knowledgebank.irri.org/training/fact-sheets/pest-management/diseases/item/sheath-blight)

Universiti Malaya

Some rice varieties, including Tetep, Tadukan, Teqing, and Jasmine 85 originated from Vietnam, Philippine, China and IRRI, respectively, have been identified to have a relatively high resistance towards this pathogen (Joshi et al., 2016). Although many rice cultivars have been examined, none of them has been identified to be totally resistant to *R. solani* (Persaud et al., 2021; Rogayah et al., 2017). SB resistance has been identified to be a typical polygenic quantitative trait and approximately 50 SB resistance QTL have been reported in all 12 chromosomes of numerous rice species (Joshi et al., 2016). It is controlled by few major genes and several minor genes in some rice varieties (Badri et al., 2017). Z. Li et al. (1995) identified first QTL linked to molecular marker RG118 using F_{2-3} population of Lemont/Teqing. Two major QTLs, *qshb7.3* and *qshb9.2*, were located on chromosomes 7 and 9 have also been identified using BC_1F_2 mapping populations from the cross BPT5104/ARC10531(Yadav et al., 2015). SB resistance governed by several minor genes or QTLs has a limited effect, and the pyramiding of such QTLs is expected to result in substantially increased resistance in pyramided cultivars. More examples of reported QTLs for SB are presented in Table 2.4.

Table 2.4: Examples of reported QTLs for sheath blight resistance in rice

Chr. No.	Locus	Marker interval or nearest marker	Rice variety
1	qSBR1-1	Hvssr68-RM306 (RM1232 - Hvssr68)	Tetep × HP2216
2	qSB-2	C624x	Teqing × Lemont
3	qSB-3	RM3856	WSS2 × Hinohikari
4	qSB-4-2	RZ590x	Teqing × Lemont
5	qSB-5	Y1049	Teqing × Lemont
6	qSB6-2	RZ508	Teqing × Lemont
7	qshb7.3	RM205	ARC10531 × BPT-5204 ×
8	qSBR-8-1	RM210-Hvssr47	Tetep × HP2216
9	qShB9-2	RM245	Jasmine 85 × Lemont
10	qSB-10	RG561	Teqing × Lemont
11	qSBR11-3	RM536–RM20	Tetep × HP2216
12	qSB-12	G1106	Teqing × Lemont

Source: Dey et al. (2019)

Universiti Malaya

2.4.3 Blast

Rice blast is the most serious fungal disease, contributing to rice losses up to 80% (Miah et al., 2017; Simkhada & Thapa, 2022). The disease is triggered by *M. oryzae*, which has many different names include rice blast fungus, rice seedling blight, rice rotten neck, oval leaf spot of graminea, pitting disease, Johnson's spot, and rye grass blast (Miah et al., 2017). *Magnaporthe oryzae* is ascomycetes which are filamentous and can propagate both sexually and asexually. The fungus conidia are translucent, two-septate, mildly darkened and obclavate, pointed at the apex (Figure 2.4) (Miah et al., 2017; Shikari et al., 2019). Blast was first detected among Taiwanese varieties that were brought in during the Second World War in Malaya in 1945 (Van, 1967). This disease is globally widespread and has more than 1,700 isolates from 40 countries (Miah et al., 2017; Shikari et al., 2019). Numerous studies have been performed using molecular markers and pathogenicity assessments for diverse varieties of rice to identify *M. oryzae* population structure all over the world (Mutiga et al., 2017; Tuan et al., 2020; Yadav et al., 2019).

Figure 2.4: Lesion caused by blast disease (Source: http://www.knowledgebank.irri.org/training/fact-sheets/pest-management/diseases/item/blast-leaf-collar)

Universiti Malaya

Rice blast resistance is a complex process, and more than 100 resistance genes have been identified (Annegowda et al., 2021; Miah et al., 2017). The use of resistant varieties would provide better management compared to other control methods, because it is affordable, but it may take a long time to produce a desired resistant variety. According to Miah et al. (2017), the future direction will be focused on the production of rice varieties with durable resistance to blast. Characterisation of blast fungus genetic diversity and recognition of various lineages and pathotypes is required.

According to Annegowda et al. (2021), breeding for blast resistance in rice may be broadly classified into four categories- conventional breeding methods, marker-dependent breeding methods, breeding approaches requiring genetic transformation, and genome editing. Numerous blast-resistant cultivars have been developed through conventional and molecular breeding. To manage blast disease effectively, breeding efforts should be emphasised on utilising the broad spectrum of resistance genes as well as pyramiding genes and QTLs of which the most effective method for disease control is to incorporate both qualitative and quantitative genes in resistant variety (Miah et al., 2013). For examples, through MAS, blast resistant genes *Pi1*, *Pi2*, *Pi33* introgressed into Russian rice varieties (Usatov et al., 2016) and *Pizt*, *Pi2*, *Pigm*, *Pi40*, *Pi9*, *Piz* introgressed into Yangdao 6 (Wu et al., 2017). Gene pyramiding has been successfully used for accumulating different blast resistance genes such as *Pi21*, *Pi34* and *Pi35* (Yasuda et al., 2015). More examples of reported resistance genes for blast are presented in Table 2.5.

Table 2.5: Examples of reported blast resistance genes in rice

Chr. No.	Resistance gene	Linked microsatellite marker	Rice variety
1	*Pitp(t)*	RM246	CO39 and Tetep
1	*Pi37*	RM302, RM212, FPSM1, FPSM2, FPSM4	C101PKT, CO39 and AS20-1 crossed with cultivar St. No. 1
2	*Pi-b*	RM166, RM138, RM208, RM266, RM138	Tohoku IL9 and Sasanishiki

Universiti Malaya

Table 2.5, continued

Chr. No.	Resistance gene	Linked microsatellite marker	Rice variety
6	*Pi-9*	RM136	Cultivar TP309
8	*Pi33*	RM72, RM44	IR64 × Azucena and Azucena × Bala
8	*Pi36*	RM5647	Aichi Asahi and Lijiangxintuanheigu (LTH) cross with Q61
11	*Pi-ks*	RM224	Near-isogenic lines C101LAC and C101A5
11	*Pi38*	RM206, RM21	CO39 and Tadukan
12	*Pita*	OSM89, RM155, RM7102	Yashiro-mochi and Tsuyuake

Source: Miah et al. (2013)

2.5 Major Grain Quality Traits in Rice

Rice quality is determined by a combination of subjective and objective factors, with consideration given to various factors such as size and shape of rice grains, fragrance, milling quality, and other eating and cooking characteristics (Custodio et al., 2019; Dela Cruz & Khush, 2000). Based on past studies, the physical and chemical properties of rice grains, such as amylose content, gelatinisation temperature, and gel consistency, are closely related to the quality of eating and cooking (Lanceras et al., 2000; Sultana et al., 2022). The desired quality characteristics of rice vary according to geographical area and ethnic group. For example, countries that consume *indica* rice, such as Malaysia and India, prefer long grain rice with a moderate amylose content while countries that consume *japonica* rice, such as Japan, prefer short grain rice with low amylose content (Verma et al., 2015).

2.5.1 Fragrance

Fragrance is one of the main characteristics of high-quality rice, and its inheritance is influenced by genetic and environmental factors. A total of 114 volatile compounds

Universiti Malaya

have been identified as the chemical component responsible for the production of fragrances, including 13 hydrocarbons, 13 alcohols, 14 acids, 14 ketones, 16 aldehydes, 8 esters, 5 phenols along with others (Ramtekey et al., 2021; Yajima et al., 1979). Tanchotikul and Hsieh (1991) reported that 2-acetyl 1-pyrroline (2-AP) is a major chemical component for fragrance production, and it is considered to be the most important component in providing distinctive aroma to Basmati rice and rice Jasmine. 2-AP can be found in all parts of fragrant rice plants except root (Lorieux et al., 1996; Okpala et al., 2020) and its concentration can reach 100 times lower in non-fragrant rice varieties (Yahya et al., 2011).

Genetic analysis showed that the fragrance recessive gene (*fgr*) on chromosome 8 is the responsible gene in the inheritance of fragrance characteristics as well as controlling the concentration of 2-AP in rice. This gene is closely located to the RFLP marker - RG28 with genetic distance 4.5 cM (Lorieux et al., 1996). 2-AP accumulation in fragrant rice can be explained by the presence of mutations that causing loss of function of *fgr* gene products. Several polymerase chain reaction (PCR)-based markers have been developed based on the RG28 locus that can distinguish between the genotypes of fragrant and non-fragrant rice varieties (Cordeiro et al., 2002; Golestan Hashemi et al., 2015; Madhav et al., 2010).

Bradbury et al. (2005) reported that there were significant polymorphisms in the coding region of fragrant genotypes comparative to non-fragrant genotypes for a single gene homologous to the gene encoding betaine aldehyde dehydrogenase 2 (BAD2) enzyme. Non-fragrant rice varieties possess a copy of the gene encoding BAD2 that appears to be fully functional, whereas fragrant varieties possess a copy of the gene encoding bad2 that contains a deletion and single nucleotide polymorphisms (SNPs), resulting in a frame shift that generates a premature stop codon that presumably disables the BAD2 enzyme (Borah et al., 2022; Roy, 2015). The full-length BAD2 protein encoded

by the bad2 inhibits 2-AP biosynthesis in non-fragrant rice (Chen et al., 2008; Golestan Hashemi et al., 2015; Qian et al., 2022). Bradbury et al. (2005) sequenced 17 genes in the *fgr* gene region and found that the bad2 recessive gene on chromosome 8 is most likely *fgr* gene because it has 8-bp deletion [5'-GATTATGG] and three SNPs in exon 7 compared with the badh2 functional gene.

Researchers have sought to develop molecular markers capable of assisting breeders in selecting fragrant genotypes because fragrance characteristics of rice being controlled by a recessive gene and the natural feature of the fragrance is difficult to assess (Lau et al., 2016; Prodhan & Qingyao, 2020). Molecular markers that are tightly linked to fragrance genes can facilitate the initial selection of fragrance characteristics and identify the nature of the locus, which may be homozygous or heterozygous. A set of molecular markers that work for rice fragrance has been successfully developed, in which both the *BADH2* and *badh2* alleles can be detected simultaneously in single PCR amplification. A set of functional molecular markers developed in the study of Bradbury et al. (2005) consists of four primers, i.e. External Antisense Primer (EAP), External Sense Primer (ESP), Internal Fragrant Antisense Primer (IFAP) and Internal Nonfragrant Sense Primer (INSP). Figure 2.5 shows the positions of the four primers (Bradbury et al., 2005).

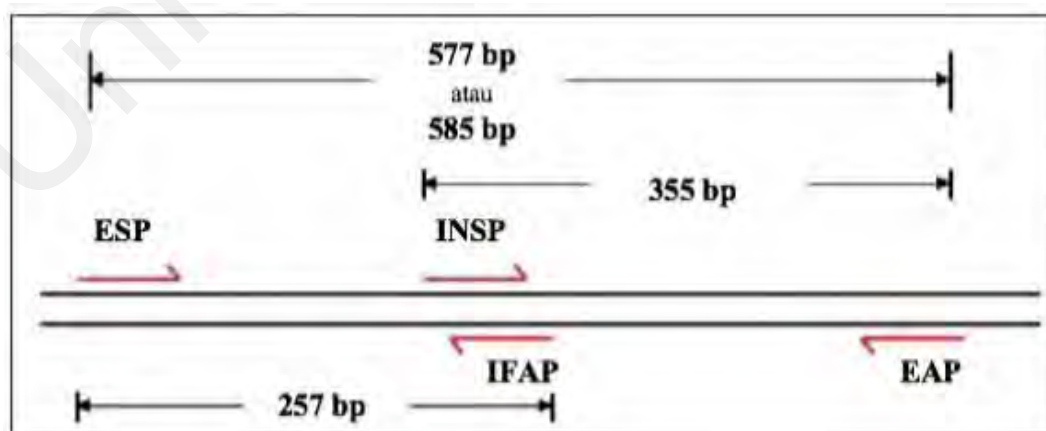

Figure 2.5: The location of four primers, EAP, ESP, IFAP and INSP used to identify fragrance characteristics in rice

Universiti Malaya

There are three possibilities that can be derived from the genotype results from multiplex PCR products of these four primers, i.e. homozygous dominant (non-fragrant), homozygous recessive (fragrant) and heterozygous (non-fragrant). The ability of this set of molecular markers detecting heterozygous genotypes makes it an ideal marker for this trait (Cheng et al., 2017). The discovery of this set of markers will benefit rice breeding programme of high-quality fragrant rice, especially in the MAS breeding programme (Golestan Hashemi et al., 2013; Sreenivasulu et al., 2022).

Previously, several conventional sensory-based methods were used in the rice breeding programme to distinguish fragrant and non-fragrant paddy (Moshi, 2016). For example, chemical methods are used which involved inhalation of leaf tissue or grains after being incorporated into a 0.1 M solution of potassium hydroxide (KOH) (Cheng et al., 2017; Sood & Siddiq, 1978). However, this approach can cause damage to the nasal cavity of the assessor. Furthermore, gas chromatography-mass spectrometry effectively evaluates the content of 2-AP, but it is costly and time-consuming (Chen et al., 2006; Verma & Srivastav, 2022). These issues can be overcome by the aid of molecular methods (Butardo et al., 2019).

2.5.2 Amylose Content

Improvement on the quality of rice starch, particularly amylose content percentage reduction on high yield varieties, is significant in producing high-value rice. Amylose content is directly related to the volume expansion, water absorption, and textural properties of cooked rice (Juliano, 1985). Amylose content is the indicator of rice starch quality and considered to be the major determinant in rice processing and cooking quality. Starch contains 90% of total dry weight of rice and has a significant impact on cooking and eating quality (Li & Liu, 2019).

Universiti Malaya

Starch is a major component in rice endosperm with two classes of polymers, including amylose and amylopectin. Amylose is a lightly branched linear α-1,4 molecule with a degree of polymerisation of 1000 to 5000 glucose unit, while amylopectin is a larger polymer unit containing frequent α-1,6 branching linkages (He et al., 2006; Jiang et al., 2004; Juliano, 1985; Unnevehr et al., 1992; Williams et al., 1958). Figure 2.6 (A) and (B) shows the structure, of amylose and amylopectin, respectively.

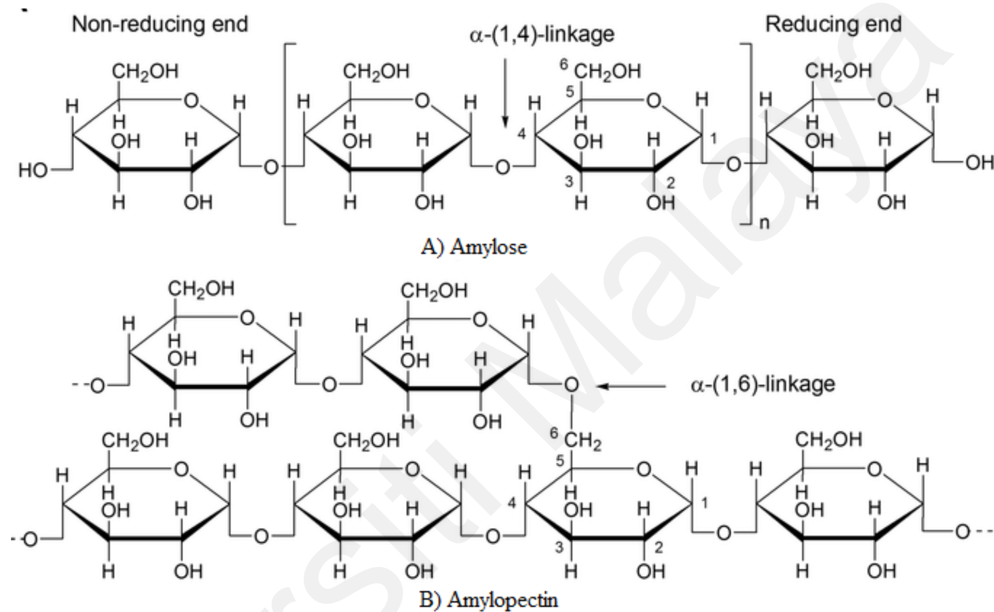

Figure 2.6: (A) Amylose structure, (B) amylopectin structure. Source: Martin & Smith, (1995)

Amylose content was determined by measuring the ratio of amylose to total starch, which varied between cultivars. The mean of amylose content of *indica* and *japonica* rice were recorded 20-30% and 15-22%, respectively (Hsu et al., 2014; Liu et al., 2009). Generally, rice amylose content can be categorised to five classes – waxy (0-2%), very low amylose content (3-9%), low (10-19%), intermediate (20-24%) and high (more than 24%). Rice with a low amylose content is soft and sticky, while rice with a high amylose content is hard, dry, and flaky (Juliano, 1971). The intermediate amylose content (20-25%) is very popular in the tropics and has a high demand because it produces rice that is soft but not sticky. Rice with amylose content of more than 20% is found to be less

Universiti Malaya

popular because of its hard and dry texture when cooked.

Specific genetic studies on amylose content trait have previously found that it is controlled by an allelic series at a single locus with major effects and by one or more modifier genes with minor effects (Ayres et al., 1997). The inheritance of this trait is complex due to cytoplasmic effects, epistasis, quantitative properties of triploid tissue of endosperm (Pooni et al., 1993), and environmental factors (Zhang et al., 2005). According to Juliano and Pascual (1980), environmental factors can affect the amylose content of the majority of cultivars by up to 6%. High amylose content levels were reported to be controlled by genes expressed either in complete dominance or incomplete dominance (partial dominance). Therefore, the inability to identify heterozygotes with conventional methods frequently leads to inefficiency in traditional breeding (McKenzie & Rutger, 1983; Pooni et al., 1993).

There are several nucleotide polymorphisms in the *Wx* gene that have been successfully identified in previous studies, including the single sequence repeat (SSR or microsatellite) $(CT)_n$ polymorphism at the *Wx* locus, i.e. the marker closely located to the *Wx* gene that contains different number of CT repetitions as well as G-T SNPs located at the 5′-leader intron splice site (Bligh et al., 1995; Wang et al., 1995; Cheng et al., 2015). The other two identified SNPs were located at exon 6 and exon 10 reported to have a high correlation between amylose content and SSR *Wx* and G-T SNP (Larkin & Park, 2003).

Bligh et al. (1995) identified a total of eight *Wx* alleles with different $(CT)_n$ repeats accounted for 80% of the amylose content variation. Wang et al. (1995) reported that the amylose content in rice endosperm could be associated with post-transcriptional regulation of the *Wx* gene. Wx protein levels are related to level of mature *Wx* messenger ribonucleic acid (mRNA) associated with intron cutting stage 1 of pre-MRNA (Cai et al., 1998; Isshiki et al., 1998). Cultivars with a high amylose content have fully processed *Granule-Bound Starch Synthase (GBSS)* mRNA levels, whereas cultivars with a low

amylose content have either partially or fully processed *GBSS* mRNA with the leader intron (Bligh et al., 1995; Wang et al., 1995).

Traditional rice breeding methods are ineffective in improving amylose content because this trait is controlled by one major gene and several other minor genes that are influenced by environmental factors. The shortcomings of traditional breeding programmes can be overcome by molecular or MAS improvement programmes, and selection on major genes can be carried out effectively in early population generations. Ayres et al. (1997) reported that a single nucleotide base change at 5'- leader intron splice sites are able to interfere with mRNA processing. This is based on their study of 89 non-waxy varieties native to the United States, where all 30 varieties with low amylose content have sequences AGTTATA while 59 varieties with moderate and high amylose content has the AGGTATA sequence. They also found that G-T mutations are capable in explaining a total of 79.7% variation in amylose content on all varieties studied. Similarly, Jayamani et al. (2007) found that G-T mutations accounted for as much as 79.2% of the variation in amylose content across all varieties tested. Cheng et al. (2012) reported that the AGGTATA and AGTTATA sequences at the 5'- leader intron splice site are closely related with the presence of the wx^a allele and the wx^b allele, respectively. The use of SSR *Wx* is able to overcome the shortcomings of other methods in determining the level of amylose content because of its ability in detecting allelic diversification effectively.

2.6 Molecular Approaches for Rice Improvement

Over the past decades, there are numerous successful uses of advanced molecular and genomic methods such as polymerase chain reaction (PCR), electrophoresis, and molecular marker systems to understand the genetic basis of important traits for crop improvement (Miah et al., 2017). PCR was invented in mid-1980's and has become a crucial tool for molecular biology research (Cheng et al., 2015). It is one of the most

Universiti Malaya

common nucleic acid tests and considered the gold standard for the identification of plant pathogens, characterizing by high precision, sensitivity, rapid detection and high-throughput detection capability (Lau & Botella, 2017; Tahamtan & Ardebili, 2020).

Multiplex PCR, a variant of PCR, is a technique that utilises two or more pairs of specific primers to amplify multiple target sequences simultaneously in a single PCR reaction (Meenakshi & Salgotra, 2020). First described in 1988, multiplex PCR has steadily gained popularity due to its ability to save time and effort in the laboratory by providing a rapid and competent approach that eliminates the tedious process of screening individual markers (Meenakshi & Salgotra, 2020). It has also been proven to be a more effective tool for detecting rice resistance genes (Hajira et al., 2016). Several multiplex PCR systems have been developed for rice to detect key genes and markers associated with agronomic performance (J. Z. Li et al., 2017), grain quality (Cheng et al., 2015), and disease resistance (Bangratz et al., 2020; Hajira et al., 2016; Mohd Hanafiah et al., 2021; Yap et al., 2016). Recent study by Bangratz et al., (2020) developed multiplex PCR systems as detection molecular tools for five bacterial pathogens and have been shown to be useful for significant advancement in disease monitoring in the field, leading to effective disease control and food safety.

Multiplex genotyping is a dependable, high-throughput, rapid, and low-cost method particularly for genotyping multiple resistance genes in rice (Yap et al., 2016). In this post-genomic era, where functional markers are extensively available for breeders to use, a simple and low-cost genotyping method is greatly needed, mainly for those working in developing and underdeveloped countries with restricted resources. Among a variety of genotyping methods, gel- and capillary-based electrophoresis are considered the most widely used methods for analyzing PCR amplification products in genotyping for crop studies. Agarose gel electrophoresis has been the mainstay method used since 1970s in separating moderate size of DNA fragments 50-500 bp (Mitchenall et al., 2018;

Universiti Malaya

Stellwagen, 2009). While traditional gel electrophoresis using standard agarose is considered a low-throughput technique with several potential disadvantages (such as the requirement for DNA staining with ethidium bromide), the technique has been enhanced through the development of specially formulated high-resolution agarose capable of efficiently separating small DNA fragments (Cheng et al., 2015; Mitchenall et al., 2018). For instance, the MetaPhor® agarose (Lonza) has been shown to be capable of separating fragments with a size difference of 20 bp (Cheng et al., 2015; Mohd Hanafiah et al., 2021). Additionally, non-mutagenic alternative DNA staining reagents, such as SYBR-Green and SYBR-Gold, have been developed to replace the use of ethidium bromide, which has been long considered as a drawback for gel-based electrophoresis (Mohd Hanafiah et al., 2021; Motohashi, 2019).

Capillary electrophoresis is a versatile and effective genotyping technique that is capable of showing accurate and consistent allele sizes with minimal manual intervention and least size error (Lau & Latif, 2019). Over the last two decades, capillary electrophoresis has been widely used for the identification of molecular markers in rice research, such as SSR and SNP (Kim et al., 2016; Mishra et al., 2022; R. Vemireddy et al., 2007). In the early 2010s, a capillary-based SNP genotyping technology - the allele-specific primer (ASP) PCR - was developed with promising findings in rice genotyping (De Wever et al., 2019; Hirotsu et al., 2010). Similar to gel electrophoresis, capillary electrophoresis can also be used for multiplexing. However, it requires specialised equipment and fluorescently labelled probes, which are often costly (Aloui et al., 2015; Qi et al., 2019). This is perhaps the primary reason why capillary electrophoresis has not been the predominant analytical technique in small- or medium-scale laboratories where gel electrophoresis is easily available (Gupta et al., 2010; Mitchenall et al., 2018).

The past three decades have observed the vast utilisation of molecular marker systems in rice improvement, particularly in breeding programmes involving MAS (Miah et al.,

Universiti Malaya

2017). MAS has proved to be an effective approach in breeding programme to improve disease resistance in rice genotypes by introgressing resistance genes with desirable traits (Hsu et al., 2020; Sabar et al., 2016; Wang et al., 2021). Molecular marker which was initially established primarily to determine the identification of individual varieties has eventually emerged as one of the most useful genomic tools facilitating conventional breeding programme (Mazid et al., 2013). Table 2.6 presents the characteristics of several widely used molecular marker systems. This technology can significantly speed up the detection and selection procedure of target loci (Molla et al., 2019), especially in identifying multiple alleles for biotic and abiotic stress resistance (Dixit et al., 2020; Mubassir et al., 2016; Sombunjitt et al., 2017).

Besides, molecular markers may be used for the assessment of genetic diversity, genotype fingerprinting, hybrid separation from selfed progeny and other applications (Ashkani et al., 2015). Among the available markers, SSR and SNPs are the two most commonly used PCR-based markers for genotyping and classifying rice varieties (Chandra et al., 2017; Cheng et al., 2015). Marker-assisted breeding is an extremely effective and accurate approach whereas conventional breeding is restricted because it requires longer time and is often influenced by environmental changes (Sombunjitt et al., 2017). It is crucial to emphasise that the breeding of new crop varieties through induced polyploidy may have unfavourable outcomes, caused in part by genetic instability following polyploidization (Cheng et al., 2022).

Universiti Malaya

Table 2.6: Comparative characteristics of various widely used molecular marker systems for plant genome analyses

	Isozyme	RFLP	RAPD	AFLP	SSR	SNP
Abundance	Low	Medium	Very high	Very high	High	Very high
Types of polymorphisms	Amino acid change in polypeptide	Single base change, insertion, deletion, inversion	Single base change, insertion, deletion, insertion	Single base change, insertion, deletion, insertion	Repeat length variation	Single base change
DNA quality	-	High	Medium	High	Medium	Medium
DNA sequence information	-	Not required	Not required	Not required	Required	Required
Level of polymorphism	Low	Medium	High	High	High	High
Inheritance	Co-dominance	Co-dominance	Dominance	Dominance	Co-dominance	Co-dominance
Reproducibility	Medium	High	Low	Medium	High	High
Technical complexity	Medium	High	Low	Medium	Low	Medium
Developmental cost	Medium	High	Low	Low	High in start	High
Species transferability	High	Medium	High	High	Medium	Low
Automation	Low	Low	Medium	Medium	High	High
Possibility of tagging an individual locus	Yes	Yes	Only after conversion	Only after conversion	Yes	Yes
Multilocus markers	No	No	Yes	Yes	No	No
Main method of analysis	Staining of proteins	Hybridization	PCR	PCR	PCR	> 20 approaches

AFLP- amplified fragment length polymorphism; RAPD- random amplified polymorphic DNA; RFLP- restriction fragment length polymorphism SNP- single nucleotide polymorphism; SSR- simple sequence repeat Sources: Khlestkina & Salina, (2006); Park et al. (2009)

Universiti Malaya

2.7 Sustainable Rice Production

A sustainable increase in rice production is feasible with the development and advancement of technology through rice research (Peng et al., 2009). It is important to sustain rice production to achieve food security. According to Zhang (2007), there are several challenges to achieving sustainable rice production including, among others, the increasingly severe occurrence of diseases and pests.

Climate change is threatening a diverse range of plant species all around the world, including rice and other Poaceae members for example wheat, maize, sorghum (*S. bicolor*), and sugarcane (*Saccharum officinarum*). These in-demand monoculture crops suffer major loss of yield and quality from biotic and abiotic stresses, thus threatening future food and nutrition security (Cheng et al., 2022). Globally, sustainable crop production often aims to optimise production through sustainable management of biological processes, biodiversity, and ecosystem services, taking into account several important factors such as economic, political, social, and environment (Bennett et al., 2015; Brodt et al., 2011). In a narrower sense, sustainable rice productioncan be achieved by increasing the production per unit area using ecologically regenerative approaches that involve minimal inputs (Mohd Hanafiah et al., 2020; Sherwood & Uphoff, 2000; Tester & Langridge, 2010).

At present, sustainable agriculture systems are focused largely on the use of recyclable and renewable resources such as wind, solar, and biomass (Bos & Broeze, 2020; Chel & Kaushik, 2011). However, the utilisation of renewable resources in growing rice is still generally limited (Shiming & Gliessman, 2017). The key obstacle to the adoption of these resources is the capital and/or construction costs, which could be resolved with subsidies for rice farmers (Mohd Hanafiah et al., 2020; Siebrecht, 2020).

Promoting the use of renewable resources in rice fields can improve long-term

Universiti Malaya

environmental stewardship, especially with regard to maintaining soil quality (Alvarez et al., 2021; Chel & Kaushik, 2011). A dynamic rice production system should allow producers to choose and adopt the best combinations of practices based on their local environmental conditions and production constraints, achieving high levels of output with minimal inputs. One remarkable example is the system of rice intensification (SRI), which recommends sustainable agronomic practices, including the use of compost or organic fertilisers (Deb, 2020; Godfray et al., 2010). To promote the use of sustainable farming approaches by low- and medium-income rice farmers, some existing agricultural policies should be revised or renewed. The new policies should abolish subsidies that cause farmers to overuse resources (Theriault et al., 2018). For example, the incentives to promote the use of fertilisers to improve crop yields should be eliminated (Balázs et al., 2021; Tilman et al., 2002).

Universiti Malaya

CHAPTER 3: METHODOLOGY

The first part of this study involved a systematic review using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) protocol to identify the critical research gaps and future directions in rice research, as well as the potential utilisation of weedy rice in modern rice breeding programmes. The second part of the study involved the development of multiplex polymerase chain reaction (PCR)-assays for the identification of multiple genes controlling key traits in rice (including blast resistance, sheath blight (SB) resistance, bacterial leaf blight (BLB) resistance, fragrance, and amylose content), mainly for local varieties. The multiplex assays developed were then be validated and used to screen weedy rice biotypes from various regions in Malaysia. Greenhouse pathogenicity test screening was performed to validate the results from the molecular analyses.

3.1 Systematic Review

A comprehensive review was conducted to identify research gaps, future directions, and the potential utilisation of weedy rice. Online search were performed on February 2019 to collect published scientific articles, case studies, and reviews based on specific keywords via the Google Scholar search database, and other sources including various institution websites. Keywords, including, among others, ‘rice breeding”, “rice genetics”, “climate change”, “food security”, “green revolution’, “modern rice”, “molecular breeding”, “molecular markers”, “underutilised grains”, “weedy rice” were used in the selection of relevant articles. The search also included on literature relevant to trending topics in agriculture, such as climate change and food security.

The systematic review of collected literature was performed in accordance with the PRISMA model's guidelines for depicting the flow of information (Figure 3.1). The

Universiti Malaya

PRISMA model was utilised because it contains extensive information on the records thatwere identified, included, and excluded from the study after screening, as well as the reasons for their exclusion from the final study (prisma-statement.org).

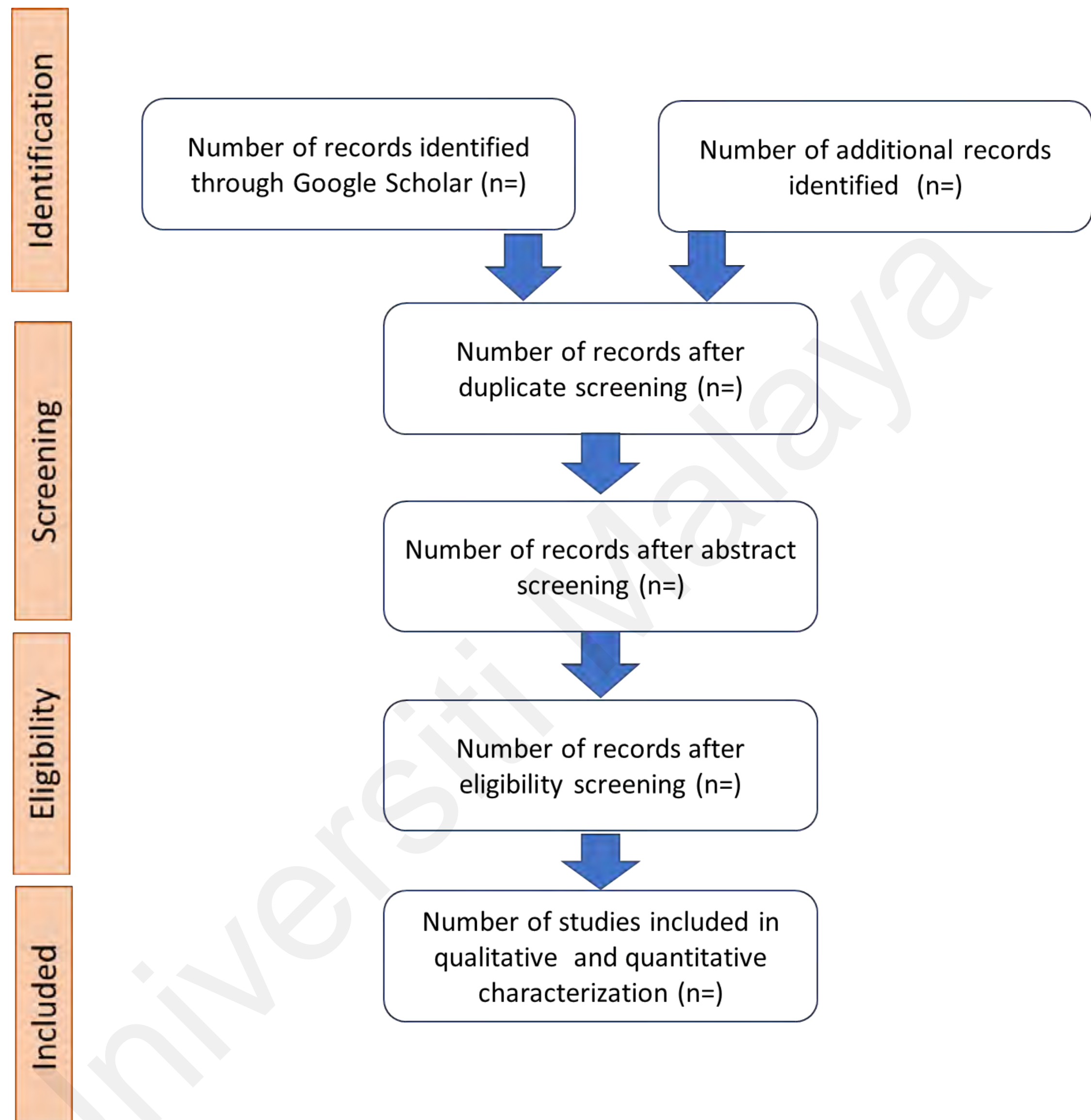

Figure 3.1: **General flow of systematic review of literature search from identification, screening, eligibility and included based on PRISMA flow diagram. The flow was adapted from https://www.prisma-statement.org accessed on February 2019**

3.2 Plant Materials

A total of 14 rice varieties, including 11 local cultivars and 3 international varieties with a wide range of morphological and physiological characteristics, were used in this study. Seeds were obtained from the Malaysian Agricultural Research and Development Institute (MARDI),Seberang Perai. Local check varieties for biotic stresses and quality traits used include MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, and MRQ74. Rice plants were grown in a greenhouse in Rimba Ilmu, Universiti Malaya (3.1311° N, 101.6578° E). Each of plants was watered every day and fertilised with nitrogen, phosphorus and potassium (NPK) fertiliser once per month.

A total of 100 weedy rice biotypes were also used for molecular analyses. Sampling of weedy rice was conducted during harvesting periods from January to June of 2019 at rice fields in Pekan, Pahang (3°56'N, 103°36'E), Sungai Burung (3°49'N, 101°16'E), Sungai Leman (3°54'N, 101°08'E), and Sungai Besar (3°46'N, 101°20'E), Selangor, and Seberang Perak, Perak (4°07'N, 100°86'E). Weedy rice was determined when it possessed at least one of these characteristics: pigmented pericarp, black or furrowed hull, shattered seeds, presence of awn, and tall stature (>100 cm). Seeds of the samples were then hand-threshed directly in the field and immediately placed in a paper bag separately for each individual. Seed samples were cleaned and separated from extraneous materials, air-dried in the greenhouse for 3 d, equally separated into two bags and stored in -20 °C freezer to prevent after-ripening.

3.3 Identification and Validation of Molecular Markers

A total of 8, 11, and 9 allele-specific markers associated with BLB, SB, and blast resistance, respectively, were identified and selected from literature using PubMed and Gene in National Center for Biotechnology Information (NCBI) databases

(https://www.ncbi.nlm.nih.gov). The key selection criteria included the inclusion in previous rice breeding programmes, degree of polymorphisms and the availability of rice genotyping data in the Gramene databases (https://www.gramene.org). For fragrance and amylose content traits, gene-specific primers, *fgr*-SNP and *Waxy* previously validated to show functional polymorphisms on local cultivars by Cheng et al. (2015), were used to detect *fgr* and *Wx* genes in rice, respectively. The details of the selected primers are presented in Table 3.1. All primers were purchased from Integrated DNA Technologies (IDT) in Malaysia. The identified markers associated with targeted traits were then be used to screen weedy rice populations collected from various regions in Malaysia.

3.4 Genomic DNA Extraction and Quality Check

Genomic deoxyribonucleic acid (DNA) was extracted from approximately 25 mg of 4-week-old rice leaves of each selected varieties using FavorPrep™ Plant Genomic DNA Extraction Mini Kit according to the manufacturer's protocol (Favorgen, Taiwan). Firstly, plant tissue was ground under liquid nitrogen to a fine powder and transferred to a new microcentrifuge tube. Secondly, 400 µL of FAPG1 Buffer and 8 µL of RNase A stock solution (50 mg/ml) were added and then vortex vigorously. The mixture then incubated at room temperature for 2 min and then at 65°C for 10 ~ 20 min and inverted 2-3 times during incubation. Thirdly, 130 µl of FAPG2 Buffer was added to the mixture. The mixture was then vortex to mix well and incubated on ice for 5 min. Fourth, Filter Column was placed to Collection Tube and the entire mixture from the previous step was transferred to the Filter Column. The Filter Column was then centrifuged at full speed (14,000 rpm) for 3 min. Fifth, the clarified lysate (supernatant) was transferred from the Collection Tube to a new microcentrifuge tube and the volume of clarified lysate was adjusted. Sixth, 1.5 volume of FAPG3 Buffer (ethanol added) was added to the clarified

Table 3.1: Details of the selected genes and primers

Trait	Gene variants/ QTLs	Primer	Forward primer	Reverse primer	Expected size (bp)	References
Blast resistance	*Pi-1*	MRG4766	ATTGCTGCAAAGTGGGAGAC	AAGTGGAGGCAGTTCACCAC	104	Guan et al. (2019)
	Pi-2	AP22	GTGCATGAGTCCAGCTCAAA	GTGTACTCCCATGGCTGCTC	143	Fu et al. (2012)
	Pi-7	RM229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	116	Hasan et al. (2018)
	Pi-kh	RM206	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	147	Tanweer et al. (2015)
	Pi-b	RM208	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC	173	
	Piz, Pi2, Pi9	RM6836	TGTTGCATATGGTGCTATTTGA	GATACGGCTTCTAGGCCAAA	240	Miah et al. (2016)
	Piz	RM8225	ATGCGTGTTCAGAAATTAGG	TTGTTGTATACCTCATCGACAG	221	
	Pi	RM168	TGCTGCTTGCCTGCTTCCTTT	GAAACGAATCAATCCACGGC	116	Ashkani et al. (2011)
	Pi	RM5961	GTATGCTCCTCCTCACCTGC	ACATGCGACGTGATGTGAAC	129	
Fragrance	*Fgr*	*fgr*-SNP	EAP -AGTGCTTTACAAAGTCCCGC ESP -TTGTTTGGAGCTTGCTGATG IFAP -CATAGGAGCAGCTGAAATATATACC INSP -CTGGTAAAAAGATTATGGCTTCA		250,350	Cheng et al. (2015)

Universiti Malaya

Table 3.1, continued

Trait	Gene variants/ QTLs	Primer	Forward primer	Reverse primer	Expected size (bp)	References
Sheath blight resistance	QRlH11	RM209	ATATGAGTTGCTGTCGTGCG	CAACTTGCATCCTCCCCTCC	134	Vidya and Ramalingam (2018)
	qSBR11-1	RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	157	
	qSBR9-1	RM257	CAGTTCCGAGCAAGAGTACTC	GGATCGGACGTGGCATATG	147	Hossain et al. (2016)
	QRlh7b	RM478	CAGCTGGGGAAGAGAGAGAG	TCAGAAACTAAACGCACCCC	205	
	qSBR11-3/QRlh11	RM202	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA	189	
	qshb9.2	RM205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	122	
		RM426	ATGAGATGAGTTCAAGGCCC	AACTCTGTACCTCCATCGCC	150	
		RM 6971	TTTGCGAACTAGACAAGGCC	GCGTCATTCTCGACGAGC	202	
	qSB9-2	RM215	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTGTAG	148	Shamim et al. (2017)
		RM245	ATGCCGCCAGTGAATAGC	CTGAGAATCCAATTATCTGGGG	150	
	qSB12-1.	RM227	ACCTTTCGTCATAAAGACGAG	GATTGGAGAGAAAAGAAGCC	124	

Table 3.1, continued

Trait	Gene variants/ QTLs	Primer	Forward primer	Reverse primer	Expected size (bp)	References
Bacterial leaf blight resistance	*Xa13*	Xa13-prom	GGCCATGGCTCAGTGTTTAT	GAGCTCCAGCTCTCTCCAAATG	R=450, S= 220	Hajira et al. (2016)
	Xa5	Xa5FM-R	AGCTCGCCATTCAAGTTCTT GAG	TGACTTGGTTCTCCAAGGCTT	R=424,134, S=424, 313	
		Xa5FM-S	GTCTGGAATTTGCTCGCGTT CG	TGGTAAAGTAGATACCTTATCA AACTGGA	R=424,134, S=424, 313	
	Xa21	pTA248	AGACGCGGAAGGGTGGTTC CCGGA	AGACGCGGTAATCGAAAGATG AAA	R=950, S=660; R=1200, S=800	Hajira et al. (2016); Kadu et al. (2018)
	Xa21	Xa21	GCTATTTCCTGATCCAGCAT ATCTGATC	GATCGGTATAACAGCAAAACT ATTTCC	R=595, S=467	Yap et al. (2016)
	Xa13	Xa13	TACCTCCTGATATGTGAGGT AGTGAGAG	AGAGAGAGGTAACTTGAAGAA AGGGAT	R=381, S=391	
	Xa7	Xa7F/Xa7-IR	GGTCGGAAGGTGAGAAAGA GGAGG	GCATGTCTGTGTCGATTCGTCC GTACGA	R=179, R=87	
	Xa7	Xa7F/Xa7-2R		GAGAGCGAACGTGGAGGCTTC TT		
	Xa5	RM13	TCCAACATGGCAAGAGAGA G	GGTGGCATTCGATTCCAG	219	Singh et al. (2015)
	Xa5	RM122	GAGTCGATGTAATGTCATCA GTGC	GAAGGAGGTATCGCTTTGTTGG AC	240, 230	Acharya et al. (2018)
Amylose content	*Wx*	*Wx*-SSR	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG	110,130	Cheng et al. (2015)

Universiti Malaya

lysate and mixed well by pipetting. Seventh, FAPG Column was placed to a new Collection tube and up to 750 µl of the sample mixture was carefully transferred to the FAPG Column then centrifuged at full speed (18,000 x g or 14,000 rpm) for 1 min. The flow-through was discarded and the FAPG Column was placed back to the Collection Tube. Next, 400 µl of Wash Buffer W1 (ethanol added) was added to the FAPG Column and then centrifuged at full speed (18,000 x g or 14,000 rpm) for 30 s.

The flow-through was discarded and the FAPG Column was placed back to the Collection Tube. Then, 650 µl of Wash Buffer W2 (ethanol added) was added to FAPG Column and centrifuged at full speed (18,000 x g or 14,000 rpm) for 30 s. The flow-through was discarded and the FAPG Column was placed back to the Collection Tube. This step was repeated for one more washing. Next, the Collection Tube was centrifuged at full speed (18,000 x g or 14,000 rpm) for an additional 3 min to dry the FAPG column completely. Finally, the FAPG Column was combined with Elution Tube, purified DNA was eluted with 50-200 µl of pre-heated Elution Buffer by adding it to the membrane center of the FAPG Column. Then, the Elution Tube was centrifuged at full speed (18,000 x g or 14,000 rpm) for 1 min to elute purified DNA which then stored at -20°C.

The purity of the extracted DNA samples was evaluated using NanoDrop 2000 UV-Vis spectrophotometer (Thermofisher Scientific, USA), while the quality of the samples was assessed using a 1% agarose gel stained with SYBR® Green (Sigma Aldrich, Germany). A 1 kb DNA ladder (Bioron, Germany) was used as marker to determine the fragment size. 3 µl of loading dye and 5 µl of genomic DNA samples were loaded into the gel. The gel electrophoresed at 100 V for 60 min. The gel was visualised under AlphaImager Mini Imaging System (ProteinSimple, USA).

Universiti Malaya

3.5 Amplification of Targeted Genes

Fragrant, >25% amylose and non-fragrant, <25% amylose checks include MRQ74 and MR219, respectively. Resistant check of MR219, Mahsuri Mutant and Pulut Hitam 9 were used for disease resistance trait of BLB, Blast and SB, respectively. The details of local checks and expected size for each of the traits are shown in Table 3.2.

Table 3.2: Local checks and expected size of PCR products

Trait	Primer	Positive control	Description	Expected product size (bp)	Negative control	Description	Expected product size (bp)
Amylose levels	*Wx*-SSR	MRQ74	>25% Amylose	~110	MR219	<25% amylose	~130
Fragrance	*fgr*-SNP	MRQ74	Fragrant	~250	MR219	Non-fragrant	~350
Bacterial leaf blight	pTA248	MR219	Resistant	~700	Ria	Susceptible	~600
Blast	RM6836	Mahsuri Mutant	Resistant	~230	MR219	Susceptible	~250
	RM8225	Mahsuri Mutant	Resistant	~200	MR219	Susceptible	~220
Sheath blight	RM202	Pulut Hitam 9	Resistant	~160	MR219	Susceptible	~180

3.5.1 Uniplex PCR

Uniplex PCR was performed on all selected allele-specific markers using 10 µL premixed ready-to-use 2X Go Taq Green PCR master mix (Promega, USA), <50 ng template DNA, 0.4 µM of each primer in 20 µL reaction with a thermal profile modified from Cheng et al. (2015) of 95°C for 4 min (initial denaturation), followed by 34 cycles of denaturation at 94°C for 0.75 min, annealing at 55°C for 0.75 min, extension at 72°C for 0.75 min and a final extension of 5 min at 72°C.

3.5.2 Development and Optimization of Multiplex PCR

A total of eight multiplex PCRs were performed with two or more primer pairs associated with at least two targeted traits (Table 3.3), whereby positive and negative controls had different banding patterns. For the multiplex PCR, 10 µL premixed ready-to-use 2X Go Taq Green PCR master mix (Promega, USA), <50 ng template DNA, 0.4

Universiti Malaya

μM of each primer in 20 μL reaction with thermal protocol of 94 °C for 5 min (initial denaturation), followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension of 5 min at 72°C (McCouch et al., 2002). The amplification was performed using Veriti 96-well thermal cycler (Applied Biosystems, USA).

Table 3.3: Details on multiplex assays developed

Number of primer sets	Multiplex developed	Primer (type)	Traits of interest
Two primer sets	Multiplex-1	*Wx*-SSR (SSR) *fgr*-SNP (SNP)	Amylose content Fragrance
	Multiplex-2	*Wx*-SSR (SSR) pTA248 (STS)	Amylose content BLB resistance
	Multiplex-3	*fgr*-SNP (SNP) pTA248 (STS)	Fragrance BLB resistance
	Multiplex-4	*fgr*-SNP (SNP) RM8225 (SSR)	Fragrance Blast resistance
	Multiplex-5	*fgr*-SNP (SNP) RM202 (SSR)	Fragrance SB resistance
Three primer sets	Multiplex-6	RM202 (SSR) RM6836 (SSR) pTA248 (STS)	SB resistance Blast resistance BLB resistance
	Multiplex-7	RM202 (SSR) RM8225 (SSR) pTA248 (STS)	SB resistance Blast resistance BLB resistance
Four primer sets	Multiplex-8	*fgr*-SNP (SNP) RM8225 (SSR) RM202 (SSR) *Wx*-SSR (SSR)	Fragrance Blast resistance SB resistance Amylose content

BLB- bacterial leaf blight, SB- sheath blight

3.6 Analysis of Polymorphisms

3.6.1 Gel Electrophoresis

The uniplex products were separated by 3% standard agarose gel in 1X TAE buffer (Cheng et al., 2015) at 100 V for 150 min, stained with SYBR safe (Invitrogen, USA). The size of the amplicons was estimated by comparisons to 50-bp (SMOBIO, Taiwan) and 100-bp DNA ladders (Bioron, Germany). The gel was prepared by a slow sprinkle of 4.5 g agarose (Hydragene, USA) into 150 ml 1x TAE buffer with rapid stirring. The agarose was soaked in the buffer for 15 min before heating to prevent the solution from

Universiti Malaya

foaming during heating in microwave. Samples were visualized under AlphaImager Mini Imaging System (ProteinSimple, USA). To validate the results of uniplex PCRs, amplified products from control varieties were sent to a commercial sequencing facility and sequenced using the ABI3100 DNA sequencer (Applied Biosystems, USA).

On the other hand, multiplex products were separated by 4% high-resolution agarose gel (Gene Xpress, Malaysia) in 1X TAE buffer at 120 V for 180 min, stained with SYBR safe (Invitrogen, USA). Following several tests with various agarose percentages, 4% high resolution agarose was used because the gel lower than 3.5% barely separated the alleles. Similar to uniplex analysis, the size of the amplicons was estimated by comparisons to 50-bp (SMOBIO, Taiwan) and 100-bp DNA ladders (Bioron, Germany). The high-resolution gel was prepared by a slow sprinkle of 6 g of high-resolution agarose (Gene Xpress, Malaysia) into 150 ml of 1x TAE buffer with rapid stirring. Multiplex products were also visualised under AlphaImager Mini Imaging System (ProteinSimple, USA). Figure 3.2 shows the general overview of gel electrophoresis-based uniplex and multiplex PCRs performed in this study.

Universiti Malaya

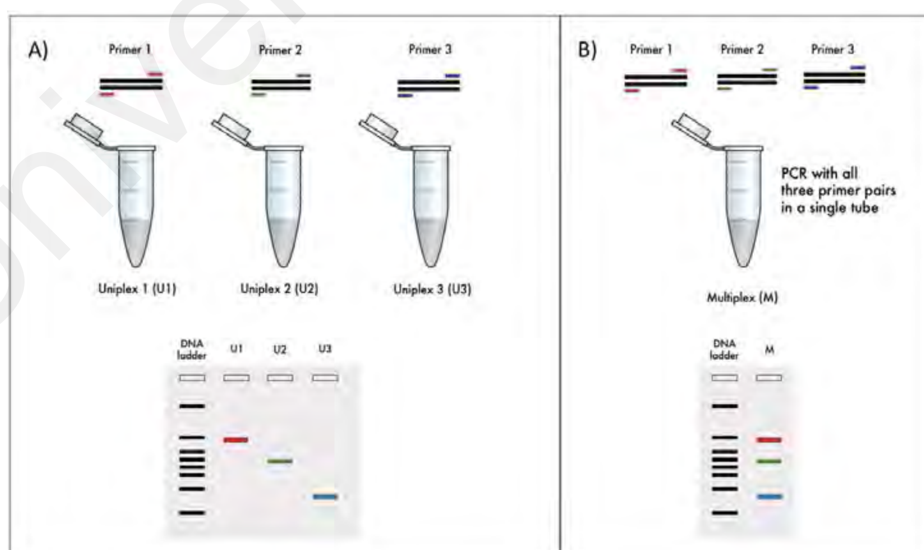

Figure 3.2: Overview of gel electrophoresis-based using three different primers. Individual DNA sample was screened with single primer set per reaction as Uniplex (A) and multiplex (B) PCR

3.6.2 Capillary Electrophoresis and Sequencing

From the results of high-resolution gel electrophoresis, multiplex products of three varieties of rice (MR219, Mahsuri Mutant and Pulut Hitam 9) reflecting a wide range of genotypes, were used to evaluate allelic variation of RM202 (associated with SB) and RM6836 (associated with blast) using capillary fluorescence electrophoresis. Fluorescently fragments were detected using ABI3100 Genetic Analyzer (Applied Biosystems, USA) multi-colour fluorescence-based DNA analysis system with capillary electrophoresis.

The peak values of each primer were scored as resistant or susceptible for all genotypes. Fluorescence HEX marked (green) RM6836 and NED marked (yellow) RM202, which are the molecular markers tightly linked to the blast resistance and SB resistance genes, respectively, were added to the PCR reaction mixture at the level of 0.4 uM in order to obtain peaks at capillary electrophoresis. The results were interpreted using the GeneMapper 4.0 analysis software (Applied Biosystems, USA) to size the alleles to the nearest base pair.

To validate the results obtained from both high-resolution gel and capillary analyses, a set of amplified products was sent to a sequencing company where the sequencing reactions were run on an ABI3100 DNA sequencer (Applied Biosystems, USA).

3.7 Genotyping of Weedy Rice Biotypes

A total of 100 weedy rice biotypes collected across Malaysia were screened using one agarose-based multiplex PCR assays (Multiplex-7 in Table 3.3) developed in this study. Genomic DNA from 4-week-old fresh leaves were extracted from each individual weedy biotype based on manufacturer's protocol (Favorgen, Taiwan) as described in Section 3.4. The results from the molecular analysis were confirmed by pathogenicity test using 15% of the weedy rice biotypes.

Universiti Malaya

3.8 Phenotyping for Biotic Stress Resistance

3.8.1 Blast Resistance

3.8.1.1 Inoculum Preparation of *Magnaporthe oryzae*

For growing the pathotype of *M. oryzae*, potato dextrose agar (PDA) (39 g/l) was prepared by mixing with distilled water and then autoclaved., The solution was poured into 9-cm-diameter Petri dishes in the laminar flow cabinet. To avoid contamination, dishes were sealed properly. Slants with fungal growth provided by MARDI Seberang Perai were chosen and used as master cultures. The contaminated slants were removed. Conidia were induced by scraping the mycelia on the culture with a toothbrush. To induce sporulation, the colonies were exposed to fluorescent light at room temperature and covered with wet cotton muslin cloth for 5 days. The inoculums were made following Hayashi et al. (2009). The aerial mycelia were gently rubbed with a water-soaked paintbrush. The brush was then soaked in sterile distilled water. The spore suspensions were filtered through nylon gauze mesh and adjusted with a haemocytometer to a concentration of 1 X 10^5 spores/ml. Before inoculation, 0.02 % Tween 20 was added to the suspension to improve spore adhesion to the plant. Figure 3.3 shows the inoculum preparation procedures to make the conidial suspension.

3.8.1.2 Inoculation of *Magnaporthe oryzae*

A total of 15 weedy rice biotypes were selected for screening based on genotype data. The selected weedy rice and local rice checks, Mahsuri Mutant and MR219, were screened against a highly virulent strain of *Magnaporthe oryzae*. Inoculum was prepared following protocol by Hayashi et al. (2009). Seedlings at 5 leaf stage were inoculated by spraying with a conidial suspension of 10^5 (100,000) conidia per ml determined by a hemacytometer. A drop of Tween 20 at final concentration approximately 0.02% was

added to aid in adhesion of the inoculum to the leaves. Reactions towards blast disease were classified according to standard evaluation system by International Rice Research Institute (IRRI, 1996) by scoring 0-2 being resistant, 3 being moderately resistant and 4-5 being susceptible. The susceptible cultivar variety was used as the control check.

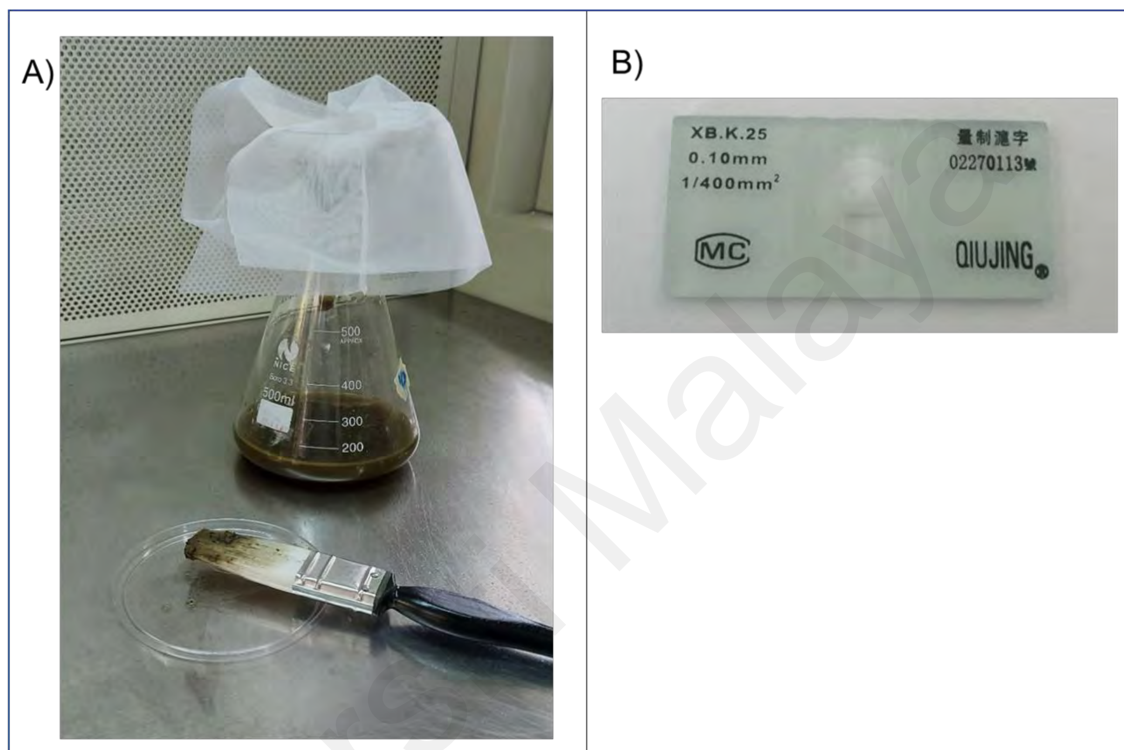

Figure 3.3: Inoculum preparation of *Magnaporthe oryzae* for rice blast pathogenicity screening. A) Washing off mycelia in sterile water for spore suspension and B) using a haemocytometer to adjust to a concentration of 1 X 10^5 spores per ml under light microscope before inoculation.

3.8.2 Sheath Blight (SB) Resistance

3.8.2.1 Inoculum Preparation of *Rhizoctonia solani*

The isolate of *R. solani* used was provided by MARDI, Seberang Perai. Inoculum was prepared following protocol by Jia et al. (2007). Sclerotia of *R. solani* was added on potato dextrose agar (PDA) (HiMedia, India) containing tetracycline (0.005%, wt/vol) and the mycelium was transferred to a new medium for growing the active mycelia. Each isolate was kept on PDA in Petri dishes between 27 to 30ºC. Inoculum consisted of 1 cm diameter

University of Malaya

round disks taken from a 3-day-old mycelia culture grown in PDA containing tetracycline (0.005%, wt/vol) in Petri dishes. Each Petri dish contained 20 ml of PDA.

3.8.2.2 Inoculation of *Rhizoctonia solani*

Microchambers were created by removing the screw caps and bottoms of 1.5-liter plastic drinking bottles. To guarantee that the presence of soilborne *R. solani* inoculum did not affecting the results of the study, the soil was first sterilised with steam. Each seedling was inoculated with a mycelial disc that was put at the base of the stem and forced up, ensuring that mycelium was in contact with the plant. After inoculation, each pot was covered with the bottle microchamber that was pushed into the soil to create a seal, allowing the bottle to function as a micro-humidity chamber (Jia et al., 2007). The cap was left off the bottle. Figure 3.4 shows bottle microchambers used after inoculation of *Rhizoctonia solani*. Plants were grown in ~30– 32°C under natural light in standard greenhouse conditions (Nadarajah et al., 2014). Reactions towards sheath blight disease were classified according to standard evaluation system by IRRI (1996) by scoring 0-2 being resistant, 3 being moderately resistant and 4-5 being susceptible and by measuring the lesion length. The susceptible cultivar variety was used as the control check.

Universiti Malaya

Figure 3.4: Bottle microchambers used after inoculation of *Rhizoctonia solani* for sheath blight pathogenicity screening

3.8.3 Bacterial leaf blight (BLB) resistance

3.8.3.1 Inoculum preparation *Xanthomonas oryzae*

Selected weedy rice biotypes were screened against *Xanthomonas oryzae* pv. *oryzae* (Xoo). The Xoo isolate used for this study was the MXO1573 isolate. This isolate was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Seberang Perai. The viscous and yellow Xoo colonies were subcultured onto nutrient agar (NA) medium and grown for 72 h at 28°C. Xoo were subcultured two days before inoculating rice plants by transferring active Xoo from the original solid NA media to a fresh solid NA media and incubating at 28 °C for an additional 48 h (Ke et al., 2017). The bacterial mass was then suspended in deionised water on the day of inoculation. The suspension was adjusted to 10^8 colony forming units (CFU)/ml from absorbance measurements (A 600 = 0.3) (Sutrisno et al., 2018). Xoo was scraped from the Petri dish

Universiti Malaya

and put to a falcon tube containing 50 mL of sterile water. The concentration of Xoo suspension was 10^8 colony forming units (CFU)/ml.

3.8.3.2 Inoculation of *Xanthomonas oryzae*

Inoculation was performed in the afternoon between 03.00 and 05.00 p.m to avoid high environmental heat and evaporation. The bacterial inoculation was carried out using the leaf clipping method described by Kauffman et al. (1973). The sterile scissors were dipped into Xoo bacterial suspension. Then, the scissors were used to cut the rice leaf at 5 cm from the leaf tip of the cultivars and weedy rice plants. Figure 3.5 shows inoculation of *X. oryzae* by leaf clipping method.

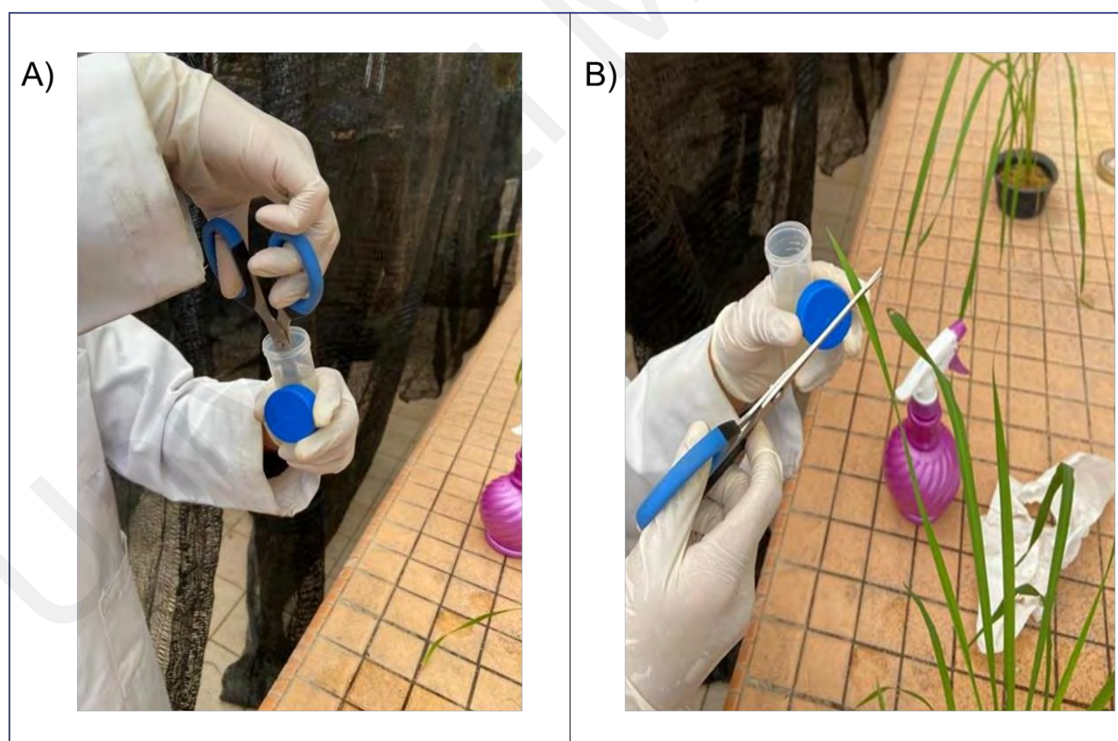

Figure 3.5: Inoculation of *Xanthomonas oryzae* v. *oryzae* (Xoo) to weedy rice plants to screen bacterial leaf blight pathogenicity. A) Dipping scissor tips into the Xoo suspension; B) Cutting the leaf tip away from the leaf.

Universiti Malaya

Reactions towards bacterial leaf blight disease were classified according to standard evaluation system by scoring 0-2 being resistant, 3 being moderately resistant and 4-5 being susceptible and by measuring the lesion length. In the greenhouse, disease severity was assessed based on lesion length measurement or estimation of diseased leaf area (Arunakumari et al., 2016). BLB lesion length was measured and the disease score was calculated as per IRRI standard evaluation system scale (IRRI, 1996).

3.9 Data Analysis

Genotype data were used to calculate observed allele number per sampling location (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Shannon's diversity index (I) using GenAlEx 6 (Peakall & Smouse, 2006). Principal Coordinate Analysis (PCoA) was also performed in GenAlEx 6 using a genetic distance matrix constructed from CS Chord distance values (Cavalli-Sforza & Edwards, 1967). Clustering patterns were analysed as they related to the genotype of biotic stresses of weedy rice biotypes and locations of sampling sites.

Universiti Malaya

CHAPTER 4: RESULTS

This chapter presents the findings from three major parts of the current study: a systematic review on rice research and development (Section 4.1), molecular analyses of major rice disease resistance and grain quality traits using uniplex and multiplex PCRs (Section 4.2), and screening of Malaysian weedy rice biotypes (Section 4.3).

4.1 Systematic Review

4.1.1 Initial Identification and Screening of Records

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) protocol was adapted to provide a comprehensive review of the literature about cultivated and weedy rice research. A total of 507 articles were identified and collected (Figure 4.1) from Google Scholar search [n = 477], as well as from other sources such as organisation websites [n = 18], and university or government websites [n = 12]. The abstracts of the articles were carefully screened, and those that did not highlight rice research and potential utilisation of weedy rice and other underutilised grains were excluded. The abstracts that met the study objective were then thoroughly reviewed, and those that did not contain information were excluded. Additionally, articles written for conferences or based on conversations were excluded, as were duplicates and conference proceedings. Articles that were deleted for reasons other than duplication were kept for future reference and re-evaluation. There were 239 articles left after initial screening and exclusion (Figure 4.1), and the literature was evaluated further during full-text analysis.

Universiti Malaya

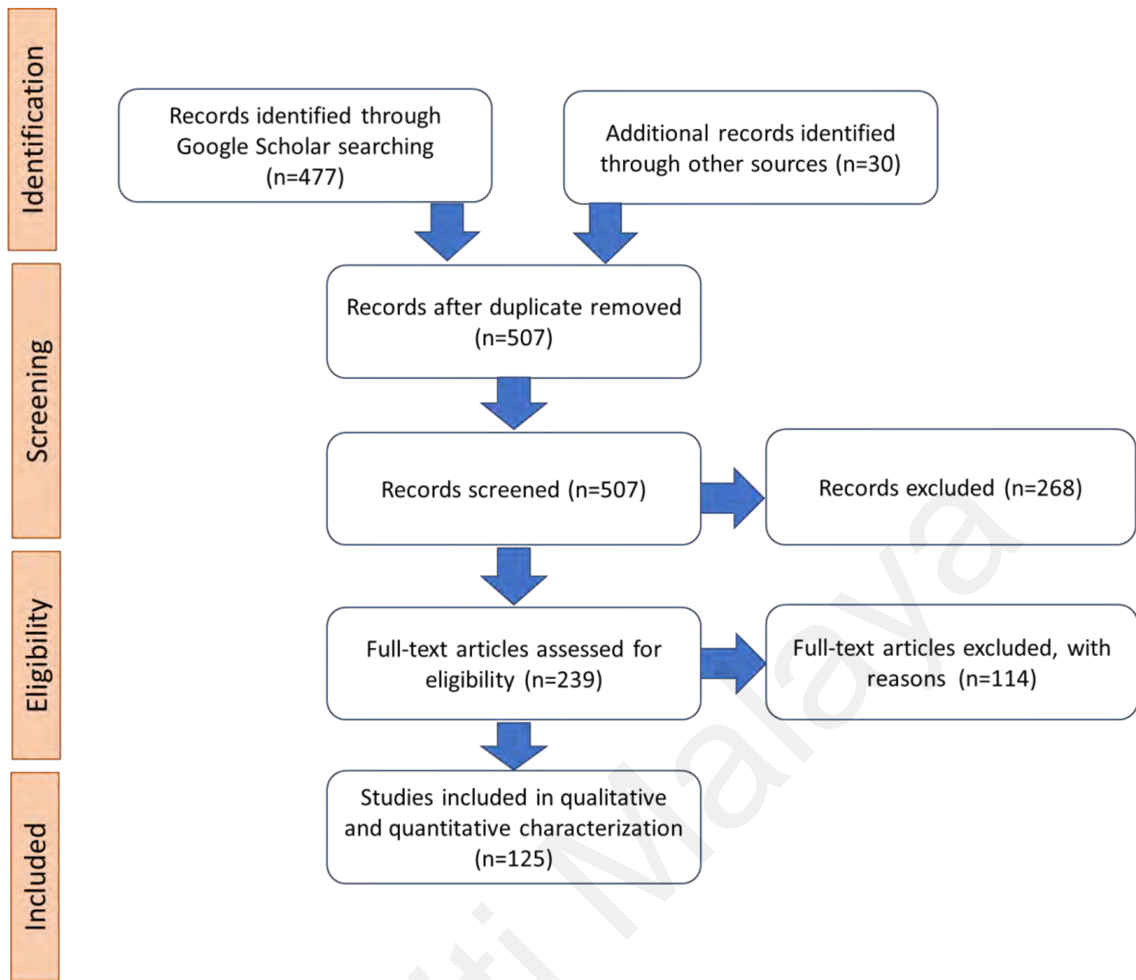

Figure 4.1: Number of articles (n) at each stage of PRISMA analysis

4.1.2 Eligibility Assessment of Selected Documents

The introduction, result analysis, and discussion chapters of selected articles were scrutinised for relevance to the study, including gaps in rice research since the green revolution, future directions of sustainable rice production, and the possible use of weedy rice and other underutilised grains (Mohd Hanafiah et al., 2020). In addition to relevance towards these key issues, selection of suitable articles was done based on the criteria list below (Table 4.1).

Universiti Malaya

Table 4.1: Criteria list for inclusion and exclusion of selected articles in eligibility assessment. Source: Salleh et al. (2020)

Criterion	Inclusion	Exclusion
Type of document	Open-access research article	Non-open access research articles, conference proceedings & background documents, editorial notes and magazine articles
Language of research	English	Non-English
Document page number	<30 pages	>30 pages

Due to relevance to the aforementioned key issues identified in relation to the study objective, 125 records were deemed eligible for review in the qualitative and quantitative analysis stage, from 239 initially screened articles. 114 articles were excluded due to their lack of relevance to the issues of interest (Figure 4.1). The majority of the articles obtained involved studies from Asia, including Malaysia, China, India Korea, and Japan.

4.1.3 Quantitative and Qualitative Analyses

Using the selected articles, quantitative and qualitative analyses were conducted in order to evaluate the records based on the major themes of the review (Figure 4.2). Rice research during the green revolution was the most frequently researched subject (32%), followed by rice research in the 21st century (20%). Other researchers were also interested in the potential use of weedy rice (13%) and underutilised grains (18%) to aid future rice research (Figure 4.2).

The major milestones in rice research since the green revolution were identified through the systematic review, as shown in Figure 4.3. Rice research advanced significantly following the completion of its genome sequence in the early 2000s (Mohd Hanafiah et al., 2020)

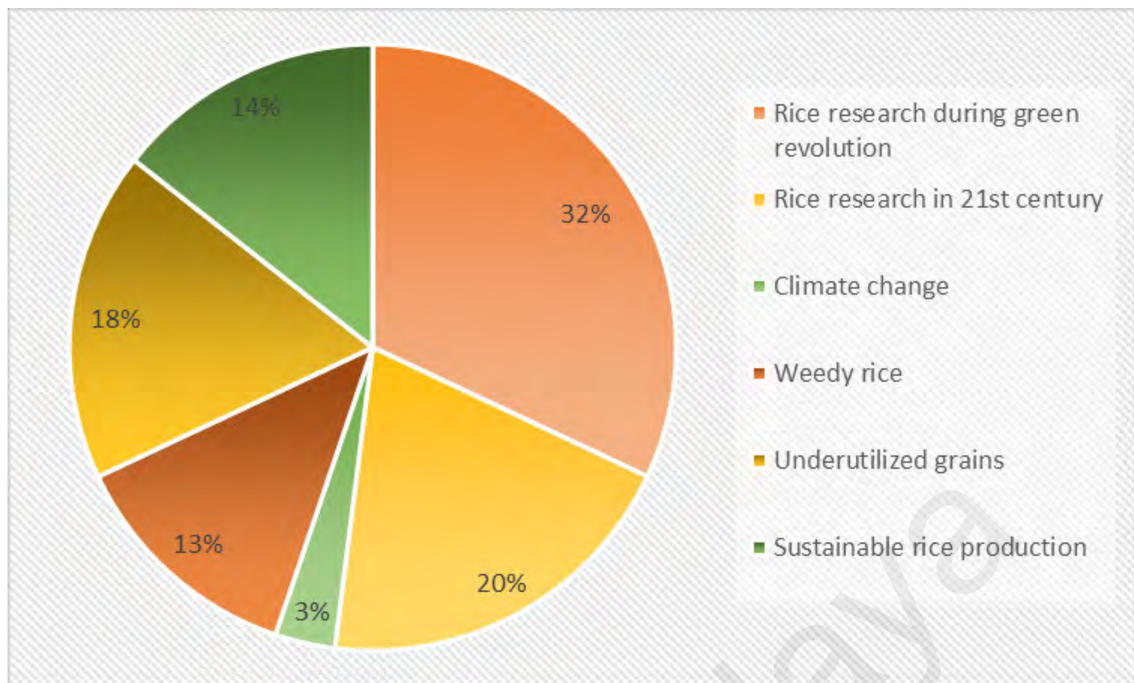

Figure 4.2: The major focus themes of research articles obtained

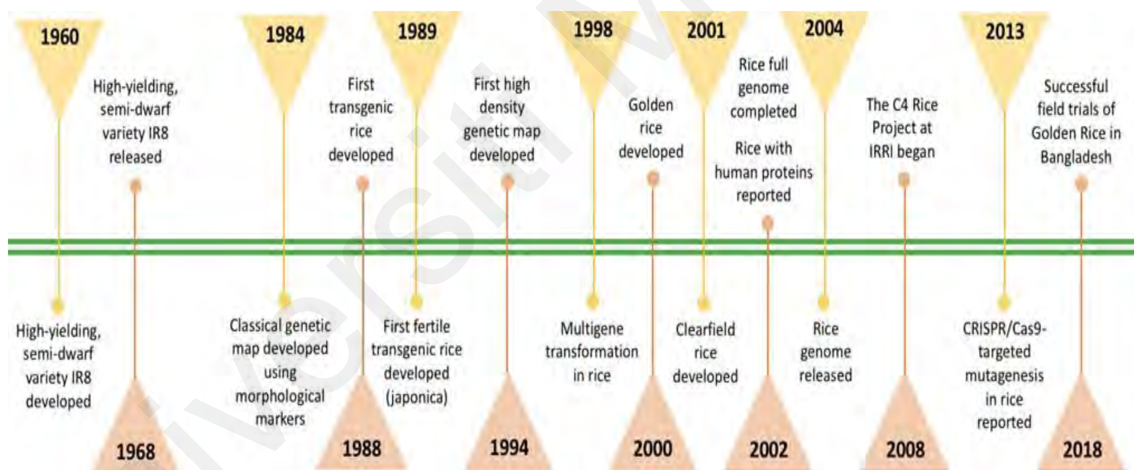

Figure 4.3: Major milestones in rice research since green revolution. Source: Mohd Hanafiah et al. (2020)

Additionally, the large-scale literature search was also used to identify the future directions in rice research. Figure 4.4 summarises the possible strategies for sustainable rice production.

University Malaya

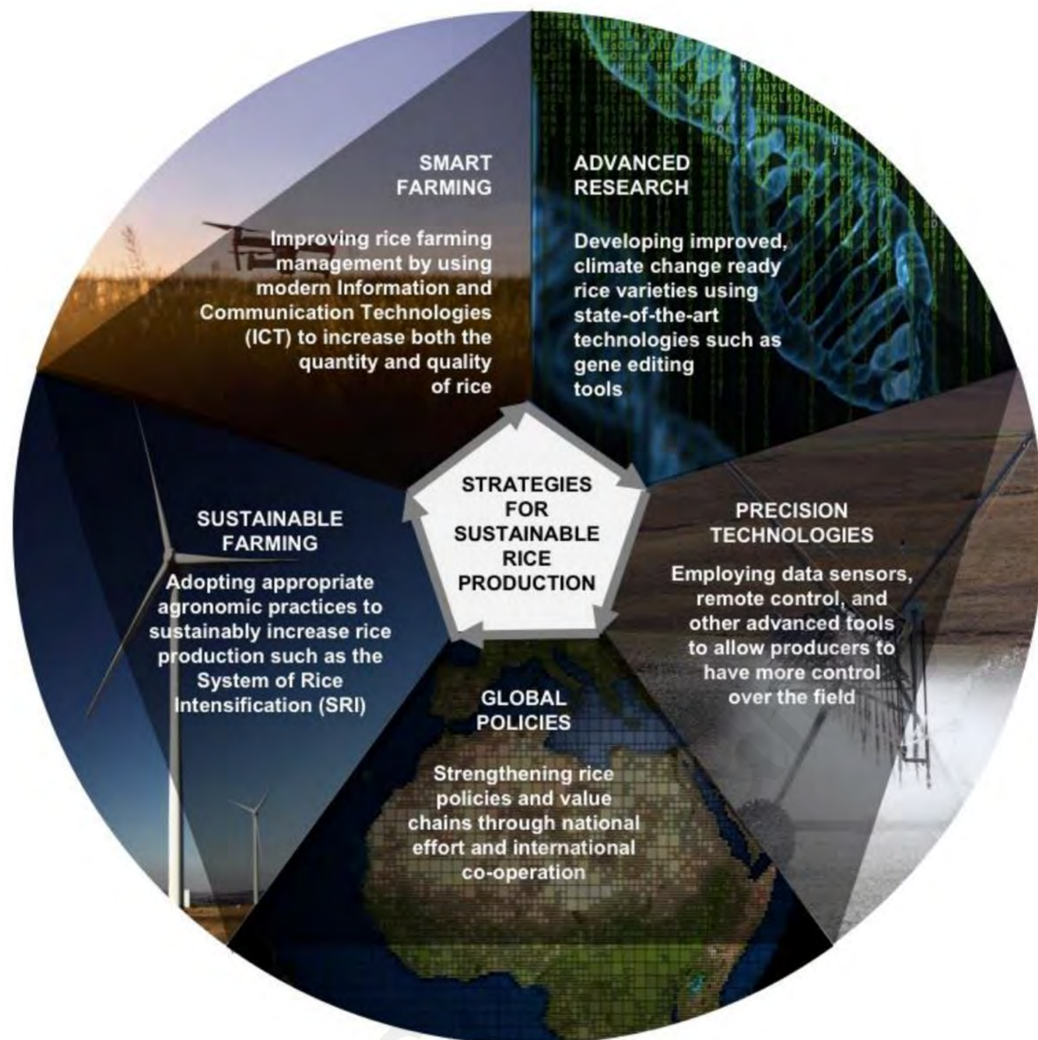

Figure 4.4: Plausible strategies to achieve sustainable rice production. Source: Mohd Hanafiah et al. (2020)

4.2 Molecular Analyses

4.2.1 DNA Quality Check and Quantification

The quality of several DNA samples extracted using FavorPrep™ Plant Genomic DNA Extraction Mini Kit according to the manufacturer's protocol (Favorgen, Taiwan) on a 1% agarose was demonstrated in Figure 4.5. The DNA purity, was determined using NanoDrop 2000 (Thermofisher Scientific, USA) based on the 260/280 ratio, with purity ranging from 1.70 to 2.00 (Särkinen et al., 2012). Table 4.2 displays the purity value of selected rice samples demonstrating that the samples were of good purity.

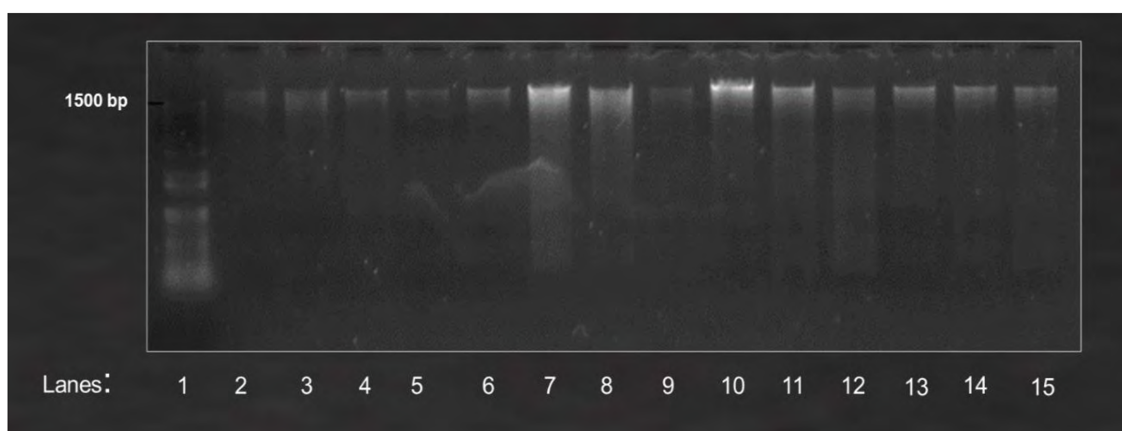

Figure 4.5: Genomic DNA samples separated using 1% agarose gel electrophoresis at 100 V for 1 h. Lane 1: 1 kb DNA ladder, Lanes 2 -15: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

Table 4.2: Purity readings of extracted DNA samples of selected rice varieties

Variety	**Nucleic acid (ng/µl)**	**260/280**
MR219	42.5	1.74
Mahsuri Mutant	51.6	1.75
Pulut Hitam 9	43.0	1.75
Ria	41.0	1.78
MR167	59.0	1.78
MR185	63.9	1.72
MR220	67.5	1.75
MR106	49.4	1.78
MRQ74	52.2	1.75
Pulut Malaysia 1	36.1	1.73
Biris	40.4	1.76
IR8	44.1	1.79
Chianung Sen Yu	33.9	1.79
Tainan 3	38.6	1.73

4.2.2 Uniplex PCR Analysis

A total of 30 sets of published allele-specific markers (Table 3.1) were selected and tested via uniplex PCR. Ten primers demonstrated functional polymorphisms on local checks (Hossain et al., 2016; Huang et al., 2017; Miah et al., 2016). Uniplex PCR products in this study were electrophoresed on 3% standard agarose gels and visualised under the AlphaImager Mini Imaging System (ProteinSimple, USA).

Universiti Malaya

4.2.2.1 Uniplex Genotyping for Blast Resistance

A total of four of the nine molecular markers linked to blast resistance (RM206, RM208, RM6836, and RM8225) were functional polymorphic based on resistant and susceptible local check Mahsuri Mutant and MR219, respectively. The amplicon size results for each of the markers tested on the selected rice varieties are shown in Table 4.3. The primer RM6836 and RM8225 were chosen for multiplex analysis because they revealed clear polymorphisms on local checks (Figure 4.6 and Figure 4.7) and amplified regions were comparable to previous research by Miah et al. (2016).

Table 4.3: Uniplex PCR product sizes for molecular markers linked to blast resistance based on agarose gel image

Varieties	Molecular markers and product sizes (bp)								
	RM206	**RM208**	**MRG476**	**AP22**	**RM229**	**RM6836**	**RM8225**	**RM168**	**RM5961**
MR219	~150	~200	~100	~140	~120	~250	~220	~100	~140
Mahsuri mutant	~170	~180	~100	~140	~120	~230	~200	~100	~140
Pulut Hitam 9	~150	~200	~100	140	~120	~230	~200	~100	~140
Ria	~170	~200	~100	~140	~140	~230	~200	~100	~140
MR167	~150	~200	~100	~140	~140	~230	~200	~100	~130
MR185	~150	~200	~100	N/A	N/A	~230	~200	~100	~140
MR220	~150	~200	~100	~140	~120	~250	~220	~100	~140
MR106	~150	~200	~100	~140	~120	~230	~200	~100	~140
MRQ74	~170	~180	~100	~140	~120	~230	~200	~100	~130
Pulut Malaysia 1	~170	~200	~100	N/A	N/A	~230	~200	~100	~140
Biris	~150	~180	~100	N/A	N/A	~250	~220	~90	~130
IR8	~150	~200	~100	~140	~120	~230	~200	~90	~140
Chianung Sen Yu	~170	~200	~100	N/A	N/A	~230	~200	~100	~140
Tainan 3	~150	~180	~100	N/A	N/A	~250	~220	~90	~130

4.2.2.2 Uniplex Genotyping for Sheath Blight (SB) Resistance

A total of four of the eleven molecular markers linked to sheath blight (SB) resistance (RM202, RM205, RM6971, RM245) were functional polymorphic based on resistant and susceptible local check, Pulut Hitam 9 and MR219, respectively. Table 4.4 shows the

Universiti Malaya

amplicon size results for each of the markers tested. RM202 marker was chosen for multiplex analysis as it demonstrated functional polymorphisms on local checks (Figure 4.8). The amplified regions for this marker were comparable to the study conducted by Hossain et al. (2016).

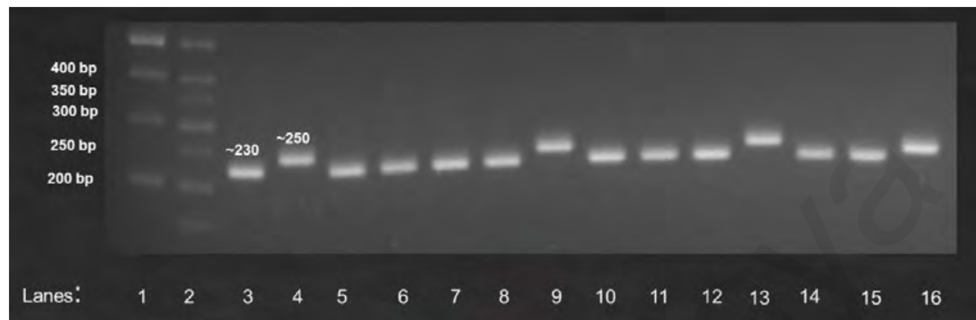

Figure 4.6: Amplified RM6836 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: Mahsuri Mutant, MR219, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

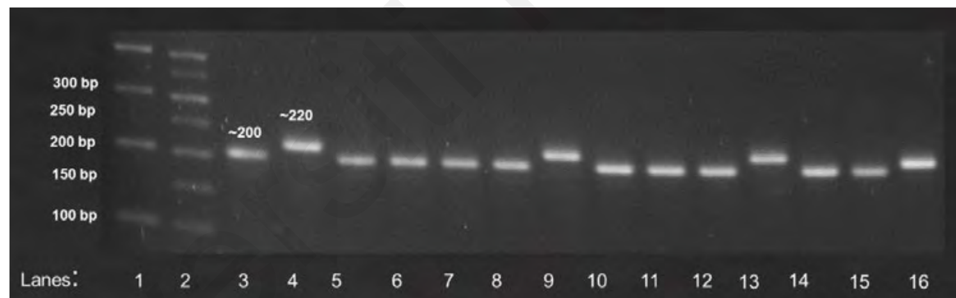

Figure 4.7: Amplified RM8225 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: Mahsuri Mutant, MR219, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

Figure 4.8: Amplified RM202 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: Pulut Hitam 9, MR219, Mahsuri Mutant, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

Table 4.4: Uniplex PCR product sizes for molecular markers linked to sheath blight (SB) resistance

Varieties	Molecular markers and product sizes (bp)										
	RM209	**RM257**	**RM478**	**RM202**	**RM224**	**RM205**	**RM426**	**RM6971**	**RM215**	**RM245**	**RM227**
MR219	~130	~150	~200	~180	~150	~100	~160	~200	~150	~150	~100
Mahsuri mutant	~130	~150	~200	~180	~150	~120	~160	~200	~150	~150	~100
Pulut Hitam 9	~130	~150	~200	~160	~150	~160	~160	~220	~150	~140	~100
Ria	~130	~150	~200	~160	~140	~120	~160	~200	~150	~150	~100
MR167	N/A	~140	~200	~180	~150	~160	~160	~220	~150	~140	~100
MR185	~130	~140	~200	~160	~140	~100	~160	~200	~150	~150	~100
MR220	~130	~140	~200	~180	~150	~100	~160	~200	~150	~150	~100
MR106	~130	~140	~200	~160	~150	~160	~160	~220	~150	~140	~100
MRQ74	~130	~140	~200	~180	~150	~120	~160	~200	~150	~150	~100
Pulut Malaysia 1	N/A	~150	~200	~160	~140	N/A	N/A	N/A	N/A	N/A	N/A
Biris	N/A	~150	~200	~180	~150	N/A	~160	N/A	N/A	N/A	N/A
IR8	~130	~140	~200	~180	~150	~120	~250	~200	~150	~150	~100
Chianung Sen Yu	~150	~140	~200	~180	~150	~120	~160	~200	~150	~140	~100
Tainan 3	N/A	~150	~200	~180	~140	N/A	~160	N/A	N/A	N/A	N/A

Universiti Malaya

4.2.2.3 Uniplex Genotyping for Bacterial Leaf Blight (BLB) Resistance

A total of two of the eight molecular markers linked to bacterial leaf blight (BLB) resistance (pTA248 and Xa7F/7-1R/7-2R) were functional polymorphic based on resistant and susceptible local check, MR219 and Ria, respectively. The amplicon size from the selected rice varieties for each of the markers tested are presented in Table 4.5. The primer pTA248, which revealed clear polymorphisms on local checks (Figure 4.9), was chosen for multiplex analysis. The amplified regions produced were found to be comparable to the findings from (Hajira et al., 2016; Kadu et al., 2018).

Table 4.5: Uniplex PCR product sizes for molecular markers linked to bacterial leaf blight (BLB) resistance

Varieties	Molecular markers and product sizes (bp)							
	Xa13-prom	Xa5FM	pTA248	Xa21	Xa13	Xa7F/7-1R/7-2R	RM13	RM122
MR219	~250	~300	~700	~500	~400	~200	~150	~240
Mahsuri mutant	~250	~300	~700	~500	~400	~200	~150	~240
Pulut Hitam 9	~250	~300	~700	~500	~400	~100	~150	~240
Ria	~250	~300	~600	~500	~400	~100	~150	~240
MR167	~250	~300	~700	~500	~400	~100	~150	~240
MR185	~250	~300	~700	~500	~400	~200	~150	~240
MR220	~250	~300	~700	~500	~400	~200	~150	~240
MR106	~250	~300	~700	~500	~400	~200	~150	~240
MRQ74	~250	~300	~700	~500	~400	~200	~150	~240
Pulut Malaysia 1	~250	~300	~600	~500	~400	~100	~150	~240
Biris	~250	~300	~600	~500	~400	~100	~130	~240
IR8	~250	~300	~700	~500	~400	~100	~150	~240
Chianung Sen Yu	~250	~300	~700	~500	~400	~200	~150	~240
Tainan 3	~250	~300	~600	~500	~400	~100, ~200	~130	~240

Universiti Malaya

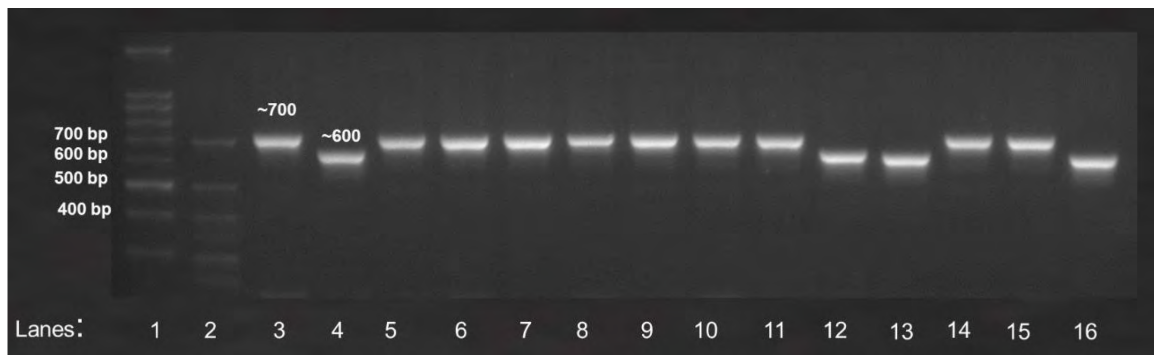

Figure 4.9: Amplified pTA248 products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: MR219, Ria, Mahsuri Mutant, Pulut Hitam 9, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

4.2.2.4 Uniplex Genotyping for Grain Quality

The current study also tested two sets of previously published allele-specific markers for fragrance and amylose content (Table 3.1) that had previously shown functional polymorphisms (Cheng et al., 2015). Figures 4.10 and 4.11 show the amplified products of *fgr*-SNP and *Wx*-SSR, respectively.

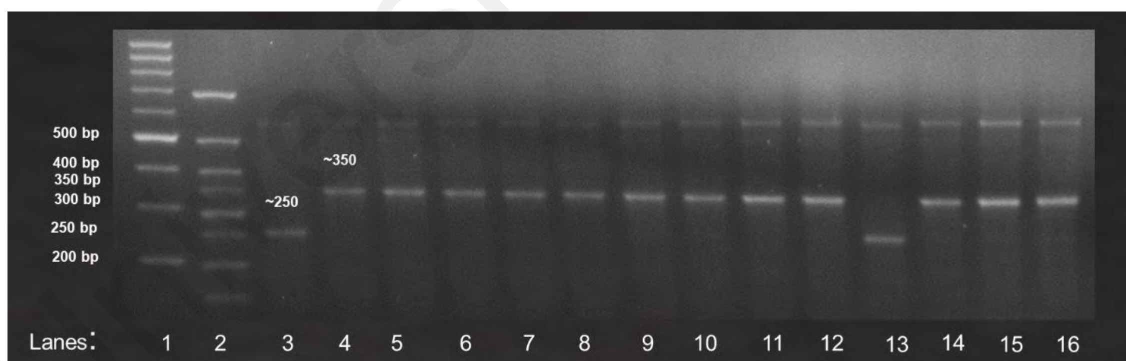

Figure 4.10: Amplified *fgr*-SNP products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 16: MRQ74, MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

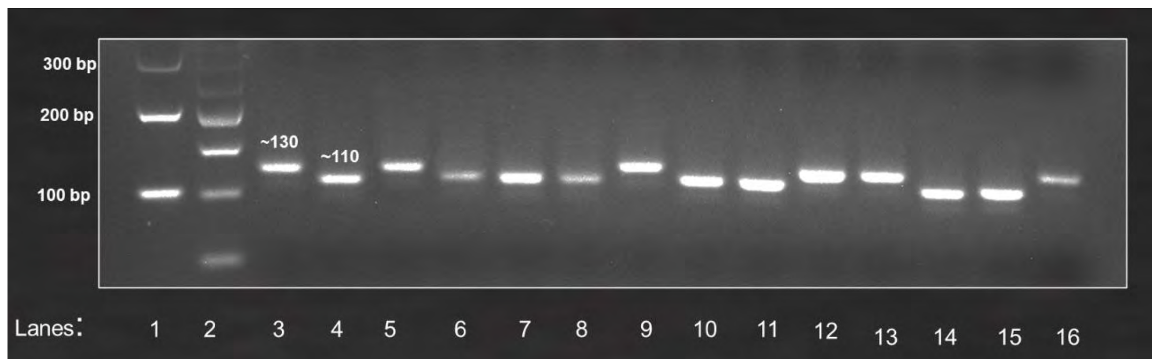

Figure 4.11: Amplified *Wx*-SSR products separated using 3% standard agarose gel electrophoresis at 100 V for 2.5 h. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

4.2.3 Multiplex PCR Analysis

A total of eight multiplex assays for genotyping multiple biotic stresses (blast, SB, and BLB) and major grain quality (fragrance and amylose content) in rice were developed, with multiplex products containing up to four primer sets identifiable by band patterns on agarose gels. These assays were developed using selected primers mentioned in Section 4.2.2.

4.2.3.1 High-resolution Agarose Multiplex Genotyping

Table 4.6 shows the molecular sizes of eight multiplex assays separated on 4% high-resolution agarose gels. The gel images produced by these assays, which involved electrophoresis at 120 V for 3 hours, are clear. The multiplex assays involved either two (Figures 4.12 -4.16), three (Figures 4.17 – 4.18), or four (Figure 4.19) sets of primers.

Universiti Malaya

Table 4.6: Description of multiplex results of fourteen selected local rice varieties

Variety	Two primer sets					Three primer sets		Four primer sets
	***Wx*-SSR** + ***fgr*- SNP**	***Wx*-SSR** + **pTA248**	***fgr*-SNP** + **pTA248**	***fgr*-SNP** + **RM8225**	***fgr*-SNP** + **RM202**	**RM202 +RM6836+ pTA248**	**RM202 +RM8225+ pTA248**	***Wx*-SSR +RM202 +RM8225 +*fgr*-SNP**
MR219	~130; ~350	~130; ~700	~350; ~700	~350; ~220	~350; ~180	~180; ~250; ~700	~180; ~220; ~700	~130; ~180; ~220; ~350
MRQ74	~110; ~250	~110; ~700	~250; ~700	~250; ~200	~250; ~180	~180; ~230; ~700	~180; ~200; ~700	~110; ~180; ~200; ~250
Ria	~110; ~350	~110; ~600	~350; ~600	~350; ~200	~350; ~160	~160; ~230; ~600	~160; ~200; ~600	~110; ~160; ~200; ~350
Mahsuri Mutant	~110; ~350	~110; ~700	~350; ~700	~350; ~200	~350; ~180	~180; ~230; ~700	~180; ~200; ~700	~110; ~180; ~200; ~350
Pulut Hitam 9	~130; ~350	~130; ~700	~350; ~700	~350; ~200	~350; ~160	~160; ~230; ~700	~160; ~200; ~700	~130; ~160; ~200; ~350
Pulut Malaysia 1	~130; ~350	~130; ~600	~350; ~600	~350; ~200	~350; ~160	~160; ~230; ~600	~160; ~200; ~600	~130; ~160; ~200; ~350
MR106	~110; ~350	~110; ~700	~350; ~700	~350; ~200	~350; ~160	~160; ~230; ~700	~160; ~200; ~700	~110; ~160; ~200; ~350
MR167	~110; ~350	~110; ~700	~350; ~700	~350; ~200	~350; ~180	~180; ~230; ~700	~180; ~200; ~700	~110; ~180; ~200; ~350
MR185	~110; ~350	~110; ~700	~350; ~700	~350; ~200	~350; ~160	~160; ~230; ~700	~160; ~200; ~700	~110; ~160; ~200; ~350
MR220	~130; ~350	~130; ~700	~350; ~700	~350; ~220	~350; ~180	~180; ~250; ~700	~180; ~220; ~700	~130; ~180; ~220; ~350
Biris	~130; ~250	~130; ~600	~250; ~600	~250; ~220	~250; ~180	~180; ~250; ~600	~180; ~220; ~600	~130; ~180; ~220; ~250
IR8	~110; ~350	~110; ~700	~350; ~700	~350; ~200	~350; ~180	~180; ~230; ~700	~180; ~200; ~700	~110; ~180; ~200; ~350
Chianung Sen Yu	~110; ~350	~110; ~700	~350; ~700	~350; ~200	~350; ~180	~180; ~230; ~700	~180; ~200; ~700	~110; ~180; ~200; ~350
Tainan 3	~130; ~350	~130; ~600	~350; ~600	~350; ~220	~350; ~180	~180; ~250; ~600	~180; ~220; ~600	~130; ~180; ~220; ~350

Universiti Malaya

Based on the multiplex products of the local checks, the ~160 bp (RM202 primer), ~230 bp (RM6836 primer), ~200 bp (RM8225 primer) and ~700 bp (pTA248 primer) amplicons denoted the presence of resistance alleles for SB, blast, and BLB, respectively. On the contrary, the ~180 bp (RM202 primer), ~250 bp (RM6836 primer), ~220 bp (RM8225) and ~600 bp (pTA248 primer) amplicons denoted the susceptible alleles for SB, blast, and BLB, respectively (Table 3.2).

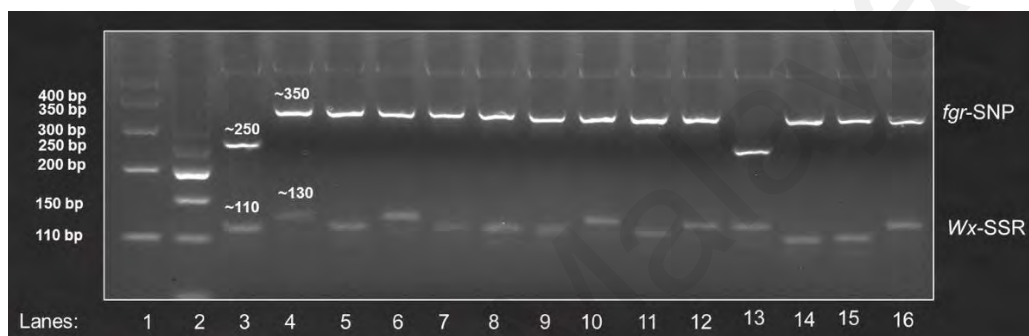

Figure 4.12 (Multiplex-1): Amplified multiplex *Wx*-SSR and *fgr*-SNP products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with amylose content [~110 bp (>25% amylose); ~130 bp (<25% amylose)] and fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MRQ74, MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

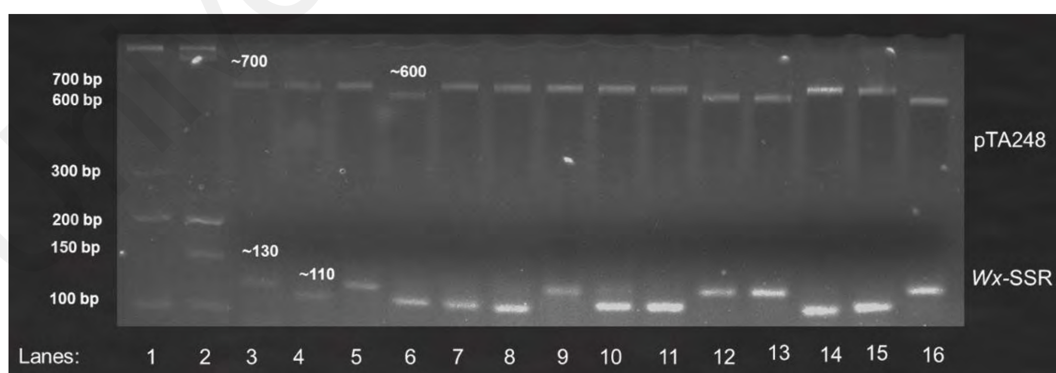

Figure 4.13 (Multiplex-2): Amplified multiplex *Wx*-SSR and pTA248 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with amylose content [~110 bp (>25% amylose); ~130 bp (<25% amylose)] and bacterial leaf blight resistance [~600 bp (susceptible); ~700 bp (resistant)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Birirs, IR8, Chianung Sen Yu, Tainan 3

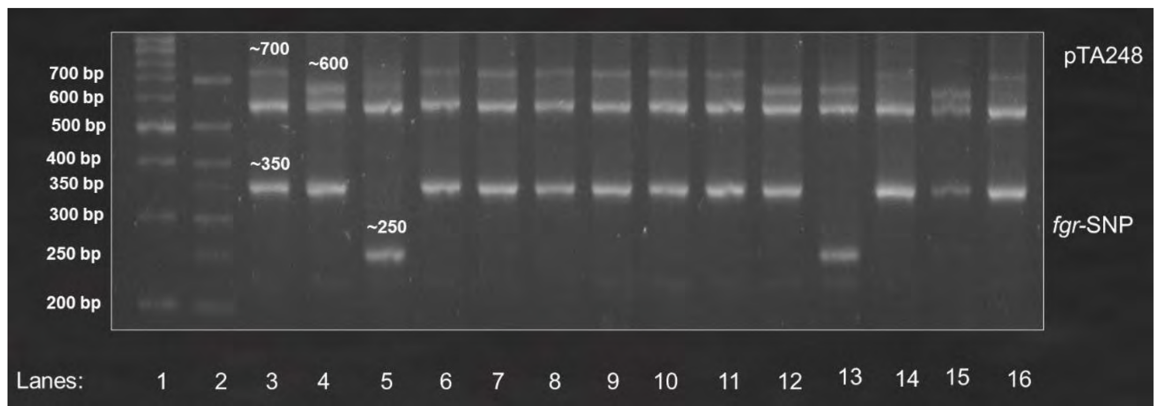

Figure 4.14 (Mutiplex-3): Amplified multiplex *fgr*-SNP and pTA248 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and bacterial leaf blight resistance [~600 bp (susceptible); ~700 bp (resistant)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Ria, MRQ74, Mahsuri Mutant, Pulut Hitam 9, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

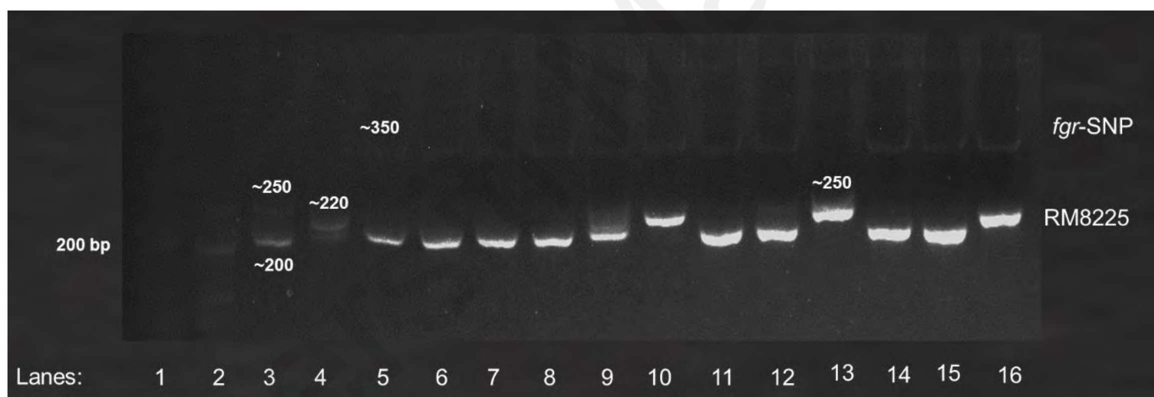

Figure 4.15 (Multiplex-4): Amplified multiplex *fgr*-SNP and RM8225 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and blast resistance [~200 bp (resistant); ~220 bp (susceptible)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MRQ74, MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia, Biris, IR8, Chianung Sen Yu, Tainan 3

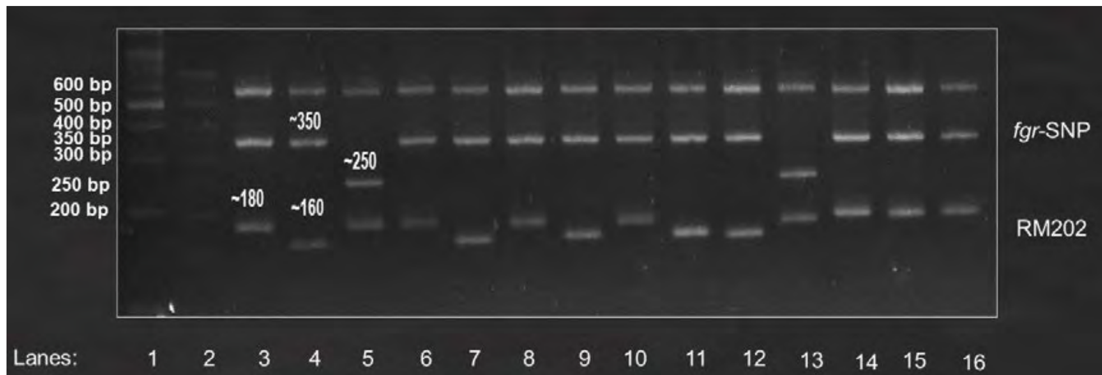

Figure 4.16 (Multiplex-5): Amplified multiplex *fgr*-SNP and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and sheath blight resistance [~160 bp (resistant); ~180 bp (susceptible)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Pulut Hitam 9, MRQ74, Mahsuri Mutant, Ria, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

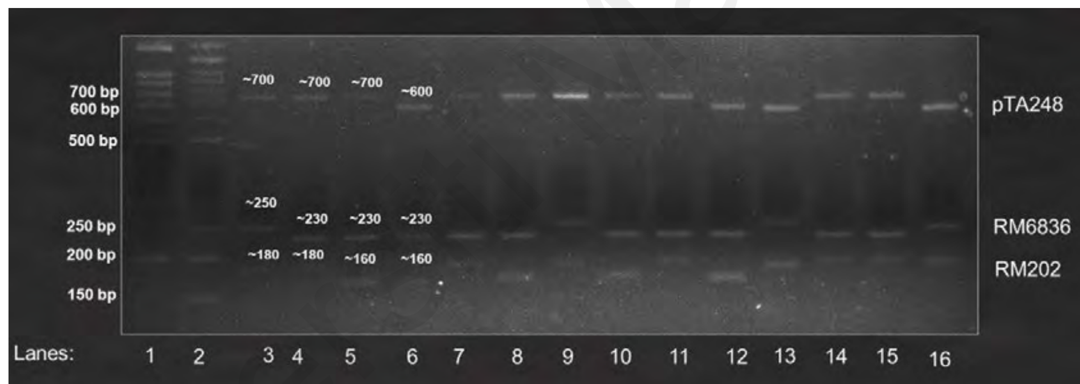

Figure 4.17 (Multiplex-6): Amplified multiplex pTA248, RM6836 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~250 bp (susceptible); ~230 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1-2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

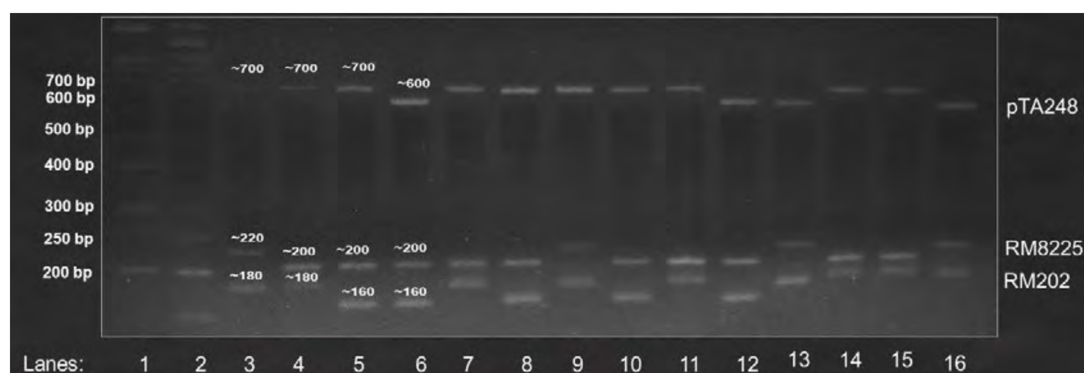

Figure 4.18 (Multiplex-7): Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MR167, MR185, MR220, MR106, MRQ74, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

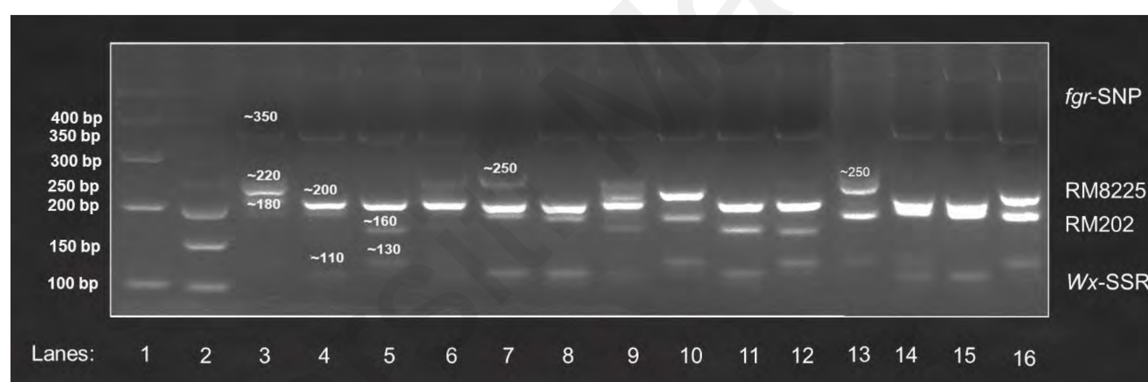

Figure 4.19 (Multiplex-8): Amplified multiplex *Wx*-SSR, RM202, RM8225 and *fgr*-SNP products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with fragrance [~250 bp (fragrant); ~350 bp (non-fragrant)] and sheath blight resistance [~160 bp (resistant); ~180 bp (susceptible)] genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 12: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, MRQ74, MR167, MR185, MR220, MR106, Pulut Malaysia 1, Biris, IR8, Chianung Sen Yu, Tainan 3

Universiti Malaya

4.2.3.2 Capillary Electrophoresis and Sequencing

The electrophenograms for HEX-RM6836 and NED-RM202 obtained from fluorescence capillary analysis for local checks MR219, Mahsuri Mutant an Pulut Hitam 9 are shown in the Figure 4.20. The results from capillary electrophoresis findings were comparable to those obtained using high-resolution gel electrophoresis. For instance, the

product sizes of MR219 labelled with HEX-RM6836 (247.08 bp) and NED-RM202 (185.18 bp) (Figure 4.20A) were comparable to those seen on the high-resolution agarose gel. It is worth noting that the primer pTA248 was excluded in capillary electrophoresis analysis as the method is only feasible for amplicons smaller than 500 bp in size (Mohd Hanafiah et al., 2021).

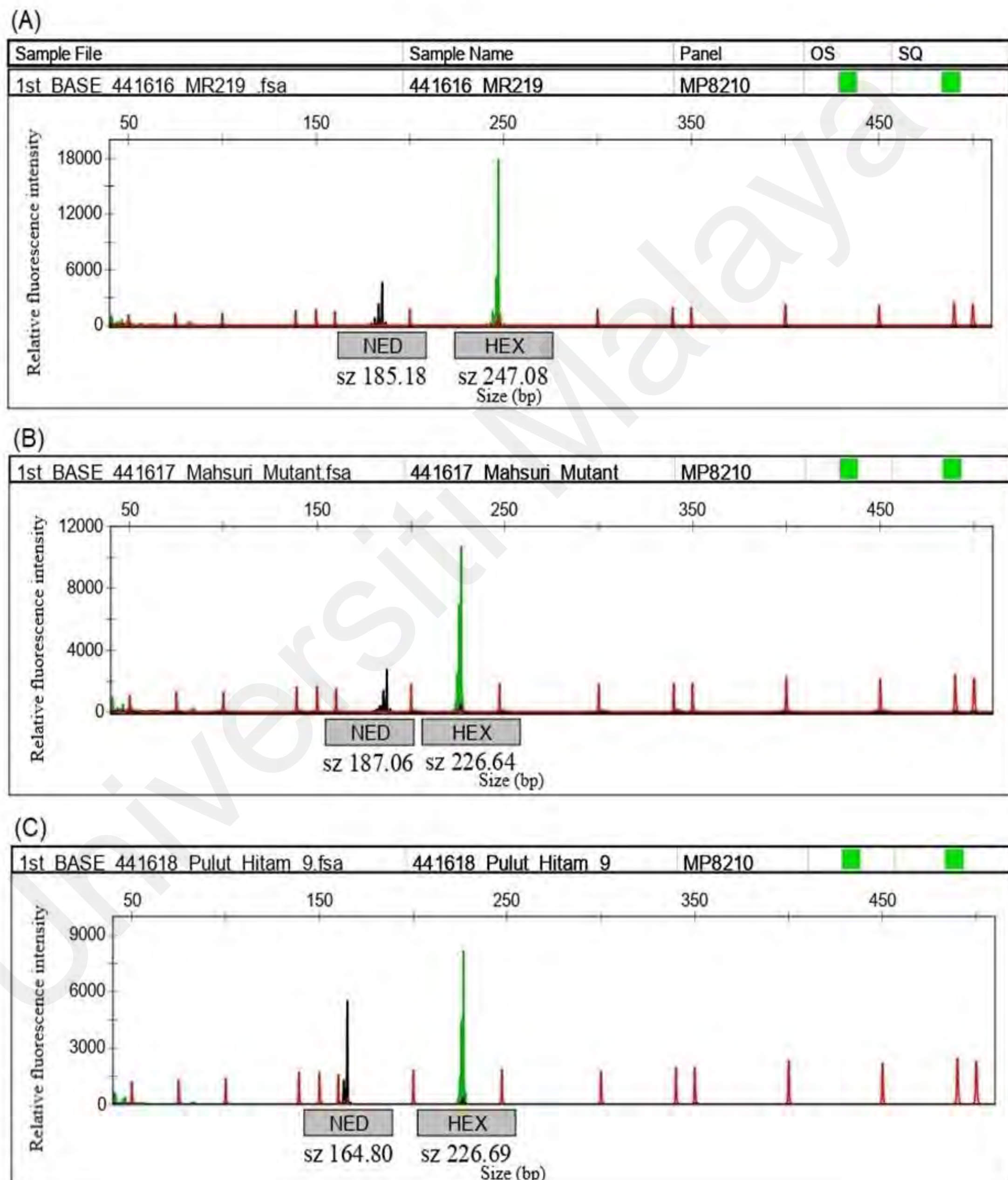

Figure 4.20: Electrophenogram results from capillary electrophoresis for local checks (A) MR219, (B) Mahsuri Mutant and (C) Pulut Hitam 9. The primer peaks of RM6836 and RM202 were presented as HEX (green) and NED (black), respectively. The red peaks represent internal size standard 500-ROX. The x- and y-axis represent size (bp) of the PCR products in bases and relative fluorescence intensity, respectively

Universiti Malaya

PCR products of local checks were also sequenced to validate the results from electrophoresis-based genotyping. The results from sequencing confirmed the presence or absence of resistance genes in the check varieties, as demonstrated by high-resolution agarose gel and capillary analyses (Tables 4.6 and 4.7; Supplementary Table S1). Similar to the findings reported by Miah et al. (2016), motifs $(TCT)_7$ and $(TCT)_{15}$ were detected from the amplified products of RM6836, representing resistance and susceptibility alleles for blast disease, respectively. On the other hand, two motifs detected from the amplified products of RM202, $(CT)_{16}$ and $(CT)_{28}$, were linked to resistance and susceptibility to SB disease, respectively (Hossain et al., 2016) (Table 4.7).

Table 4.7: Comparison of capillary electrophoresis and sequencing results to determine resistant (R) or susceptible (S) response of RM6836 and RM202 towards blast and sheath blight (SB), respectively

Capillary electrophoresis				Sequencing			
RM6836		RM202		RM6836		RM202	
Product size (bp)	Blast response	Product size (bp)	SB response	Motif size $(TCT)_n$	Blast response	Motif size $(CT)_n$	SB response
247.08	S	185.18	S	15	S	28	S
226.64	R	187.06	S	7	R	28	S
226.69	R	164.80	R	7	R	16	R

4.3 Multiplex Screening of Weedy Rice Biotypes for Biotic Stresses

A total of 100 weedy rice biotypes were screened using Multiplex-7 (Table 3.3) for genotyping multiple biotic stresses (i.e. blast, SB, and BLB) (Table 3.3). Table 4.8 shows the multiplex products of 100 weedy rice biotypes separated on 4% high-resolution agarose gels. The gel images produced by these assays, are clear (Figures 4.21-4.28).

Table 4.8: Description of multiplex results of 100 weedy rice biotypes (ID) and its association with sheath blight (SB), blast and bacterial leaf blight (BLB). Molecular size representing susceptible, resistant and heterozygous was labelled as S, R and H, respectively

ID	Multiplex product size	Multiplex description			ID	Multiplex product size	Multiplex description		
	RM202 +RM8225 +pTA248	SB	Blast	BLB		RM202 +RM8225 +pTA248	SB	Blast	BLB
WR01	~180;~220;~700	S	S	R	WR51	~180;~220;~700	S	S	R
WR02	~160, ~180;~220;~700	H	S	R	WR52	~180;~220;~700	S	S	R
WR03	~180;~220;~700	S	S	R	WR53	~180;~200;~700	S	R	R
WR04	~160,~180;~220; ~600	H	S	S	WR54	~180;~220;~700	S	S	R
WR05	~180;~220;~600	S	S	S	WR55	~180;~220;~700	S	S	R
WR06	~160;~220,~700	R	S	R	WR56	~180;~200;~700	S	R	R
WR07	~160, ~180;~220; ~700	H	S	R	WR57	~180;~220;~700	S	S	R
WR08	~160;~220;~700	S	S	R	WR58	~180;~200;~700	S	R	R
WR09	~180;~220;~700	S	S	R	WR59	~160,~180;~220; ~700	H	S	R
WR10	~180;~220;~700	S	S	R	WR60	~180;~200;~700	S	R	R
WR11	~180;~220;~700	S	S	R	WR61	~180;~220;~700	S	S	R
WR12	~180;~220;~700	S	S	R	WR62	~160;~220;~700	R	S	R
WR13	~180;~220;~700	S	S	R	WR63	~180;~220;~700	S	S	R
WR14	~180;~220	S	S	NA	WR64	~180;~220;~700	S	S	R
WR15	~180;~220;~700	S	S	R	WR65	~180;~200;~700	S	R	R
WR16	~160,~180;~220; ~700	H	S	R	WR66	~160,~180;~200; ~700	H	R	R
WR17	~180;~220;~600	S	S	S	WR67	~180;~220;~700	S	S	R
WR18	~180;~200;~220; ~700	S	H	R	WR68	~180;~220;~700	S	S	R
WR19	~160;~220;~700	R	S	R	WR69	~180;~220;~700	S	S	R
WR20	~180;~200;~700	S	R	R	WR70	~180;~220;~700	S	S	R
WR21	~160;~200;~700	R	R	R	WR71	~180;~200;~700	S	R	R
WR22	~180;~220;~600	S	S	S	WR72	~180;~220;~700	S	S	R
WR23	~180;~220;~700	S	S	R	WR73	~180;~200;~700	S	R	R
WR24	~180;~220;~700	S	S	R	WR74	~180;~220;~700	S	S	R
WR25	~180;~200;~700	S	R	R	WR75	~180;~220;~700	S	S	R
WR26	~180;~200;~700	S	R	R	WR76	~160;~220;~700	R	S	R
WR27	~180;~220;~700	S	S	R	WR77	~180;~220;~700	S	S	R
WR28	~180;~220;~700	S	S	R	WR78	~180;~220;~700	S	S	R
WR29	~180;~220;~700	S	S	R	WR79	~180;~220;~700	S	S	R
WR30	~180;200;700	S	R	R	WR80	~180;~220;~700	S	S	R
WR31	180;220;600	S	S	S	WR81	~160;~220;~700	R	S	R
WR32	180;200;700	S	R	R	WR82	~180;~~220;~700	S	S	R
WR33	~180;~200;~700	S	R	R	WR83	~180;~~220;~700	S	S	R
WR34	~180;~220;~700	S	S	R	WR84	~160;~180;~220; ~700	H	S	R

Table 4.8, continued

ID	Multiplex product size	Multiplex description			ID	Multiplex product size	Multiplex description		
	RM202 +RM8225 +pTA248	SB	Blast	BLB		RM202 +RM8225 +pTA248	SB	Blast	BLB
WR35	~160,~180;~220; ~700	H	S	R	WR85	~160;~220;~700	R	S	R
WR36	~160;~200;~700	R	S	R	WR86	~160;~200;~700	R	R	R
WR37	~180;~200;~700	S	R	R	WR87	~160,~180;~220;7 00	H	S	R
WR38	~180;~220;~700	S	S	R	WR88	~180;~220;~700	S	S	R
WR39	~180;~220;~700	S	S	R	WR89	~160;~200;~700	R	R	R
WR40	~180;~220;~700	S	S	R	WR90	~160,~180;~220;7 00	H	S	R
WR41	~180;~220;~600	S	S	S	WR91	~160,~180;~220;7 00	H	S	R
WR42	~180;~220;~700	S	S	R	WR92	~160;~200;~700	R	R	R
WR43	~180;~220;~700	S	S	R	WR93	~180;~220;~700	S	S	R
WR44	~180;~220;~700	S	S	R	WR94	~180;~220;~700	S	S	R
WR45	~180;~220;~700	S	S	R	WR95	~180;~200,~220; ~700	S	H	R
WR46	180;220;700	S	S	R	WR96	~180;~220;~700	S	S	R
WR47	~180;~220;~700	S	S	R	WR97	~160;~220;~700	R	S	R
WR48	~160,~180;~220; ~700	H	S	R	WR98	~180;~220;~600	S	S	S
WR49	~160,~180;~220; ~700	H	S	R	WR99	~180;~220;~700	S	S	R
WR50	~180;~220;~700	S	S	R	WR10 0	~180;~220;~700	S	S	R

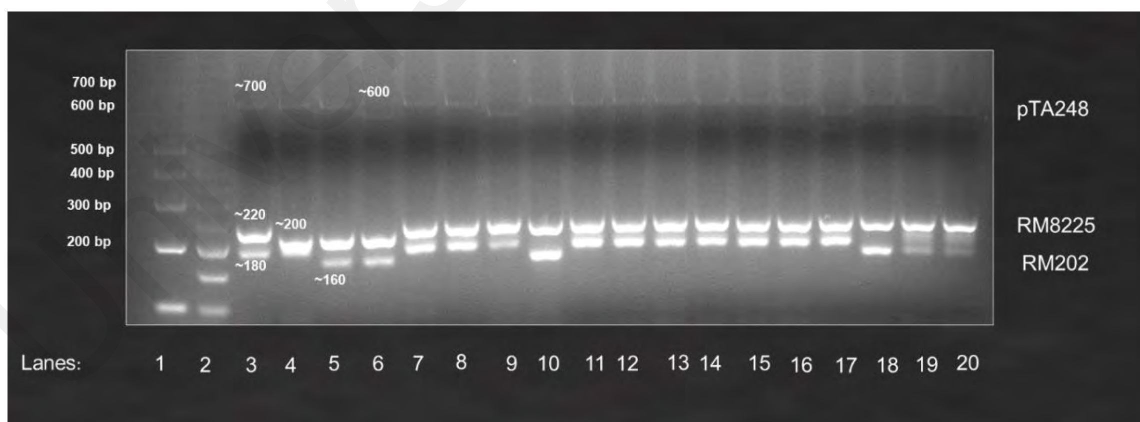

Figure 4.21: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR1, WR03, WR05, WR06, WR09, WR10, WR11, WR12, WR13, WR15, WR17, WR19, WR02, WR04

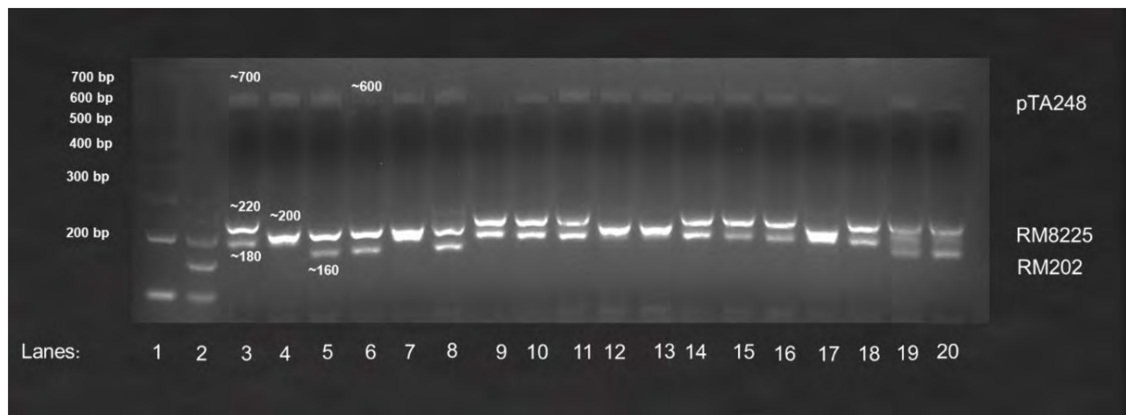

Figure 4.22: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR20, WR21, WR22, WR23, WR24, WR25, WR26, WR27, WR28, WR29, WR30, WR31, WR07, WR16

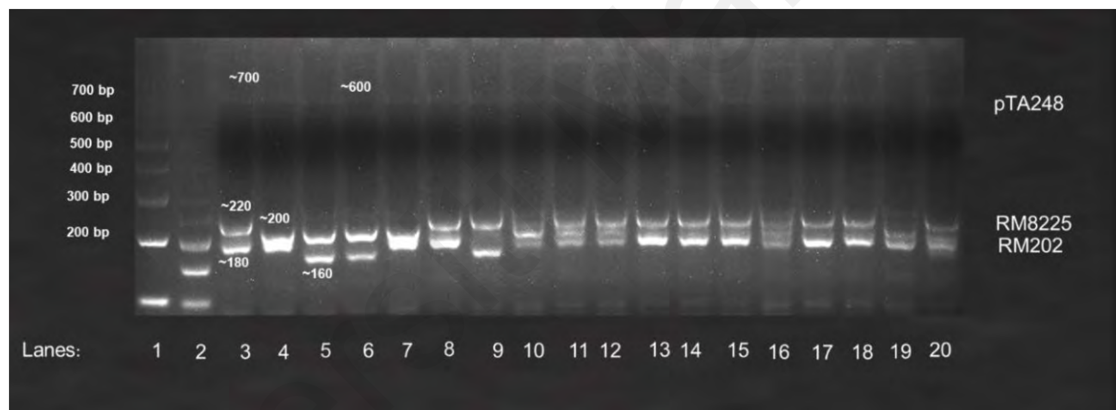

Figure 4.23: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR33, WR34, WR36, WR37, WR38, WR39, WR40, WR41, WR42, WR43, WR44, WR45, WR32, WR35

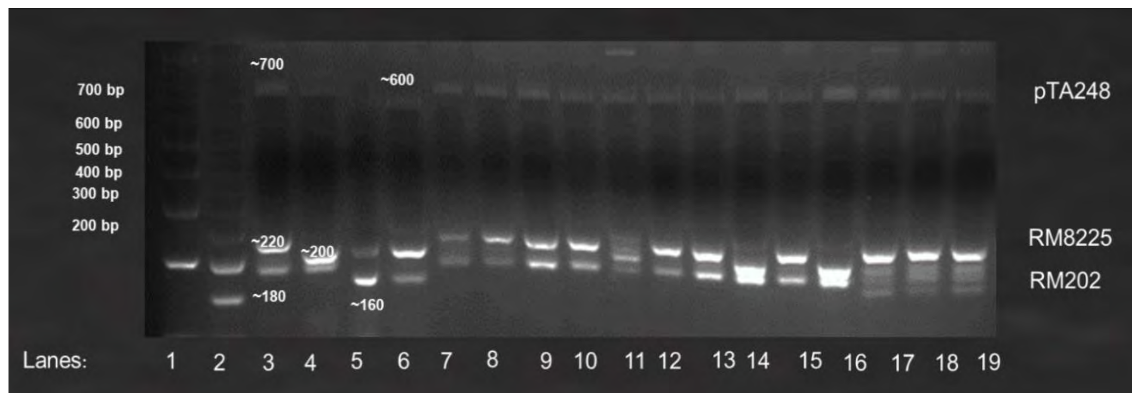

Figure 4.24: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 19: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR47, WR50, WR51, WR52, WR53, WR54, WR55, WR56, WR57, WR58, WR48, WR49, WR59

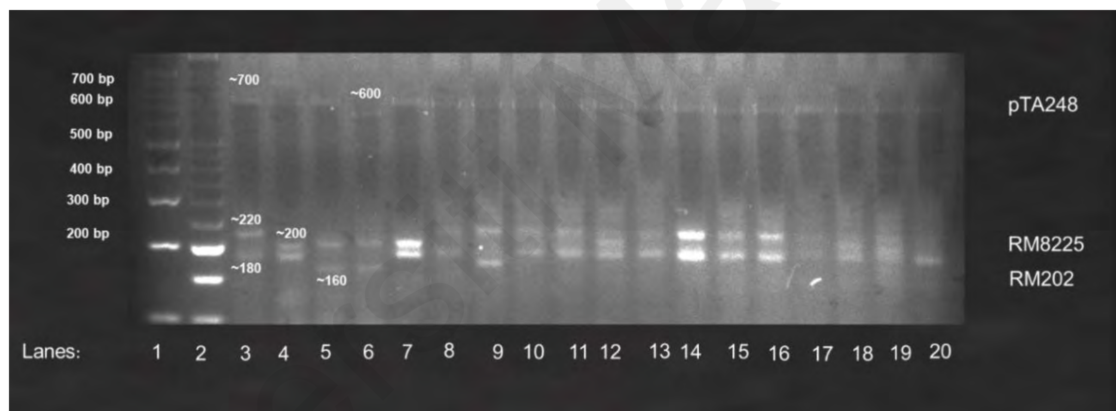

Figure 4.25: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR60, WR61, WR62, WR63, WR64, WR65, WR67, WR68, WR69, WR70, WR72, WR73, WR66, WR71

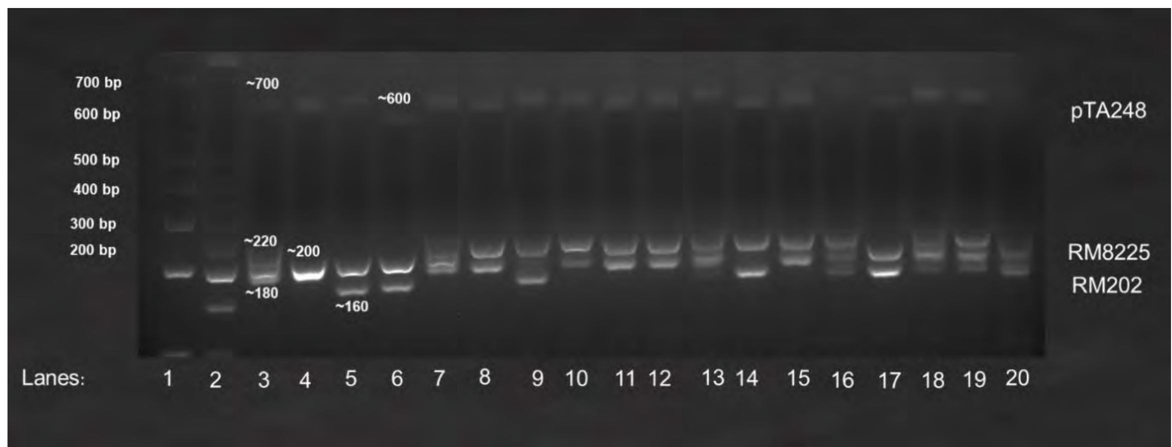

Figure 4.26: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 20: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR74, WR75, WR76, WR77, WR78, WR79, WR80, WR81, WR83, WR85, WR86, WR84, WR87

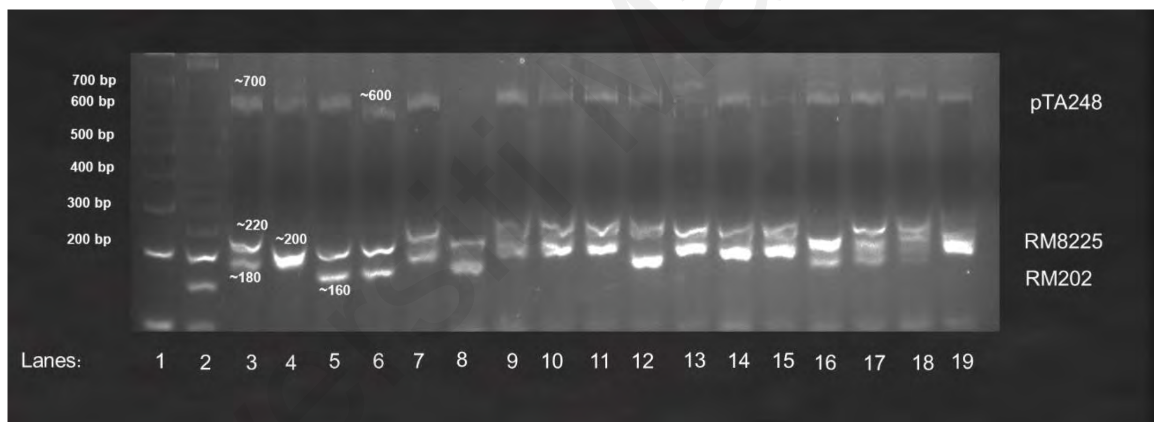

Figure 4.27: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 19: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR88, WR92,WR93, WR94, WR96, WR97, WR98, WR99, WR100, WR89, WR90, WR91, WR95

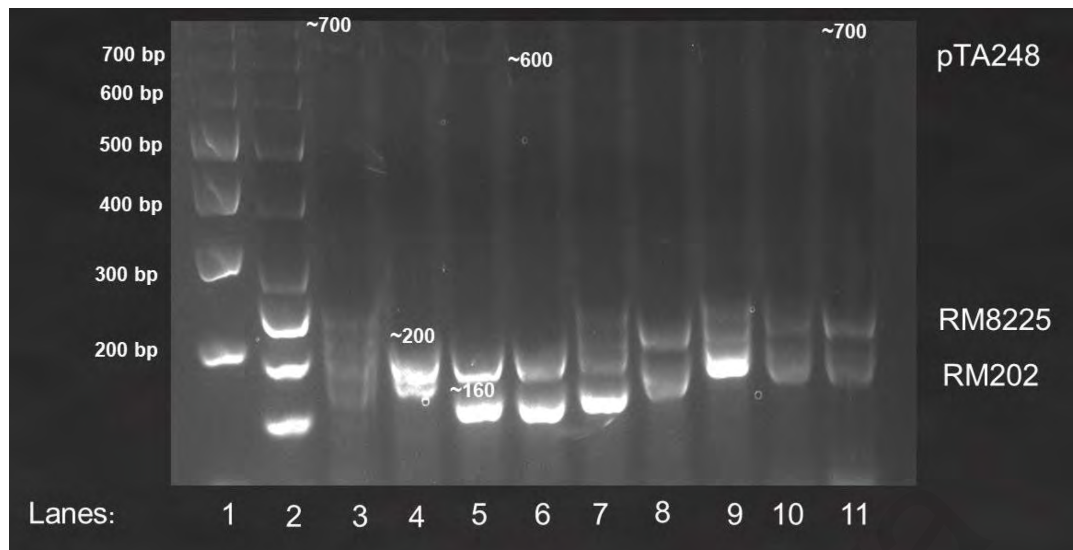

Figure 4.28: Amplified multiplex pTA248, RM8225 and RM202 products separated using 4% high-resolution agarose gel electrophoresis at 120 V for 3 h associated with bacterial leaf blight [~600 bp (susceptible); ~700 bp (resistance)], blast [~220 bp (susceptible); ~200 bp (resistance)], and sheath blight [~180 bp (susceptible); ~160 bp (resistance)] resistance genes, respectively. Lanes 1 - 2: 100 bp and 50 bp ladders; Lanes 3 - 11: MR219, Mahsuri Mutant, Pulut Hitam 9, Ria, WR8, WR14, WR18, WR46, WR82

Screening of three markers associated with three respective diseases (blast, SB, and BLB) on weedy rice samples from five different locations showed Malaysian weedy rice has various genetic responses towards rice diseases (Figure 4.29). The genotyping of markers in Multiplex-7 on 100 weedy rice biotypes revealed that 12%, 19% and 89% of the biotypes showed resistance to SB, blast and BLB, respectively (Figure 4.29). Heterozygosity is at the highest (14%) in SB compared to blast (3%) and BLB (4%) (Figure 4.29).

The allelic frequency of markers (RM202, RM8225 and pTA248) for 100 weedy rice biotypes collected at different locations are shown in the Figure 4.30. Allelic frequency ratio of 180bp:160bp for SB and 220bp:200bp for blast for all locations showed leniency towards susceptibility of weedy rice to the diseases.

On the other hand, allelic frequency ratio for BLB showed higher frequency towards resistant genotype (Figure 4.30). All biotypes collected in Sungai Besar and Sungai Leman, Selangor, had 700 bp product sizes, indicating resistance to BLB.

Universiti Malaya

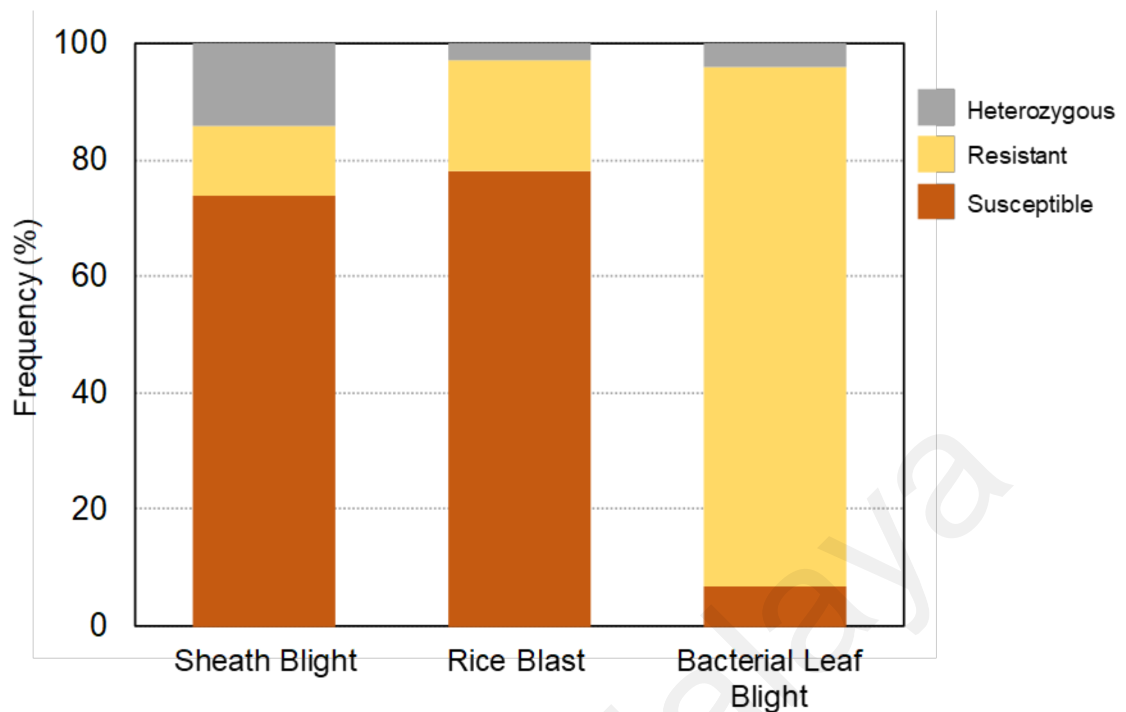

Figure 4.29: Frequency distribution of weedy rice biotypes associated with major rice diseases in Malaysia. Resistant and susceptible genotypes were determined based on molecular screening

Genetic diversity of Malaysian weedy rice based on these disease-associated markers was comparable among locations (Table 4.9). Pekan detected highest diversity for BLB (He=0.287, I=0.462). while Sungai Burung and Seberang Perak recorded highest diversity for blast (He=0.430, I=0.621) and sheath blight (He=0.408, I=0.598), respectively. Sungai Leman, on the other hands showed only single genotype for all markers. Among the weedy rice biotypes studied, the highest Shannon's information index was 0.62 for blast disease detected by marker RM8225 in Sungai Burung, Selangor. Observed heterozygosity (Ho) values were low in the sampled weedy rice with an average of 0.062 across all locations (Table 4.9).

Universiti Malaya

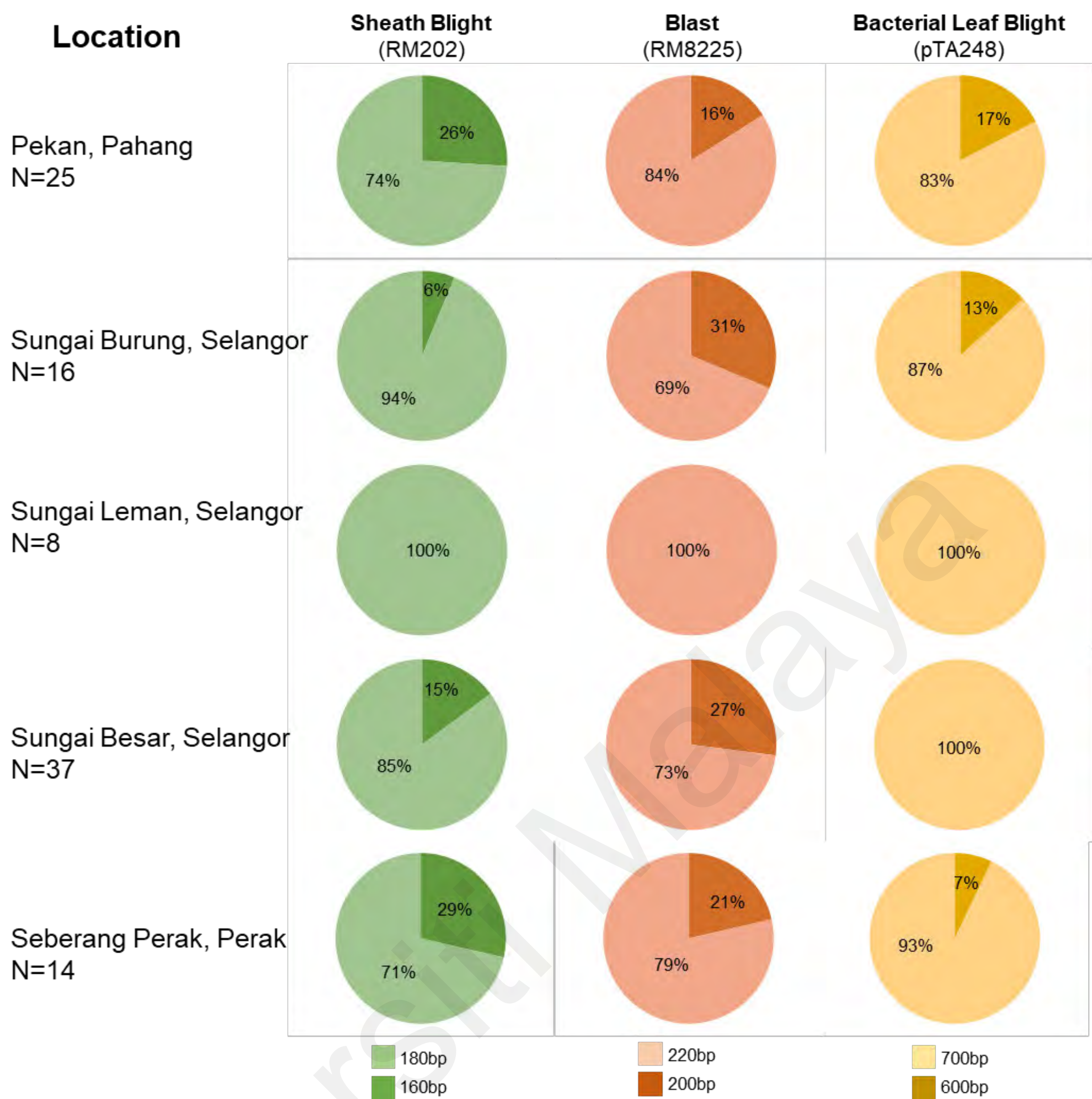

Figure 4.30: Allelic frequency of weedy rice samples for markers associated with major rice diseases in Malaysia

University Malaya

Table 4.9: Genetic diversity of weedy rice samples based on three (3) loci associated with sheath blight (SB), rice blast, and bacterial leaf blight (BLB) diseases at five (5) different rice fields

Location	Diseases	N	Na	Ne	I	Ho	He	uHe
Pekan	SB	25	2.000	1.625	0.573	0.120	0.385	0.393
	Blast		2.000	1.368	0.440	0.080	0.269	0.274
	BLB		2.000	1.403	0.462	0.000	0.287	0.294
Sungai Burung	SB	16	2.000	1.133	0.234	0.000	0.117	0.121
	Blast		2.000	1.753	0.621	0.000	0.430	0.444
	BLB		2.000	1.301	0.393	0.000	0.231	0.239
Sungai Leman	SB	8	1.000	1.000	0.000	0.000	0.000	0.000
	Blast		1.000	1.000	0.000	0.000	0.000	0.000
	BLB		1.000	1.000	0.000	0.000	0.000	0.000
Sungai Besar	SB	37	2.000	1.339	0.420	0.027	0.253	0.257
	Blast		2.000	1.651	0.584	0.000	0.394	0.400
	BLB		1.000	1.000	0.000	0.000	0.000	0.000
Seberang Perak	SB	14	2.000	1.690	0.598	0.286	0.408	0.423
	Blast		2.000	1.508	0.520	0.000	0.337	0.349
	BLB		2.000	1.153	0.257	0.000	0.133	0.138
Mean total		100	2.663	0.118	0.071	0.062	0.020	0.043

N: Sample Size; Na: Number of alleles: Ne, number of effective alleles; I: Shannon's information index; Ho: observed heterozygosity; He: expected heterozygosity; uHe: unbiased expected heterozygosity

To validate the genotyping results, greenhouse phenotyping based on disease scoring index was carried out using the Standard Evaluation System (SES) created by the International Rice Research Institute (IRRI) (Figure 4.31). Two of the fifteen weedy rice biotypes tested (WR15 and WR20) with disease score of 5 for SB (Figure 4.32) were pre-determined in as susceptible in genotyping screening. On the other hand, two of fifteen weedy rice biotypes (WR15 and WR19) scored 4 for rice blast and were also pre-determined as susceptible using the developed multiplex assay. As for BLB, thirteen of the fifteen selected biotypes were resistant in genotyping screening, and the phenotyping screening scoring ranged from 1 to 3, with only one biotype WR20 receiving a score of 3.

Universiti Malaya

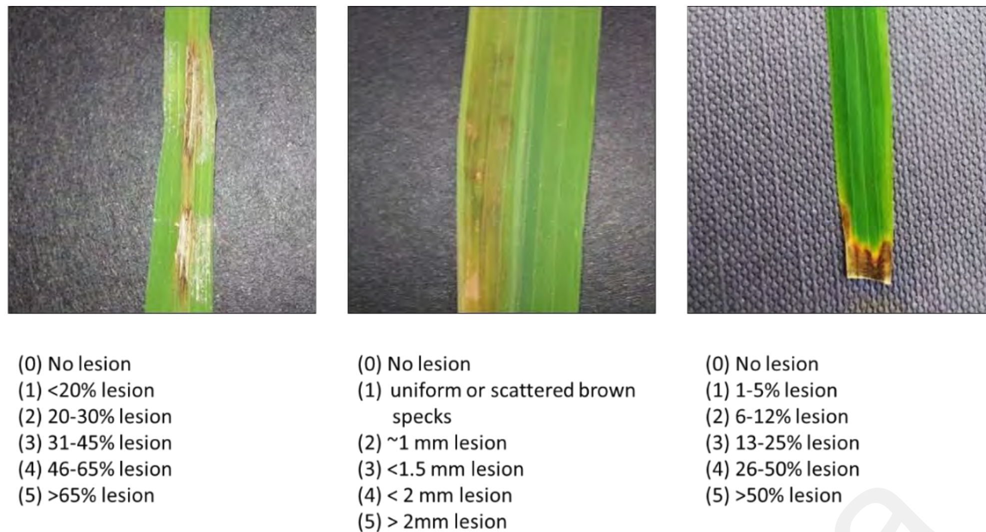

Figure 4.31: Symptoms after phenotyping for biotic stresses and disease scoring scale on selected weedy rice samples for (A) sheath blight, (B) blast, and (C) bacterial leaf blight

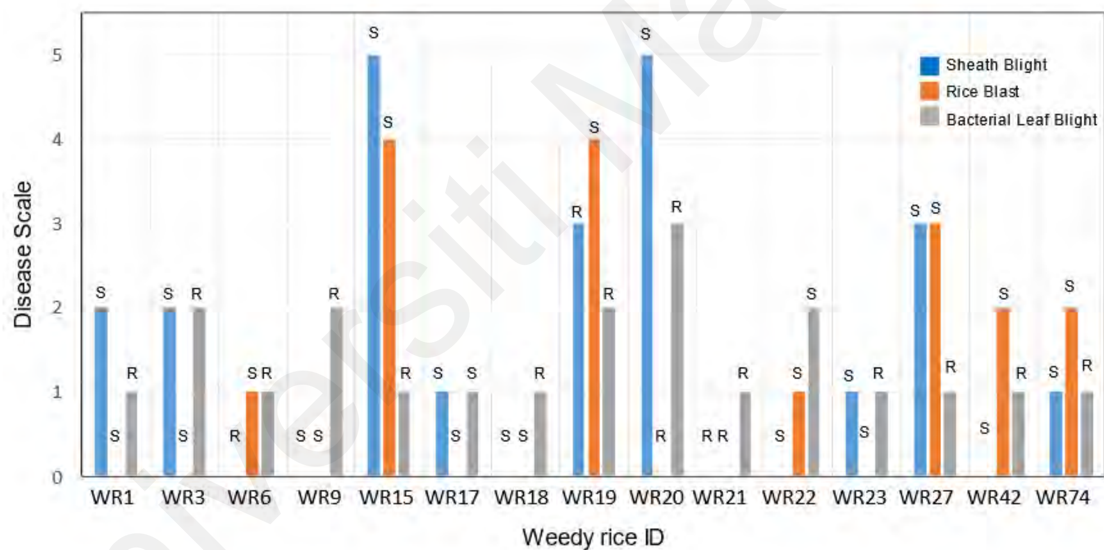

Figure 4.32: Disease score for selected weedy rice samples for sheath blight (blue), rice blast (orange), and bacterial leaf blight (grey) diseases. Predetermined susceptible (S) and resistant (R) weedy rice based on genotype scoring were indicated above the bar

The Principal Coordinate Analysis (PCoA) scatter plot shows separation of current most cultivated rice in Peninsular Malaysia (Malaysian elite *indica* cultivars MR219 and MR220), which formed Cluster 1, a tight cluster, with majority of weedy rice biotypes (Figure 4.33). Although the Malaysian weedy rice samples were found to be dispersed

Universiti Malaya

across the plot, certain patterns were evident, such as the closer positioning of biotypes near to cultivated rice varieties, sharing common characteristics of which susceptible to blast and SB and resistance to BLB (Tables 4.6; 4.8). Cluster 2 was formed with 16 weedy rice biotypes with five rice varieties (Mahsuri Mutant, MRQ74, MR167, IR8 and Chianung Sen Yu) of which shown to be resistant to BLB and blast, susceptible to SB (Tables 4.6; 4.8). Cluster 3 involved 3% of weedy rice biotypes (WR21, WR86 and WR92) of which showing resistant to all biotic stresses studied in multiplex genotyping together with Pulut Hitam 9, MR106 and MR185. Some weedy rice biotypes (14%, 4% and 3% for SB, BLB and blast respectively) shown heterozygous results in genotyping which could not be found in rice cultivars (Figure 4.29; Table 4.8).

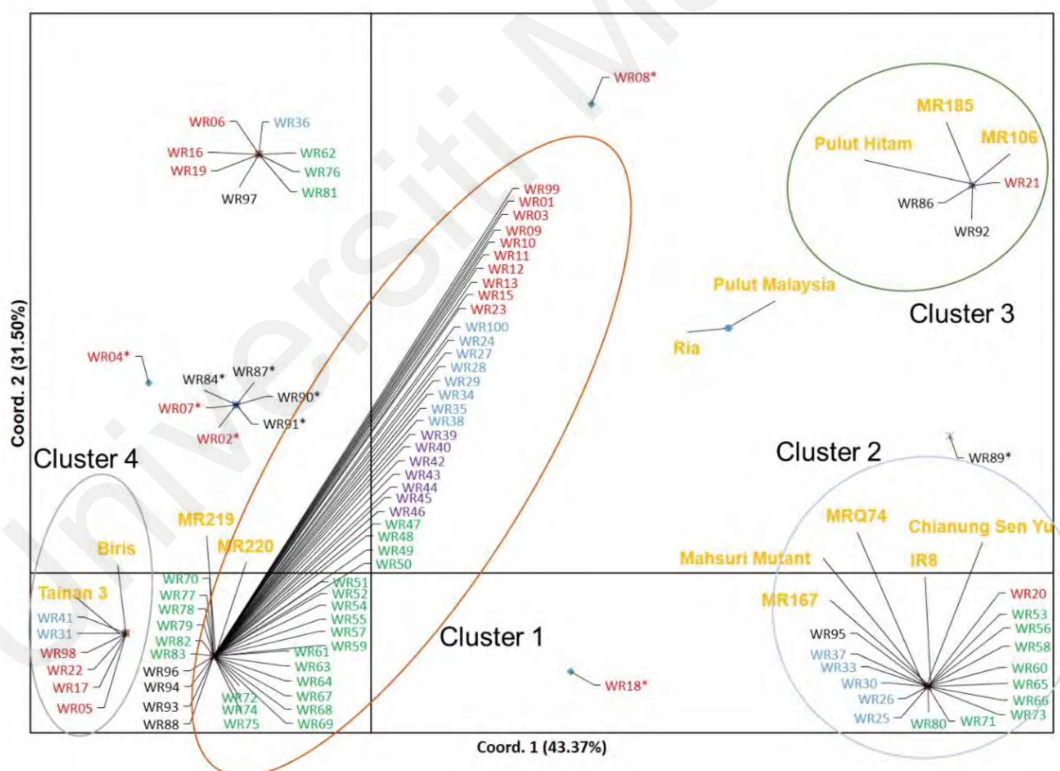

Figure 4.33: Principal coordinate analysis (PCoA) showing the genetic distances between weedy rice samples from different locations and selected cultivated rice. Weedy rice biotypes from Pekan (Pahang), Sungai Burung (Selangor), Sungai Leman (Selangor), Sungai Besar (Selangor), and Seberang Perak (Perak) are represented by fonts in red, blue, purple, green, and black, while cultivated rice are represented by font in yellow. Weedy rice lines marked with an asterisk have heterozygosity in at least one of the loci

Universiti Malaya

CHAPTER 5: DISCUSSION

Rice, a diploid species in Poaceae, is feeding more than 50% of the world's population and providing an important source of revenue for most people in Asia and Africa (Nawaz et al., 2022). It is one of the ancient polyploids derived from at least one event of whole genome duplication and diploidisation, resulting in extensive gene loss and genome reorganisation (Cheng et al., 2022). The last half-century has witnessed a slew of significant scientific innovations for rice research (Figure 4.3) (Mohd Hanafiah et al., 2020), ranging from the development of its high-yielding semi-dwarf varieties IR8 to more sophisticated studies at the molecular level. The completion of the rice genome in the early 2000s has not only led to significant advances in its genetics and breeding (Figure 4.3) (Mohd Hanafiah et al., 2020), but it has also paved the way for the sequencing of more complex crop genomes, including two other major cereal crops wheat and maize (Jackson, 2016).

Although rice is an excellent model crop (Hirochika et al., 2004), its genetic diversity has been reduced due mainly to monoculture farming since the green revolution (Mohd Hanafiah et al., 2020; Pingali, 2019). Rice breeders should begin growing more genetically unique cultivars in a single field (Varshney et al., 2018). While the presence of weedy rice has been a challenge to rice production throughout the world, recent studies demonstrated that it has novel sources of resistance to certain rice diseases, including blast and sheath blight (SB) (Jia et al., 2022). Therefore, exploiting the potential of weedy rice can be beneficial for breeding and evolutionary studies of rice (Mohd Hanafiah et al., 2020).

5.1 Rice Research Development for Food Security in the Face of Climate Change

To keep up with rising worldwide demand for rice, researchers must come up with reliable means to increase its production per capita (Mohd Hanafiah et al., 2021).

Universiti Malaya

Furthermore, researchers have to continually develop more durable and high-quality rice varieties to address the ever-changing or fluctuating demand for rice (Papademetriou, 2000). Asia is the primary rice producer, accounting for 90% of global rice production and consumption (H. Yu et al., 2020). This could be the reason for rice studies in Asia accounted for the majority of the publications included in the systematic review, including Malaysia, China, India, Korea, and Japan (Section 4.1).

Figure 4.3 shows the major milestones in rice research since the green revolution. The success of the development of semi-dwarf variety IR8 during the 1960s was acknowledged by rice breeders worldwide (Mohd Hanafiah et al., 2020; Peng et al., 2021). The "miracle seeds" saved millions of lives throughout the world, particularly countries in Asia such as China and India (Hargrove & Cabanilla, 1979). After the inception of the green revolution, many breeding projects utilised semi-dwarf varieties as the donors for various food crops such as wheat (Syme, 1970) and maize (Welch, 1983). However, growing these modern varieties require the excessive utilisation of chemical pesticides and fertilisers, as well as the dependency on efficient irrigation systems (Mohd Hanafiah et al., 2020).

The green revolution has also led to an increase in monoculture, causing modern varieties to be more susceptible to changing environments, including biotic and abiotic stresses. Monoculture has been widely reported to decrease crop genetic diversity,making crops more susceptible to agricultural threats, especially disease and pest infestation (Cheng, 2018; Cheng et al., 2022; Massawe et al., 2016). Hence, crop diversification could be the key to maintain rice genetic diversity. Underutilised species,such as weedy rice and ancient grains, should be developed along with major crops to ensure the future of food security (Massawe et al., 2016; Mustafa et al., 2019). Closing this gap is crucial and requires collaborative efforts between different actors, including researchers, producers, governments, and consumers (Fischer, 2019; Mohd Hanafiah et al., 2020).

Universiti Malaya

Sustainable rice production can be achieved by increasing the production per unit area using ecologically regenerative approaches that involve minimal inputs (Mohd Hanafiah et al., 2020; Sherwood & Uphoff, 2000; Tester & Langridge, 2010). Several main strategies that can potentially contribute to sustainable rice production are presented in Figure 4.4, which include advanced research, smart and sustainable farming, precision technologies, and strengthening global policies. Smart farming has recently become the norm in some countries (such as the United States and Japan) in rice production (Pivoto et al., 2018). Precision technologies, as opposed to smart farming (which includes connected technologies that integrate all agricultural operations), focus on precise measurements using specific sensors or equipment, providing economic flexibility that is easier to implement (McBride & Daberkow, 2003; Mohd Hanafiah et al., 2020).

The strategies used for sustainable rice production should offer socio-economic benefits to both the producers and society for all social classes (Mohd Hanafiah et al., 2020). Some existing agricultural policies should either be revised or renewed to promote the use of sustainable farming approaches, especially for low- and medium-income farmers. Alternatively, policymakers can provide farmers with incentives to use natural resources wisely. It is important for policymakers to interact with other actors such as farmers, producers, and researchers in order to understand the current needs in building a resilient future in the agricultural sector (Sethuraman et al., 2021).

5.2 Development of Multiplex PCR Assays for Screening of Important Traits in Rice

Advanced research is one of the strategies for sustainable rice production (Figure 4.4). While the rapid development of new technologies such as genotyping procedures might

Universiti Malaya

be advantageous for modern breeding programmes, some of them can be expensive, time-consuming, and difficult to use in laboratories with limited resources (Mohd Hanafiah et al., 2021). To improve rice breeding efforts, particularly in resource-constrained environments such as those in developing and undeveloped nations, it is essential to identify and promote the most cost-effective means of screening the desired characteristics (Mohd Hanafiah et al., 2021).

Rice is known to be vulnerable to a variety of pathogenic species and diseases. Rice blast, SB, and bacterial leaf blight (BLB) are the most prevalent and widespread, lowering the quality and quantity of rice cultivars produced significantly. For example, a field condition substantially damaged by blast disease may lose roughly 50% of rice output, which is predicted to destroy enough rice crops each year to feed over 60 million people (Shahriar et al., 2020). This disease leads to production constraint in many parts of the world, including Malaysia (NurulNahar et al., 2020). Similarly to blast, BLB and SB have also been causing crop losses, accounting for 8.75% and 8.51% losses in China and the Indo-Gangetic Plain, respectively during the past ten years (Savary et al., 2019). According to Lau and Botella (2017), massive grain losses occur mostly as a result of numerous pests and diseases, which are specifically severe in developing countries.

It is worth noting that the improvement in the quality of life in many developing countries has resulted in a shift in rice demand, with many consumers willing to pay more for more palatable rice, such as fragrance rice and healthy rice (Zhou et al., 2020). One way to develop more desirable rice varieties is through the utilisation of functional markers linked to genes of interest in modern breeding programmes. In fact, rice breeders today frequently strive to improve multiple key traits (such as agronomic, quality, and resistance) within a single breeding programme, which oftentimes requires a large number of samples (Ahmar et al., 2020; Mohd Hanafiah et al., 2021).

Universiti Malaya

In order to find suitable allele-specific markers for local rice breeding programmes, the current study tested and validated a total of 30 published markers for BLB, SB, blast resistance, fragrance, and amylose content genes (Table 3.1) that have been effectively used in various recent rice breeding programmes. The primary selection criteria include biological (i.e., inclusion of resistant and susceptible checks), technical (i.e., functional polymorphisms on local checks), and practical (i.e., efficacy in breeding programmes) aspects (Mohd Hanafiah et al., 2021; Platten et al., 2019). A set of control varieties and local traditional and improved rice varieties (Tables 3.2) with varying disease resistance and grain quality backgrounds were subjected to uniplex PCRs. Of the 30 selected markers, six showed clear polymorphisms in uniplex PCRs and consistent with the previous studies (Table 3.1, Figures 4.6- 4.11) (Cheng et al., 2015; Hajira et al., 2016; Hossain et al., 2016; Miah et al., 2016), and hence, these markers (RM6836, RM8225 RM202, pTA248, *fgr*-SNP and *Wx*-SSR) were used for the development of multiplex PCR assays.

Even though, multiplex may have several drawbacks including good quality DNA may be required, requirement for optimising multiple primers and potential ineffective in treating various populations, developed novel multiplex PCR assays from this study are reliable to aid breeding initiatives for developing countries that have limited resources to carry out large-scale genotyping as well as breeding for high-quality and resistant rice varieties.

Although different types of molecular markers were used, including SSR, SNP, and STS (Table 3.3), the PCR cycle reported by McCouch et al. (2002) to screen SSRs was efficient to be used in the developed multiplex PCR assays in this study. This demonstrates that a universal PCR cycle is found to detect the desired traits in a single tube, implying that the same protocol can be applied to other genes containing functional molecular markers.

Universiti Malaya

This study developed eight agarose gel-based multiplex assays that can detect up to four genes in a single PCR run (Table 3.3, Figures 4.12- 4.19). The multiplex results, either from high-resolution agarose gel electrophoresis (Section 4.2.3.1) or capillary electrophoresis and sequencing (Section 4.2.3.2), were found to be comparable and compatible with previous research (Cheng et al., 2015; Hajira et al., 2016; Hossain et al., 2016; Miah et al., 2016).

Capillary electrophoresis genotyping has been shown to be more sensitive than agarose gel electrophoresis because it can differentiate PCR products that differ by less than 5 bp (Aloui et al., 2015; Stewart et al., 2011). However, the cost of using capillary electrophoresis to get comparable findings is substantially greater than that of using high-resolution agarose gel-based electrophoresis, especially since conventional capillary electrophoresis requires the use of costly equipment and fluorescent-labeled primers (Pan et al., 2018). Each sample analysed by high-resolution agarose gel electrophoresis in this study cost about USD 0.30 (when the same gel was reused ten times), whereas capillary electrophoresis cost USD 7.60. It is worth noting that the cost of sequencing per sample was approximately USD 6.00. Additionally, it should be highlighted that the same high-resolution agarose gel may be easily reused up to 15 times without degrading the results' quality. Nonetheless, capillary electrophoresis or direct sequencing would be a better alternative if funding is not a constraint, given that these advanced genotyping technologies allow for fast screening for alleles that differ by as little as 1 bp (Lian et al., 2021; Mansor et al., 2015).

5.3. Weedy Rice as Potential Genetic Resources for Rice Breeding Programme

Although abundant information is available on the traits of weedy rice, information on the disease resistance traits of weedy rice is limited (Jia & Gealy, 2018). It is well known that weedy rice has high adaptability towards environment which enable them to survive

Universiti Malaya

in harsh conditions and disease infestation (Mohd Hanafiah et al., 2020; Nadir et al., 2017). Therefore, the useful genes for biotic stress especially for disease resistance which have been maintained in weedy rice can be explored for breeding (Jia & Gealy, 2018). Figure 5.1 showed weedy rice in a BLB infected fields possessed various degrees of infection from highly susceptible to strong resistance towards BLB. Therefore, it is critical to investigate weedy rice genetic background to be utilised for rice improvement, which has received little attention (He et al., 2017; Mohd Hanafiah et al., 2020). Weedy rice has a strong competitive ability for resources compared to cultivated rice, and it is seen as a severe threat to rice production in many leading rice-producing countries (Jia et al., 2022; Singh et al., 2013).

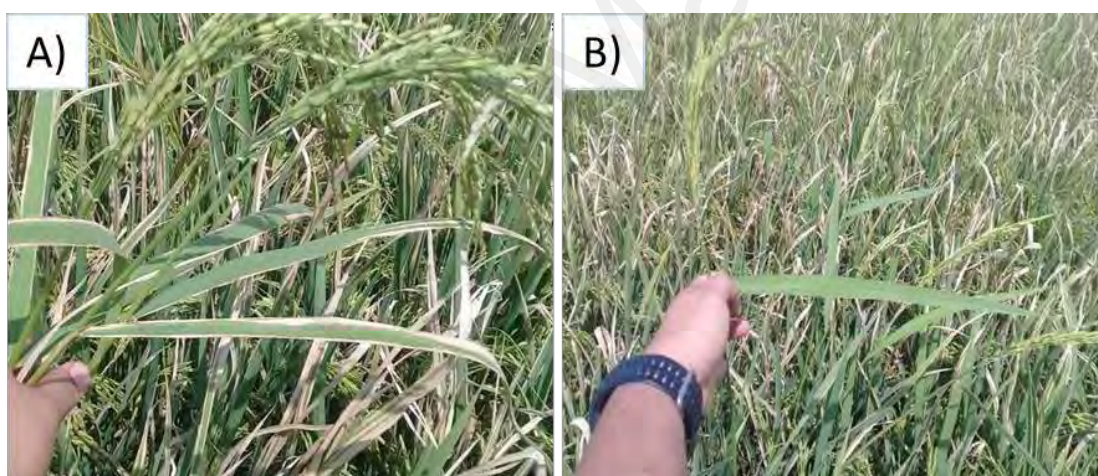

Figure 5.1: Observations in field showed weedy rice(s) in bacterial leaf blight (BLB) infected fields possessed various degrees of infection from A) highly susceptible to B) strong resistance to BLB. This indicates rich genetic diversity in weedy rice as a potential to provide useful genetic resources for rice breeding programme

Weedy rice has been reported to have a broad-spectrum resistance to rice blast (Nadir et al., 2017). For example, 28 QTLs for novel blast resistance were identified from weedy rice ecotypes of the United States (Liu et al., 2015). Similarly, in this study, resistance to rice blast was found in 19% of the Malaysian weedy rice biotypes (Figure 4.29).

SB resistance genotype also varies in the US weedy rice, with *indica* types being more resistant than *japonica* types (Goad et al., 2020). Novel QTL conferring SB resistance

University of Malaya

was detected in two weedy rice populations using black hull and straw hull awned biotypes as the genetic background (Goad et al., 2020). Furthermore, the height of weedy rice can help prevent SB damage that causes rice stem, leaf, and sheath injury (Jia & Gealy, 2018). Similarly, in this study, resistance to sheath blight was found in 12% of the Malaysian weedy rice biotypes (Figure 4.29).

This study also identified 89% of the weedy rice possessed resistant gene to BLB. This indicate that weedy rice can be an effective option to screen the beneficial specific genes and crossing-over with rice cultivar to produce new BLB-resistant rice variety (Mohd Hanafiah et al., 2022). Majority of the weedy rice biotypes showed presence of resistance gene detected by molecular marker, pTA248 thus would be a good resistance trait to be explored deeper particularly using larger weedy rice samples to study on the genetic diversity and their potential utilisation in breeding programme. To date, there are more than 42 resistance genes that have been identified and used in rice plant breeding (Shu et al., 2021). Among the BLB resistance genes identified, *Xa21*, originally introgressed from an accession of wild rice, *O. longistaminata* mapped to chromosome 11 (Ronald et al., 1992; Song et al., 1995), is a major one conferring broad spectrum resistance against many virulent isolates of the pathogen (Hajira et al., 2016). Another example is *Xa27*, one of the major resistance gene identified from wild rice, *O. minuta*. *Xa27* confers a broad-spectrum resistance against Xoo that originated from Asian regions (Bimolata et al., 2013). Based on DNA polymorphism analysis of *Xa27* in two species of wild rice, *O. nivara* and *O. sativa*, this resistance gene may offer adaptive trait mechanism that can be preserved across the breeding process. This study showed that weedy rice in Malaysia has resistant towards BLB which can be further investigated in rice breeding programme (Mohd Hanafiah et al., 2021).

Heterozygosity was also observed in all three biotic stresses genotyping among the weedy rice biotypes indicating potential crop-weed outcrossing. According to Xia et al.

(2011), outcrossing rates have a major impact on population heterozygosity, which may influence how weedy rice evolves. In other words, outcrossing can significantly increase heterozygosity in weedy rice populations.

The multiplex assay (Multiplex-7; Table 3.3) developed in this study showed a potential of using multiplex technique to screen various weedy rice biotypes in a short period of time. The rapid screening can accelerate the utilisation of potential genes/loci of weedy rice for rice cultivar improvement programmes (Table 4.8). Additionally, this multiplex PCR method provides a rapid and efficient method for detecting different resistance genes of three major rice diseases, which will aid in pyramiding genes to increase the durability of SB, BLB and blast resistance. Multiplex PCR has come a long way since its inception in 1988 and has been successfully employed in numerous gene-based selections in various crop improvement programmes (Mohd Hanafiah et al., 2021). Several multiplex PCR systems have been developed to detect key genes and markers related to grain quality (Cheng et al., 2015), and disease resistance in rice (Bangratz et al., 2020; Yap et al., 2016). These examples show that multiplex PCR provides a quick, low-cost, and efficient method for pyramiding genes in rice breeding programmes. Furthermore, this strategy offers advantages over traditional breeding, which may take longer and be more difficult to achieve due to dominance and epistasis of alleles providing disease resistance (Yap et al., 2016).

Weedy rice in Pekan, Sungai Burung, and Seberang Perak displayed the highest genetic variations for BLB, blast and SB, respectively (Table 4.9). These genetic variations showed that weedy rice in these locations are highly diverse for genes associated with rice diseases. Selection of weedy rice biotypes from these locations for breeding program needs a careful consideration. Expected heterozygosity (He) is a common statistic for assessing genetic variation within populations of which its accuracy and precision on the estimation of genetic diversity decreases when individuals are related or inbred, due to

Universiti Malaya

increased dependence among allele copies in the sample (Harris & DeGiorgio, 2017). On the other hand, weedy rice in Sungai Leman and Sungai Besar only have resistant biotypes (He=0.00) for BLB (Table 4.9). This mono-allelic event specific for BLB in these two locations provide potential of selection of weedy rice on BLB specific genes for rice breeding. Low observed heterozygosity (Ho=0.062) for genotypes associated with major rice diseases in weedy rice for this study (Table 4.9) is consistent with a low selfing rate for the weedy rice as previously reported for weedy rice in Malaysia (Ho=0.065) (Song et al. 2014) and in the USA (Ho=0.02).

The higher the Shannon's Index, the greater the genetic diversity in a population (Jasim Aljumaili et al., 2018). Shannon's Information Index (I) in Sungai Leman was zero for all three diseases (Table 4.9). However, only eight samples of weedy rice were collected from this particular location (N=8), suggesting that this number of samples may not adequately represent the population's wide genetic diversity (Shrestha et al., 2022). To gain insight into the genetic basis of this trait in weedy rice, a larger sample size, a greater number of markers, or high-throughput sequencing techniques will be required in the future to capture the genetic variance associated with major rice diseases.

Universiti Malaya

CHAPTER 6: CONCLUSION

6.1 Conclusion

Rice, as one of the world's major crops with a small genome size, has deservedly garnered a lot of attention from researchers worldwide. However, the systematic review conducted in this study pointed that rice has lost genetic diversity since the green revolution due to monoculture farming, which is heavily reliant on inputs such as water, chemical fertilisers, and pesticides. Yields in many major rice-producing countries, including China, have been reported to be plateauing, and the yield gap between rice fields and research stations that provide actual and potential yield, respectively, is still an issue in many countries. Closing this gap is critical, and it will require collaborative efforts from different actors such as breeders and governments to ensure that rice production increases in a sustainable manner. Significant investment in rice research is required to revitalise breeding programmes and technology transfer schemes throughout the world, especially in developing nations, in order to provide producers with improved varieties, as well as the necessary knowledge of technologies and skills through appropriate programmes.

To save cost of genotyping in local rice breeding programmes with limited funding, simple and reliable agarose-based genotyping assays were developed in this study to analyse multiple key genes in a single PCR tube, which can determine up to four major biotic stresses (blast, BLB, and SB) and grain quality (fragrance and amylose content). These practical assays are particularly valuable for researchers in developing countries who are interested in mass genotyping or work in facilities with limited infrastructures (Mohd Hanafiah et al., 2021). However, advanced methods such as capillary electrophoresis and sequencing are less labour intensive because they are fully automated, though skilled researchers are required to operate the equipment (Lian et al., 2021;

Universiti Malaya

Sarkozy & Guttman, 2022). It is worth noting that research on understanding the polyploid origin can set the platform for more successful development of rice and its polyploids in future (Cheng et al., 2022).

Screening of weedy rice biotypes using one of the developed gel-based multiplex assays in this study will allow simultaneous analysis of functional polymorphisms BLB, SB, and blast resistance genes. This would play an important role in detecting potential use of weedy rice biotypes with resistance genes for future development of rice cultivars with enhanced resistance. Exploring the potential of weedy rice is critical, and more research is needed to identify the suitable biotypes for future breeding programmes. In addition, weedy rice which is the conspecific weed with the cultivated rice can be the best candidate to be added in the germplasm for future rice breeding programmes. The close genetic relationship with cultivated rice will give further advantage for the weedy rice to be included as potential genetic resources in the rice breeding programmes.

6.2 Recommendations for Future Research

A larger sample size, a greater number of markers, or high-throughput sequencing techniques are recommended for future studies to gain a better understanding of the genetic basis of important traits in both commercial and weedy rice. Additionally, if the study is focused on comparing the selected parameters across several different locations, a similar sample size from each location may result in a better overall finding.

Universiti Malaya

REFERENCES

Abraham, A., Mathew, A. K., Sindhu, R., Pandey, A., & Binod, P. (2016). Potential of rice straw for bio-refining: An overview. *Bioresource Technology*, *215*, 29-36.

Abraham, C. T., & Jose, N. (2015). Weedy rice invasion and its management. *Indian Journal of Weed Science*, 47(3), 216-223.

Acharya, A., Adhikari, N., Amgain, R., Poudel, A., Yadav, R., & Poudyal, K. (2018). Identification of rice genotypes resistant to bacterial leaf blight disease using SSR markers. *Journal of the Institute of Agriculture and Animal Science*, *35*(1), 113- 120.

Adnan, N., & Nordin, S. M. (2020). How COVID 19 effect Malaysian paddy industry? Adoption of green fertilizer a potential resolution. *Environment, Development and Sustainability*, *23*, 8089–8129.

Agostinetto, D., Oliveira, C., Langaro, A. C., Silva, J. D. G. d., & Barbieri, G. F. (2022). Differences in biochemical, physiological and molecular response mechanisms of rice, weedy rice and barnyardgrass subjected to drought. *Pesquisa AgropecuáriaTropical*, *52*.

Ahmar, S., Gill, R. A., Jung, K.-H., Faheem, A., Qasim, M. U., Mubeen, M., & Zhou, W. (2020). Conventional and molecular techniques from simple breeding to speed breeding in crop plants: Recent advances and future outlook. *International Journal of Molecular Sciences*, *21*(7), Article#2590.

Ajala, A., & Gana, A. (2015). Analysis of challenges facing rice processing in Nigeria. *Journal of Food Processing*, *2015*.

Aloui, C., Sut, C., Cognasse, F., Granados, V., Hassine, M., Chakroun, T., … Laradi, S. (2015). Development of a highly resolutive method, using a double quadruplex tetra-primer-ARMS-PCR coupled with capillary electrophoresis to study CD40LG polymorphisms. *Molecular and Cellular Probes*, *29*(6), 335-342.

Alvarez-Martinez, C. E., Sgro, G. G., Araujo, G. G., Paiva, M. R., Matsuyama, B. Y., Guzzo, C. R., … Farah, C. S. (2021). Secrete or perish: The role of secretion systems in Xanthomonas biology. *Computational and Structural Biotechnology Journal*, *19*, 279-302.

Alvarez, A. L., Weyers, S. L., Goemann, H. M., Peyton, B. M., & Gardner, R. D. (2021). Microalgae, soil and plants: A critical review of microalgae as renewable resources for agriculture. *Algal Research*, *54*, Article#102200.

Ameen, A., & Raza, S. (2017). Green revolution: A review. *International Journal of Advances in Scientific Research*, *3*(12), 129-137.

Annegowda, D. C., Prasannakumar, M. K., Mahesh, H. B., Siddabasappa, C. B., Devanna, P., Banakar, S. N., … Prasad, S. R. (2021). Rice Blast Disease in India: Present Status and Future Challenges. In M. Huang (Ed), *Integrated Advances in Rice Research* (pp. 157-197). London, England: IntechOpen.

Universiti Malaya

Arshad, F. M., Alias, E. F., Noh, K. M., & Tasrif, M. (2011). Food security: Self-sufficiency of rice in Malaysia. *International Journal of Molecular Sciences, 18*(2), 83-100.

Arunakumari, K., Durgarani, C., Satturu, V., Sarikonda, K., Chittoor, P., Vutukuri, B., … Jamal, M. (2016). Marker-assisted pyramiding of genes conferring resistance against bacterial blight and blast diseases into Indian rice variety MTU1010. *Rice Science*, *23*(6), 306-316.

Ashkani, S., Rafii, M., Sariah, M., Siti, N. A. A., Rusli, I., Harun, A., & Latif, M. (2011). Analysis of simple sequence repeat markers linked with blast disease resistance genes in a segregating population of rice (*Oryza sativa*). *Genetics and Molecular Research*, *10*(3), 1345-1355.

Ashkani, S., Rafii, M. Y., Shabanimofrad, M., Miah, G., Sahebi, M., Azizi, P., … Nasehi, A. (2015). Molecular breeding strategy and challenges towards improvement of blast disease resistance in rice crop. *Frontiers in Plant Science*, *6*, Article#886.

Awika, J. M. (2011). Major cereal grains production and use around the world. In *Advances in cereal science: Implications to food processing and health promotion* (pp. 1-13). ACS Publications.

Ayres, N., McClung, A., Larkin, P., Bligh, H., Jones, C., & Park, W. (1997). Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germ plasm. *Theoretical and Applied Genetics*, *94*(6), 773-781.

Azmi, M., Abdullah, M., Mislamah, B., & Baki, B. (2000). Management of weedy rice (*Oryza sativa* L.): the Malaysian experience. *Wild and Weedy Rice in Rice Ecosystems in Asia-A Review*, 91-96.

Azmi, M., & Karim, S. (2008). Weedy rice-biology, ecology and management. *Malaysian Agricultural Research and Development Institute (MARDI), Kuala Lumpur, Malaysia*, 56.

Babujee, L., & Gnanamanickam, S. (2000). Molecular tools for characterization of rice blast pathogen (*Magnaporthe grisea*) population and molecular marker-assisted breeding for disease resistance. *Current Science*, *78*(3), 248-257.

Badri, J., Dey, S., Prakasam, V., Nymagoud, S., Priyanka C, Shaik, H., … Subba Rao, L. V. (2017). Allelic variation of sheath blight QTLs among genotypes promising for sheath blight tolerance. *Journal of Rice Research*, *10*(1), 36-39.

Baki, B., & Mispan, M. (2010). Spatio-temporal distribution pattern of new biotypes of weedy rice (*Oryza sativa* L.) in Selangor north-west project, Malaysia. *Korean Journal of Weed Science*, *30*(2), 68-83.

Balázs, B., Kelemen, E., Centofanti, T., Vasconcelos, M. W., & Iannetta, P. P. (2021). Policy interventions promoting sustainable food-and feed-systems: A delphi study of legume production and consumption. *Sustainability*, *13*(14), Article#7597.

Universiti Malaya

Bandumula, N. (2017). Rice production in Asia: Key to global food security. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *88*(4),1323-1328.

Bangratz, M., Wonni, I., Kini, K., Sondo, M., Brugidou, C., Béna, G., … Silué, D. (2020). Design of a new multiplex PCR assay for rice pathogenic bacteria detection and its application to infer disease incidence and detect co-infection in rice fields in Burkina Faso. *PLOS One*, *15*(4), Article#e0232115.

Bao, J., Zhuo, C., Zhang, D., Li, Y., Hu, F., Li, H., … He, H. (2021). Potential applicability of a cyanobacterium as a biofertilizer and biopesticide in rice fields. *Plant and Soil*, *463*(1), 97-112.

Barbhuiya, A. (2017). *Identification of gene clusters in rice genome* [Dissertation, Lovely Professional University]. Punjab, India.

Bennett, E. M., Cramer, W., Begossi, A., Cundill, G., Díaz, S., Egoh, B. N., … Lazos, E. (2015). Linking biodiversity, ecosystem services, and human well-being: Three challenges for designing research for sustainability. *Current Opinion in Environmental Sustainability*, *14*, 76-85.

Bevilacqua, C. B., Basu, S., Pereira, A., Tseng, T.-M., Zimmer, P. D., & Burgos, N. R. (2015). Analysis of stress-responsive gene expression in cultivated and weedy rice differing in cold stress tolerance. *PLOS One*, *10*(7).

Bigini, V., Camerlengo, F., Botticella, E., Sestili, F., & Savatin, D. V. (2021). Biotechnological resources to increase disease-resistance by improving plant immunity: A sustainable approach to save cereal crop production. *Plants*, *10*(6), Article#1146.

Bimolata, W., Kumar, A., Sundaram, R. M., Laha, G. S., Qureshi, I. A., Reddy, G. A., & Ghazi, I. A. (2013). Analysis of nucleotide diversity among alleles of the major bacterial blight resistance gene *Xa27* in cultivars of rice (*Oryza sativa*) and its wild relatives. *Planta*, *238*(2), 293-305.

Biswal, A. K., Shamim, M., Cruzado, K., Soriano, G., Ghatak, A., Toleco, M., & Vikram, P. (2017). Role of biotechnology in rice production. In *Rice Production Worldwide* (pp. 487-547). Springer.

Biswas, P. L., Nath, U. K., Ghosal, S., Goswami, G., Uddin, M., Ali, O. M., … Hossain, A. (2021). Introgression of bacterial blight resistance genes in the rice cultivar Ciherang: Response against *Xanthomonas oryzae* pv. *oryzae* in the f6 generation. *Plants*, *10*(10), Article#2048.

Bligh, H., Till, R., & Jones, C. (1995). A microsatellite sequence closely linked to the *Waxy* gene of *Oryza sativa*. *Euphytica*, *86*(2), 83-85.

Bokor, B., Santos, C. S., Kostoláni, D., Machado, J., da Silva, M. N., Carvalho, S. M., … Vasconcelos, M. W. (2021). Mitigation of climate change and environmental hazards in plants: Potential role of the beneficial metalloid silicon. *Journal of Hazardous Materials*, *416*, Article#126193.

Universiti Malaya

Borah, N., Sarma, R. N., Borah, V. V., & Behera, P. P. (2022). Status of Genomic Research in Non-basmati Aromatic Joha Rice of Assam. *International Journal of Environment and Climate Change*, *12*(10), 338-347.

Bos, H. L., & Broeze, J. (2020). Circular bio-based production systems in the context of current biomass and fossil demand. *Biofuels, Bioproducts and Biorefining*, *14*(2),187-197.

Bradbury, L. M., Henry, R. J., Jin, Q., Reinke, R. F., & Waters, D. L. (2005). A perfect marker for fragrance genotyping in rice. *Molecular Breeding*, *16*(4), 279-283.

Brodt, S., Six, J., Feenstra, G., Ingels, C., & Campbell, D. (2011). Sustainable agriculture. *Nature Education Knowledge*, *3*(1).

Butardo, V. M., Sreenivasulu, N., & Juliano, B. O. (2019). Improving rice grain quality: State-of-the-art and future prospects. *Rice Grain Quality*, *1892*, 19-55.

Bzour, M. I., Zuki, F. M., & Mispan, M. S. (2018). Introduction of imidazolinone herbicide and Clearfield® rice between weedy rice—control efficiency and environmental concerns. *Environmental Reviews*, *26*(2), 181-198.

Cai, X. L., Wang, Z. Y., Xing, Y. Y., Zhang, J. L., & Hong, M. M. (1998). Aberrant splicing of intron 1 leads to the heterogeneous 5′ UTR and decreased expression of *waxy* gene in rice cultivars of intermediate amylose content. *The Plant Journal*,*14*(4), 459-465.

Cavalli-Sforza, L. L., & Edwards, A. W. (1967). Phylogenetic analysis. Models and estimation procedures. *American Journal of Human Genetics*, *19*(3 Pt 1), 233.

Chandra, H., Yadava, Y. K., Bishnoi, P., & Kumar, P. (2017). Genetic diversity analysis using microsatellite based simple sequence repeats (SSR) markers in Basmati *indica* rice (*Oryza sativa* L.). *Vegetos- An International Journal of Plant Research*, *30*, 284-292.

Chel, A., & Kaushik, G. (2011). Renewable energy for sustainable agriculture. *Agronomy for Sustainable Development*, *31*(1), 91-118.

Chen, L. J., Lee, D. S., Song, Z. P., Suh, H. S., & Lu, B. R. (2004). Gene flow from cultivated rice (*Oryza sativa*) to its weedy and wild relatives. *Annals of Botany*, *93*(1), 67-73.

Chen, S., Wu, J., Yang, Y., Shi, W., & Xu, M. (2006). The *fgr* gene responsible for rice fragrance was restricted within 69 kb. *Plant Science*, *171*(4), 505-514.

Chen, S., Yang, Y., Shi, W., Ji, Q., He, F., Zhang, Z., … Xu, M. (2008).*Badh2*, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. *The Plant Cell*, *20*(7), 1850-1861.

Cheng, A. (2018). Shaping a sustainable food future by rediscovering long-forgotten ancient grains. *Plant Science*, *269*, 136-142.

Universiti Malaya

Cheng, A., Ismail, I., Osman, M., & Hashim, H. (2012). Simple and rapid molecular techniques for identification of amylose levels in rice varieties. *International Journal of Molecular Sciences*, *13*(5), 6156-6166.

Cheng, A., Ismail, I., Osman, M., Hashim, H., & Mohd Zainual, N. S. (2017). Rapid and targeted introgression of *fgr* gene through marker-assisted backcrossing in rice (*Oryza sativ*a L.). *Genome*, *60*(12), 1045-1050.

Cheng, A., Massawe, F., Ismail, I., Osman, M., & Hashim, H. (2015). High resolution agarose-based system for single-tube genotyping of *fgr* and *Waxy* genes in rice: MAGE to displace PAGE? *Plant Omics*, *8*(4), Article#348.

Cheng, A., Mohd Hanafiah, N., Harikrishna, J. A., Lim, P. E., Baisakh, N., & Mispan, M. S. (2022). A reappraisal of polyploidy events in grasses (Poaceae) in a rapidly changing world. *Biology*, *11*(5), Article#636.

Cheng, A., Raai, M. N., Mohd Zain, N. A., Massawe, F., Singh, A., & Wan Mohtar, W. A. A. Q. I. (2019). In search of alternative proteins: unlocking the potential of underutilized tropical legumes. *Food Security*, 1-11.

Christiaensen, L., Rutledge, Z., & Taylor, J. E. (2021). The future of work in agri-food. *Food Policy*, *99*, Article#101963.

Chukwu, S. C., Rafii, M. Y., Ramlee, S. I., Ismail, S. I., Oladosu, Y., Okporie, E., … Swaray, S. (2019a). Marker-assisted selection and gene pyramiding for resistance to bacterial leaf blight disease of rice (*Oryza sativa*L.). *Biotechnology & Biotechnological Equipment*, *33*(1), 440-455.

Chukwu, S. C., Rafii, M., Ramlee, S., Ismail, S., Hasan, M., Oladosu, Y., … Olalekan, K. (2019b). Bacterial leaf blight resistance in rice: A review of conventional breeding to molecular approach. *Molecular biology reports*, *46*(1), 1519-1532.

Clapp, J., Moseley, W. G., Burlingame, B., & Termine, P. (2021). The case for a six-dimensional food security framework. *Food Policy*, Article#102164.

Cobb, J. N., Juma, R. U., Biswas, P. S., Arbelaez, J. D., Rutkoski, J., Atlin, G., … Ng, E. H. (2019). Enhancing the rate of genetic gain in public- sector plant breeding programs: Lessons from the breeder's equation. *Theoreticaland Applied Genetics*, *132*(3), 627-645.

Cohen, S. P., & Leach, J. E. (2019). Abiotic and biotic stresses induce a core transcriptome response in rice. *Scientific Reports*, *9*(1), 1-11.

Collard, B. C., & Mackill, D. J. (2008). Marker-assisted selection: An approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1491), 557-572.

Cordeiro, G. M., Christopher, M. J., Henry, R. J., & Reinke, R. F. (2002). Identification of microsatellite markers for fragrance in rice by analysis of the rice genome sequence. *Molecular Breeding*, *9*(4), 245-250.

Cornell Alliance for Science. (2018). *US FDA Approves Golden Rice*.

Universiti Malaya

Cui, Z., Ojaghian, M., Tao, Z., Kakar, K., Zeng, J., Zhao, W., … Zhu, B. (2016). Multiplex PCR assay for simultaneous detection of six major bacterial pathogens of rice. *Journal of Applied Microbiology*, *120*(5), 1357-1367.

Custodio, M. C., Cuevas, R. P., Ynion, J., Laborte, A. G., Velasco, M. L., & Demont, M. (2019). Rice quality: How is it defined by consumers, industry, food scientists, and geneticists? *Trends in Food Science & Technology*, *92*, 122-137.

Das, G., Patra, J. K., & Baek, K.-H. (2017). Insight into MAS: A molecular tool for development of stress resistant and quality of rice through gene stacking. *Frontiers in Plant Science*, *8*, Article#985.

Das, G., & Rao, G. (2015). Molecular marker assisted gene stacking for biotic and abiotic stress resistance genes in an elite rice cultivar. *Frontiers in Plant Science*, *6*, Article#698.

De Wever, J., Everaert, H., Coppieters, F., Rottiers, H., Dewettinck, K., Lefever, S., & Messens, K. (2019). The development of a novel SNP genotyping assay to differentiate cacao clones. *Scientific Reports*, *9*(1), 1-10.

Deb, D. (2020). Is the system of rice intensification (SRI) consonant with agroecology? *Agroecology and Sustainable Food Systems*, *44*(10), 1338-1369.

Dela Cruz, N., & Khush, G. (2000). Rice grain quality evaluation procedures. In *Aromatic rices* (Vol. 3, pp. 15-28). International Rice Research Institute.

Delannay, X., McLaren, G., & Ribaut, J.-M. (2012). Fostering molecular breeding in developing countries. *Molecular Breeding*, *29*(4), 857-873.

Dey, S., Badri, J., Ram, K., Chhabra, A., & Janghel, D. (2019). Current status of rice breeding for sheath blight resistance. *International Journal of Current Microbiology and Applied Sciences*, *8*(2), 163-175.

Dixit, S., Singh, U. M., Singh, A. K., Alam, S., Venkateshwarlu, C., Nachimuthu, V. V., … Devi, M. N. (2020). Marker assisted forwardbreeding to combine multiple biotic-abiotic stress resistance/tolerance in rice. *Rice*, *13*(1), 1-15.

Duan, X., Li, X., Xue, Q., Abo-EI-Saad, M., Xu, D., & Wu, R. (1996). Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. *Nature Biotechnology*, *14*(4), Article#494.

Food and Agriculture Organization. (2018). *Rice Market Monitor: Food and agriculture organization of United Nations* (Washington, DC, Issue 1).

Food and Agriculture Organization of the United Nations, International Fund for Agricultural Development, United Nations Children's Fund, World Food Programme, & World Health Organization. (2017). *The state of food security and nutrition in the world 2017.* FAO.

Universiti Malaya

Food and Agriculture Organization of the United Nations, United Nations Children's Fund, World Food Programme, & World Health Organization. (2019). *Placing Nutrition at the Centre of Social Protection. Asia and the Pacific Regional Overview of Food Security and Nutrition*. FAO.

Fedoroff, N. V., Battisti, D. S., Beachy, R. N., Cooper, P. J., Fischhoff, D. A., Hodges, C., … Molden, D. (2010). Radically rethinking agriculture for the 21st century. *Science*, *327*(5967), 833-834.

Firdaus, R. R., Leong Tan, M., Rahmat, S. R., & Senevi Gunaratne, M. (2020). Paddy, rice and food security in Malaysia: A review of climate change impacts. *Cogent Social Sciences*, *6*(1), Article#1818373.

Fischer, R. A. (2019). Closing crop yield gaps around the world. In R. Zeigler (Ed.), *Sustaining Global Food Security: The Nexus of Science and Policy* (pp. 14). CSIRO Publishing.

Fu, C., Wu, T., Liu, W., Wang, F., Li, J., Zhu, X., … Zhu, M. (2012). Genetic improvement of resistance to blast and bacterial blight of the elite maintainer line Rongfeng B in hybrid rice (*Oryza sativa* L.) by using marker-assisted selection. *African Journal of Biotechnology*, *11*(67), 13104-13114.

Fukagawa, N. K., & Ziska, L. H. (2019). Rice: Importance for Global Nutrition. *Journal of Nutritional Science and Vitaminology*, 65 (Supplement), S2-S3.

Fukuoka, S., Saka, N., Mizukami, Y., Koga, H., Yamanouchi, U., Yoshioka, Y., … Yano, M. (2015). Gene pyramiding enhances durable blast disease resistance in rice. *Scientific Reports*, *5*, Article#7773.

Gadal, N., Shrestha, J., Poudel, M. N., & Pokharel, B. (2019). A review on production status and growing environments of rice in Nepal and in the world. *Archives of Agriculture and Environmental Science*, *4*(1), 83-87.

Gealy, D. (2005). Gene movement between rice (*Oryza sativa*) and weedy rice (*Oryza sativa*)-a US temperate rice perspective. *Crop Ferality and Volunteerism*, 323- 354.

Ghosh, A., Misra, S., Bhattacharyya, R., Sarkar, A., Singh, A. K., Tyagi, V. C., … Meena, V. S. (2020). Agriculture, dairy and fishery farming practices and greenhouse gas emission footprint: a strategic appraisal for mitigation. *Environmental Science and Pollution Research*, *27*(10), 10160-10184.

Gibson, D. J. (2009). *Grasses and grassland ecology*. Oxford University Press.

Goad, D. M., Jia, Y., Gibbons, A., Liu, Y., Gealy, D., Caicedo, A. L., & Olsen, K. M. (2020). Identification of novel QTL conferring sheath blight resistance in two weedy rice mapping populations. *Rice*, *13*(21), 1-10.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., … Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *Science*, *327*(5967), 812-818.

Universiti Malaya

Golestan Hashemi, F., Rafii, M., Ismail, M., Mahmud, T., Rahim, H., Asfaliza, R., … Latif, M. (2013). Biochemical, genetic and molecular advances of fragrance characteristics in rice. *Critical Reviews in Plant Sciences*, *32*(6), 445-457.

Golestan Hashemi, F., Rafii, M., Razi Ismail, M., Mohamed, M., Rahim, H., Latif, M., & Aslani, F. (2015). Opportunities of marker-assisted selection for rice fragrance through marker–trait association analysis of microsatellites and gene-based markers. *Plant Biology*, *17*(5), 953-961.

Global Rice Science Partnership. (2003). Rice almanac, 4th Edition. In (pp. 283). Los Banos, Philippines: International Rice Research Institute.

Guan, H., Hou, X., Jiang, Y., Srivastava, V., Mao, D., Pan, R., … Chen, Z. (2019). Feature of blast resistant near-isogenic lines using an elitemaintainer line II-32B by marker-assisted selection. *Journal of Plant Pathology*, *101*(3), 491-501.

Gull, A., Lone, A. A., & Wani, N. U. I. (2019). Biotic and Abiotic Stresses in Plants. In A. B. de Oliveira (Ed.), *Abiotic and Biotic Stress in Plants* (pp. 1-19). London, England: IntechOpen.

Gupta, V., Dorsey, G., Hubbard, A. E., Rosenthal, P. J., & Greenhouse, B. (2010). Gel versus capillary electrophoresis genotyping for categorizing treatment outcomes in two anti-malarial trials in Uganda. *Malaria Journal*, *9*(19).

Hajira, S., Sundaram, R., Laha, G., Yugander, A., Balachandran, S., Viraktamath, B., … Anila, M. (2016). A single-tube, functional marker-based multiplex PCR assay for simultaneous detection of majorbacterial blight resistance genes *Xa21*, *xa13* and *xa5* in rice. *Rice Science*, *23*(3), 144-151.

Han, B., Cui, D., Ma, X., Cao, G., Zhang, H., Koh, H. J., & Han, L. (2022). Evidence for evolution and selection of drought-resistant genes based on high-throughput resequencing in weedy rice. *Journal of Experimental Botany*, *73*(7), 1949-1962.

Hargrove, T. R., & Cabanilla, V. L. (1979). The impact of semidwarf varieties on Asian rice-breeding programs. *Bioscience*, *29*(12), 731-735.

Harris, A. M., & DeGiorgio, M. (2017). An unbiased estimator of gene diversity with improved variance for samples containing related and inbred individuals of any ploidy. *G3: Genes, Genomes, Genetics*, *7*(2), 671-691.

Harun, S. N., Hanafiah, M. M., & Aziz, N. I. H. A. (2021). An LCA-based environmental performance of rice production for developing a sustainable agri-food system in Malaysia. *Environmental Management*, *67*(1), 146-161.

Hasan, M., Rafii, M., Ismail, M., Mahmood, M., Rahim, H., Latif, M., … Malek, M. (2018). Genetic analysis of the resistance to rice blast in the BC2F1 population derived from MR263× Pongsu Seribu 1. *Biotechnology &Biotechnological Equipment*, *32*(5), 1134-1140.

Hasan, M. M. (2015). *Development of blast resistant rice variety through marker-assisted backcrossing between varieties MR263 and Pongsu Seribu 1* [Doctoral thesis, Universiti Putra Malaysia]. Seri Kembangan, Malaysia.

Universiti Malaya

Hayashi, N., Kobayashi, N., Cruz, C., & Fukuta, Y. (2009). Protocols for the sampling of diseased specimens and evaluation of blast disease in rice. *JIRCAS Working Report*, *63*, 17-33.

He, Q., Kim, K. W., & Park, Y. J. (2017). Population genomics identifies the origin and signatures of selection of Korean weedy rice. *Plant Biotechnology Journal*, *15*(3),357-366.

Hirochika, H., Guiderdoni, E., An, G., Hsing, Y.-I., Eun, M. Y., Han, C.-D., … Pereira, A. (2004). Rice mutant resources for gene discovery. *Plant Molecular Biology*, *54*(3), 325-334.

Hirotsu, N., Murakami, N., Kashiwagi, T., Ujiie, K., & Ishimaru, K. (2010). Protocol: A simple gel-free method for SNP genotyping using allele-specific primers in rice and other plant species. *Plant Methods*, *6*(1), 1-10.

Holm, L. (1997). The wild rices. In *World Weeds: Natural Histories and Distribution* (pp. 531-547). John Wiley & Sons.

Hossain, M. K., Jena, K. K., Bhuiyan, M. A. R., & Wickneswari, R. (2016). Association between QTLs and morphological traits toward sheath blight resistance in rice (*Oryza sativa* L.). *Breeding Science*, *66*(4), 613-624.

Hsu, Y.-C., Chiu, C.-H., Yap, R., Tseng, Y.-C., & Wu, Y.-P. (2020). Pyramiding bacterial blight resistance genes in Tainung82 for broad-spectrum resistance using marker-assisted selection. *International Journal of Molecular Sciences*, *21*(4), Article#1281.

Hsu, Y.-C., Tseng, M.-C., Wu, Y.-P., Lin, M.-Y., Wei, F.-J., Hwu, K.-K., … Lin, Y.-R. (2014). Genetic factors responsible for eating and cooking qualities of rice grains in a recombinant inbred population of an inter-subspecific cross. *Molecular Breeding*, *34*(2), 655-673.

Hu, X., Lu, L., Guo, Z., & Zhu, Z. (2020). Volatile compounds, affecting factors and evaluation methods for rice aroma: A review. *Trends in Food Science & Technology*, *97*, 136-146.

Hu, Y., Cong, S., & Zhang, H. (2021). Comparison of the grain quality and starch physicochemical properties between *Japonica* rice cultivars with different contents of amylose, as affected by nitrogen fertilization. *Agriculture*, *11*(7), Article#616.

Huang, Z., Young, N. D., Reagon, M., Hyma, K. E., Olsen, K. M., Jia, Y., & Caicedo, A. L. (2017). All roads lead to weediness: Patterns of genomic divergence reveal extensive recurrent weedy rice origins from South Asian *Oryza*. *MolecularEcology*, *26*(12), 3151-3167.

Intergovernmental Panel on Climate Change. (2018). *Global warming of 1.5° C*. Cambridge University Press.

International Rice Research Institute. (1996). *Standard Evaluation System for Rice. 4th edn*. International Rice Research Institute.

International Service for the Acquisition of Agri-biotech Applications. (2019). *Philippines Approves Golden Rice for Direct Use as Food and Feed, or for Processing*.

Isshiki, M., Morino, K., Nakajima, M., Okagaki, R. J., Wessler, S. R., Izawa, T., & Shimamoto, K. (1998). A naturally occurring functional allele of the rice *waxy* locus has a GT to TT mutation at the 5′ splice site of the first intron. *The Plant Journal*, *15*(1), 133-138.

Izawa, T., & Shimamoto, K. (1996). Becoming a model plant: the importance of rice to plant science. *Trends in Plant Science*, *1*(3), 95-99.

Jackson, S. A. (2016). Rice: the first crop genome. *Rice*, *9*(1), Article#14.

Jagadish, S., Muthurajan, R., Oane, R., Wheeler, T. R., Heuer, S., Bennett, J., & Craufurd, P. Q. (2010). Physiological and proteomic approaches to address heat tolerance during anthesis in rice (*Oryza sativa* L.). *Journal of Experimental Botany*, *61*(1),143-156.

Jasim Aljumaili, S., Rafii, M., Latif, M., Sakimin, S. Z., Arolu, I. W., & Miah, G. (2018). Genetic diversity of aromatic rice germplasm revealed by SSR markers. *BioMed Research International*, *2018*.

Jayamani, P., Negrao, S., Brites, C., & Oliveira, M. (2007). Potential of *Waxy* gene microsatellite and single-nucleotide polymorphisms to develop *japonica* varieties with desired amylose levels in rice (*Oryza sativa* L.). *Journal of Cereal Science*, *46*(2), 178-186.

Jia, Y., Correa-Victoria, F., McClung, A., Zhu, L., Liu, G., Wamishe, Y., … Rutger, J. (2007). Rapid determination of rice cultivar responses to the sheath blight pathogen *Rhizoctonia solani* using a micro-chamber screening method. *Plant Disease*, *91*(5), 485-489.

Jia, Y., & Gealy, D. (2018). Weedy red rice has novel sources of resistance to biotic stress. *The Crop Journal*, *6*(5), 443-450.

Jia, Y., Singh, V., Gealy, D., Liu, Y., Ma, J., Thurber, C., … Caicedo, A. (2022). Registration of two rice mapping populations using weedy rice ecotypes as a novel germplasm resource. *Journal of Plant Registrations*, *16*(1), 162-175.

Jiang, H., Dian, W., Liu, F., & Wu, P. (2004). Molecular cloning and expression analysis of three genes encoding starch synthase II in rice. *Planta*, *218*(6), 1062-1070.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., & Weeks, D. P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Research*, *41*(20), Article#e188.

Joshi, M., Singh, P. K., Waza, S. A., Singh, V., Goswami, S., Kumar, S., … Singh, U. S. (2016). Establishing an association between molecular markers and sheath blight ('*Rhizoctonia solani* Kuhn') resistance in rice.*Plant Omics*, *9*(4), Article#281.

Juliano, B. (1971). A simplified assay for milled rice amylose. *Cereal Science Today*, *16*(10), 334-360.

Universiti Malaya

Juliano, B. O. (1985). Criteria and tests for rice grain qualities. In *Rice Chemistry and Technology* (Vol. 2nd edition, pp. 443-524). American Association of Cereal Chemists.

Juliano, B. O., & Pascual, C. G. (1980). Quality characteristics of milled rice grown in different countries. *IRRI research paper series* (48).

Kadu, T. P., Kale, S. S., Chavan, N. R., Agrawal, T., & Verulkar, S. (2018). Pyramiding of three bacterial blight resistance genes in rice cultivar using marker assisted selection. *International Journal of Bio-resource and Stress Management*, *9*(2), 243-248.

Kanapeckas, K. L., Vigueira, C. C., Ortiz, A., Gettler, K. A., Burgos, N. R., Fischer, A. J., & Lawton-Rauh, A. L. (2016). Escape to ferality: the endoferal origin of weedy rice from crop rice through de-domestication. *PLOS One*, *11*(9).

Ke, Y., Hui, S., & Yuan, M. (2017). *Xanthomonas oryzae* pv. *oryzae* inoculation and growth rate on rice by leaf clipping method. *Bio-protocol*, *7*(19).

Keesstra, S. D., Bouma, J., Wallinga, J., Tittonell, P., Smith, P., Cerdà, A., … Van Der Putten, W. H. (2016). The significance of soils and soil science towards realization of the United Nations Sustainable Development Goals. *Soil*, *2*(2), 111–128.

Kennedy, G., Burlingame, B., & Nguyen, V. (2003). Nutritional contribution of rice and impact of biotechnology and biodiversity in rice-consuming countries. *Sustainable rice production for food security.* Proceedings of the 20th session of the International Rice Commission, Bangkok, Thailand. (pp. 59-70). FAO.

Khazanah Research Institute. (2019). *The status of the paddy and rice industry in Malaysia*. Khazanah Research Institute.

Khlestkina, E., & Salina, E. (2006). SNP markers: Methods of analysis, ways of development, and comparison on an example of common wheat. *Genetika*, *42*(6),725-736.

Khush, G. S. (1984). *Terminology for rice growing environments*. International Rice Research Institute.

Khush, G. S. (1987). Rice breeding: past, present and future. *Journal of Genetics*, *66*(3), 195-216.

Khush, G. S. (1995). Modern varieties—Their real contribution to food supply and equity. *GeoJournal*, *35*(3), 275-284.

Khush, G. S. (1997). Origin, dispersal, cultivation and variation of rice. *Plant Molecular Biology*, *35*(1-2), 25-34.

Khush, G. S. (2001). Green revolution: the way forward. *Nature Reviews Genetics*, *2*(10), Article#815.

Universiti Malaya

Kim, S.-R., Ramos, J., Ashikari, M., Virk, P. S., Torres, E. A., Nissila, E., … Jena, K. K. (2016). Development and validation of allele- specific SNP/indel markers for eight yield-enhancing genes using whole-genome sequencing strategy to increase yield potential of rice, *Oryza sativa* L. *Rice*, *9*(12),1-17.

Kiran, A., Wakeel, A., Mahmood, K., Mubaraka, R., & Haefele, S. M. (2022). Biofortification of staple crops to alleviate human malnutrition: Contributions and potential in developing countries. *Agronomy*, *12*(2), Article#452.

Kumagai, M., Wang, L., & Ueda, S. (2010). Genetic diversity and evolutionary relationships in genus *Oryza* revealed by using highly variable regions of chloroplast DNA. *Gene*, *462*(1-2), 44-51.

Ladha, J. K., Radanielson, A. M., Rutkoski, J. E., Buresh, R. J., Dobermann, A., Angeles, O., … Chivenge, P. (2021). Steady agronomic and genetic interventions are essential for sustaining productivity in intensive rice cropping. *Proceedings of the National Academy of Sciences*, *118*(45).

Lanceras, J. C., Huang, Z.-L., Naivikul, O., Vanavichit, A., Ruanjaichon, V., & Tragoonrung, S. (2000). Mapping of genes for cooking and eating qualities in Thai jasmine rice (KDML105). *DNA Research*, *7*(2), 93-101.

Larkin, P. D., & Park, W. D. (2003). Association of *waxy* gene single nucleotide polymorphisms with starch characteristics in rice (*Oryza sativa* L.). *Molecular Breeding*, *12*(4), 335-339.

Lau, H. Y., & Botella, J. R. (2017). Advanced DNA-based point-of-care diagnostic methods for plant diseases detection. *Frontiers in Plant Science*, *8*(2016).

Lau, W. C. P., & Latif, M. A. (2019). Current breeding approaches for developing rice with improved grain and nutritional qualities. In *Quality breeding in field crops* (pp. 199-216). Springer.

Lau, W. C. P., Latif, M. A., Y. Rafii, M., Ismail, M. R., & Puteh, A. (2016). Advances to improve the eating and cooking qualities of rice by marker-assisted breeding. *Critical Reviews in Biotechnology*, *36*(1), 87-98.

Lee, S., Jia, Y., Jia, M., Gealy, D. R., Olsen, K. M., & Caicedo, A. L. (2011). Molecular evolution of the rice blast resistance gene *Pi-ta* in invasive weedy rice in the USA.*PLOS One*, *6*(10), Article#e26260.

Li, H., & Liu, Y. (2019). Effects of variety and growth location on the chain-length distribution of rice starches. *Journal of Cereal Science*, *85*, 77-83.

Li, J. Z., Cao, Y. R., Li, M. Q., Zhao, W. L., Sun, H. Z., & Zhao, Q. Z. (2017). A multiplex PCR system for detection of wide compatibility allele *S5-n* and erect panicle allele *dep1* in rice. *Crop Breeding and Applied Biotechnology*, *17*(3), 250-258.

Li, S., Wang, Q., & Chun, J. (2017). Impact assessment of climate change on rice productivity in the Indochinese Peninsula using a regional-scale crop model. *International Journal of Climatology*, *37*(S1), 1147-1160.

Universiti Malaya

Li, Y., Xiao, J., Chen, L., Huang, X., Cheng, Z., Han, B., … Wu, C. (2018). Rice functional genomics research: Past decade and future. *Molecular Plant*, *11*(3), 359-380.

Li, Z., Pinson, S., Marchetti, M., Stansel, J., & Park, W. (1995). Characterization of quantitative trait loci (QTLs) in cultivated rice contributing to field resistance to sheath blight (*Rhizoctonia solani*). *Theoretical and Applied Genetics*, *91*(2), 382-388.

Lian, D.-S., Chen, X.-Y., Zeng, H.-S., & Wang, Y.-Y. (2021). Capillary electrophoresis based on nucleic acid analysis for diagnosing inherited diseases. *Clinical Chemistry and Laboratory Medicine*, *59*(2), 249-266.

Lin, H. C., Coe, R. A., Quick, W. P., & Bandyopadhyay, A. (2019). Climate-Resilient Future Crop: Development of C 4 Rice. In *Sustainable solutions for food security* (pp. 111-124). Springer.

Liu, L., Ma, X., Liu, S., Zhu, C., Jiang, L., Wang, Y., … & Chen, L. (2009). Identification and characterization of a novel *Waxy* allele from a Yunnan rice landrace. *Plant Molecular Biology*, *71*(6), 609-626.

Liu, Y., Qi, X., Gealy, D. R., Olsen, K. M., Caicedo, A. L., & Jia, Y. (2015). QTL analysis for resistance to blast disease in US weedy rice. *Molecular Plant-Microbe Interactions*, *28*(7), 834-844.

Lorieux, M., Petrov, M., Huang, N., Guiderdoni, E., & Ghesquière, A. (1996). Aroma in rice: Genetic analysis of a quantitative trait. *Theoretical and Applied Genetics*, *93*(7), 1145-1151.

Lu, B.-R., & Snow, A. A. (2005). Gene flow from genetically modified rice and its environmental consequences. *Bioscience*, *55*(8), 669-678.

Madhav, M., Pandey, M., Kumar, P., Sundaram, R., Prasad, G., Sudarshan, I., & Rani, N. (2010). Identification and mapping of tightly linked SSR marker for aroma trait for use in marker assisted selection in rice. *Rice Genetics Newsletter*, *25*, 38-40.

Mahmod, I. F., Saiman, M. Z., Mohamed, Z., Ishak, M. N., & Mispan, M. S. (2021). Morphological variation, distribution and relationship of weedy rice (*Oryza sativa*L.) in Peninsular Malaysia. *Weed Biology and Management*, *21*(2), 86-99.

Majeed, A., & Muhammad, Z. (2019). Salinity: A major agricultural problem—Causes, impacts on crop productivity and management strategies. In *Plant abiotic stress tolerance* (pp. 83-99). Springer.

Mansor, F., Zamri, L., & Hamzah, S. S. (2015). Electrophoretic techniques for the detection of human microsatellite d19s884. *The Malaysian Journal of Medical Sciences*, *22*(2), 18-24.

Martin, C., & Smith, A. M. (1995). Starch biosynthesis. *The Plant Cell*, *7*(7), Article#971.

Universiti Malaya

Marzi, S., Mysiak, J., Essenfelder, A. H., Pal, J. S., Vernaccini, L., Mistry, M. N., … Vousdoukas, M. (2021). Assessing futurevulnerability and risk of humanitarian crises using climate change and populationprojections within the INFORM framework. *Global Environmental Change*, *71*, Article#102393.

Massawe, F., Mayes, S., & Cheng, A. (2016). Crop diversity: An unexploited treasure trove for food security. *Trends in Plant Science*, *21*(5), 365-368.

Mazid, M. S., Rafii, M. Y., Hanafi, M. M., Rahim, H. A., & Latif, M. A. (2013). Genetic variation, heritability, divergence and biomass accumulation of rice genotypes resistant to bacterial blight revealed by quantitative traits and ISSR markers. *Physiologia Plantarum*, 149 (3), 432-447.

Mbow, C., Rosenzweig, C., Barioni, L., Benton, T., Herrero, M., & Krishnapillai, M. (2019). Food security. In: P.R. Shukla, J. Skea, E. Calvo Buendia, V. Masson-Delmotte, H.-O. Pörtner, D.C. Roberts, … J. Malley (Eds.), *Climate change and land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems*. In press.

McBride, W. D., & Daberkow, S. G. (2003). Information and the adoption of precision farming technologies. *Journal of Agribusiness*, *21*(1), 21-38.

McCouch, S. R., Teytelman, L., Xu, Y., Lobos, K. B., Clare, K., Walton, M., … Xing, Y. (2002). Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Research*, *9*(6), 199-207.

McKenzie, K., & Rutger, J. (1983). Genetic analysis of amylose content, alkali spreading score, and grain dimensions in rice *Crop Science*, *23*(2), 306-313.

Meenakshi, R., & Salgotra, R. (2020). Multiplex PCR assay for detection of major bacterial blight resistance genes *Xa21* and *xa13* in Basmati (*Oryza sativa* L.). *Research Journal of Biotechnology*, *15*(2), 40-46.

Miah, G., Rafii, M., Ismail, M., Puteh, A., Rahim, H., Asfaliza, R., & Latif, M. (2013). Blast resistance in rice: a review of conventional breeding to molecular approaches. *Molecular Biology Reports*, *40*(3), 2369-2388.

Miah, G., Rafii, M., Ismail, M., Sahebi, M., Hashemi, F., Yusuff, O., & Usman, M. (2017). Blast disease intimidation towards rice cultivation: a review of pathogen and strategies to control. *Journal of Animal and Plant Sciences*, *27*, 1058-1066.

Miah, G., Rafii, M. Y., Ismail, M. R., Puteh, A. B., Rahim, H. A., & Latif, M. A. (2016). Marker-assisted introgression of broad-spectrum blast resistance genes into the cultivated MR219 rice variety. *Journal of the Science of Food and Agriculture*, *97*(9), 2810-2818.

Miao, J., Guo, D., Zhang, J., Huang, Q., Qin, G., Zhang, X., … Qu, L.-J.(2013). Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Research*, *23*(10), 1233-1236.

Miryeganeh, M. (2021). Plants' epigenetic mechanisms and abiotic stress. *Genes*, *12*(8), Article#1106.

Universiti Malaya

Mishra, A., Singh, P. K., Bhandawat, A., Sharma, V., Sharma, V., Singh, P., … Sharma, H. (2022). Analysis of SSR and SNP markers. In *Bioinformatics* (pp. 131-144). Elsevier.

Mispan, M. S., Bzoor, M., Mahmod, I., MD-Akhir, A. H., & Zulrushdi, A. (2019). Managing weedy rice (*Oryza sativa* L.) in Malaysia: challenges and ways forward. *Journal of Research in Weed Science*, *2*(2), 149-167.

Mispan, M. S., Zhang, L., Feng, J., & Gu, X.-Y. (2013). Quantitative trait locus and haplotype analyses of wild and crop-mimic traits in US weedy rice. *G3: Genes, Genomes, Genetics*, *3*(6), 1049-1059.

Mitchenall, L. A., Hipkin, R. E., Piperakis, M. M., Burton, N. P., & Maxwell, A. (2018). A rapid high-resolution method for resolving DNA topoisomers. *BMC Research Notes*, *11*(1), 1-7.

Mohammed Zuki, I., & Kamarudin, D. (1994, May). Status and control of padi angin in the Muda area. Padi angin workshop, 19.

Mohapatra, S., Pandit, E., Barik, S., Patra, B., Meher, J., & Pradhan, S. (2017). Genetic diversity and population structure using linked SSR markers for heat stress tolerance in rice. *ORYZA-An International Journal on Rice*, *54*(2), 158-168.

Mohd Hanafiah, N., Cheng, A., Lim, P. E., Sethuraman, G., Mohd Zain, N. A., Baisakh, N., & Mispan, M. S. (2022). Novel PCR-Based Multiplex Assays for Detecting Major Quality and Biotic Stress in Commercial and Weedy Rice. *Life*, 12(10), Article#1542.

Mohd Hanafiah, N., Mispan, M. S., Lim, P. E., Baisakh, N., & Cheng, A. (2020). The 21st century agriculture: When rice research draws attention to climate variability and how weedy rice and underutilized grains come in handy. *Plants*, *9*(3), Article#365.

Mohd Hanafiah, N., Mispan, M. S., Lim, P. E., Baisakh, N., & Cheng, A. (2021). Low-cost high-resolution genotyping assay for detecting multiple biotic stress resistance genes in rice (*Oryza sativa*). *Plant Omics*, *14*(01), 57-63.

Molla, K. A., Azharudheen, T. M., Ray, S., Sarkar, S., Swain, A., Chakraborti, M., … Mukherjee, A. K. (2019). Novel biotic stress responsive candidate gene based SSR (cgSSR) markers from rice. *Euphytica*, *215*(2), Article#17.

Moshi, W. E. (2016). *Association of Betaine aldehyde dehydrogenase 2.1 (Badh2. 1) gene allele with aroma in popular traditional rice varieties in Tanzania* [Master's dissertation, Sokoine University of Agriculture]. Morogoro, Tanzania.

Motohashi, K. (2019). Development of highly sensitive and low-cost DNA agarose gel electrophoresis detection systems, and evaluation of non-mutagenic and loading dye-type DNA-staining reagents. *PLOS One*, *14*(9), Article#e0222209.

Mubassir, M., Nasiruddin, K. M., Shahin, N. H., Begum, S. N., Saha, M. K., & Rashid, A. (2016). Morpho-molecular screening for bacterial leaf blight resistance in some rice lines and varieties. *Journal of Plant Sciences*, *4*(6), 146-152.

Universiti Malaya

Mustafa, M., Mayes, S., & Massawe, F. (2019). Crop diversification through a wider use of underutilised crops: A strategy to ensure food and nutrition security in the face of climate change. In *Sustainable Solutions for Food Security* (pp. 125-149). Springer.

Muthayya, S., Sugimoto, J. D., Montgomery, S., & Maberly, G. F. (2014). An overview of global rice production, supply, trade, and consumption. *Annals of the New York Academy of Sciences*, *1324*(1), 7-14.

Mutiga, S., Rotich, F., Ganeshan, V. D., Mwongera, D., Mgonja, E., Were, V., … Feng, C. (2017). Assessment of the virulence spectrum and its association with genetic diversity in *Magnaporthe oryzae* populations from sub-Saharan Africa. *Phytopathology*, *107*(7), 852-863.

Nadarajah, K., Omar, N. S., Rosli, M. M., & Shin Tze, O. (2014). Molecular characterization and screening for sheath blight resistance using Malaysian isolates of *Rhizoctonia solani*. *BioMed Research International*, *2014*.

Nadir, S., Xiong, H.-B., Zhu, Q., Zhang, X.-L., Xu, H.-Y., Li, J., … Khan, S. (2017). Weedy rice in sustainable rice production. A review. *Agronomy Sustainable Development*, *37*(5), 46.

Nawaz, A., Rehman, A. U., Rehman, A., Ahmad, S., Siddique, K. M., & Farooq, M. (2022). Increasing sustainability for rice production systems. *Journal of Cereal Science*, *103*, Article#103400.

Nelson, G. C., Rosegrant, M. W., Palazzo, A., Gray, I., Ingersoll, C., Robertson, R., … Ringler, C. (2010). *Food security, farming, and climate change to 2050: Scenarios, results, policy options*. Washington, D.C: International Food Policy Research Institute.

Nisar, S., Rehman, A. U., Basit, A., Ahmad, B., Mateen, A., Ahmad, M. S., … Hanif, Q. (2018). Physiological characteristics of grain quality and yieldattributing traits of advance rice lines (*Oryza sativa* L.). *Biomedical Letters*, *4*(1),1-6.

NurulNahar, E., Adam, P., Mazidah, M., Roslan, I., & Rafii, Y. M. (2020). Rice blast disease in Malaysia: Options for its control. *Journal of Tropical Agriculture and Food Science*, *48*(1), 11-23.

Oka, H. (1988). *Origin of cultivated rice*. Japan Science Society Press.

Okpala, N. E., Potcho, M. P., An, T., Ahator, S. D., Duan, L., & Tang, X. (2020). Low temperature increased the biosynthesis of 2-AP, cooked rice elongation percentage and amylose content percentage in rice. *Journal of Cereal Science*, *93*, Article#102980.

Olajumoke, B., Juraimi, A. S., Uddin, M., Husni, M. H., & Alam, M. (2016). Competitive ability of cultivated rice against weedy rice biotypes: A review. *Chilean Journal of Agricultural Research*, 76(2), 243-252.

Ortega, D. L., & Tschirley, D. L. (2017). Demand for food safety in emerging and developing countries: A research agenda for Asia and Sub-Saharan Africa. *Journal of Agribusiness in Developing and Emerging Economies*, *7*(1), 21-34.

Ou, S. H. (1985). *Rice diseases*. IRRI.

Universiti Malaya

Pan, J.-Z., Fang, P., Fang, X.-X., Hu, T.-T., Fang, J., & Fang, Q. (2018). A low-cost palmtop high-speed capillary electrophoresis bioanalyzer with laser induced fluorescence detection. *Scientific Reports*, *8*(1791), 1-11.

Papademetriou, M. K. (2000). *Rice production in the Asia-Pacific region: Issues and perspectives* Bridging the rice yield gap in the Asia-Pacific region, FAO, UN, Bangkok, Thailand.

Park, Y.-J., Lee, J. K., & Kim, N.-S. (2009). Simple sequence repeat polymorphisms (SSRPs) for evaluation of molecular diversity and germplasm classification of minor crops. *Molecules*, *14*(11), 4546-4569.

Peakall, R., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*(1), 288- 295.

Peng, S., Tang, Q., & Zou, Y. (2009). Current status and challenges of rice production in China. *Plant Production Science*, 12(1), 3-8.

Peng, Y., Hu, Y., Qian, Q., & Ren, D. (2021). Progress and prospect of breeding utilization of green revolution gene *SD1* in rice. *Agriculture*, *11*(7), Article#611.

Persaud, R., McGowan, D., & Persaud, M. (2021). Managing the imminent danger of rice blast (*Pyricularia oryzae* Cav.) and sheath blight (*Rhizoctonia solani* Kühn) disease: A critical review article. *American Journal of Agriculture and Forestry*, *9*(6), 409-423.

Pingali, P. L. (2019). The green revolution and crop biodiversity. In P. Dasgupta, P. Raven & A. McIvor (Eds.), *Biological extinctions: New perspectives* (pp. 175- 192). Cambridge University Press.

Pinto, Y. M., Kok, R. A., & Baulcombe, D. C. (1999). Resistance to rice yellow mottle virus (RYMV) in cultivated African rice varieties containing RYMV transgenes. *Nature Biotechnology*, *17*(7), Article#702.

Pivoto, D., Waquil, P. D., Talamini, E., Finocchio, C. P. S., Dalla Corte, V. F., & de Vargas Mores, G. (2018). Scientific development of smart farming technologies and their application in Brazil. *Information Processing in Agriculture*, *5*(1), 21- 32.

Platten, J. D., Cobb, J. N., & Zantua, R. E. (2019). Criteria for evaluating molecular markers: Comprehensive quality metrics to improve marker-assisted selection. *PLOS One*, *14*(1), Article#e0210529.

Pooni, H., Kumar, I., & Khush, G. (1993). Genetical control of amylose content in selected crosses of *indica* rice. *Heredity*, *70*(3), 269-280.

Popp, J., Lakner, Z., Harangi-Rakos, M., & Fari, M. (2014). The effect of bioenergy expansion: Food, energy, and environment. *Renewable and Sustainable Energy Reviews*, *32*, 559-578.

Poudel, S., & Shaw, R. (2016). The relationships between climate variability and crop yield in a Mountainous environment: A case study in Lamjung District, Nepal. *Climate*, *4*(1), Article#13.

Universiti Malaya

Pradhan, S. K., Pandit, E., Pawar, S., Baksh, S. Y., Mukherjee, A. K., & Mohanty, S. P. (2019). Development of flash-flood tolerant and durable bacterial blight resistant versions of mega rice variety 'Swarna' through marker-assisted backcross breeding. *Scientific Reports*, *9*(1), 1-15.

Prodhan, Z. H., & Qingyao, S. (2020). Rice aroma: A natural gift comes with price and the way forward. *Rice Science*, *27*(2), 86-100.

Pustika, A., & Yolanda, K. (2021). Control of bacterial leaf blight and rice blast with antagonist microbial in the agroecosystem of irrigated rice. IOP Conference Series: Earth and Environmental Science, 653, Article#012137.

Qaim, M. (2020). Role of new plant breeding technologies for food security and sustainable agricultural development. *Applied Economic Perspectives and Policy*, *42*(2), 129-150.

Qi, S., Zhu, L., Wang, X., Du, J., Yang, Q., & Li, Y. (2019). Near-infrared turn-on fluorescent probe for discriminative detection of *Cys* and application in in vivo imaging. *RSC Advances*, *9*(71), 41431-41437.

Qian, L., Jin, H., Yang, Q., Zhu, L., Yu, X., Fu, X., … Yuan, F. (2022). A sequence variation in *Gmbadh2* enhances soybean aroma and is a functional marker for improving soybean flavor. *International Journal of Molecular Sciences*, *23*(8), Article#4116.

Qiu, J., Zhou, Y., Mao, L., Ye, C., Wang, W., Zhang, J., … Qian, F. (2017). Genomic variation associated with local adaptation of weedy rice during de-domestication. *Nature Communications*, *8*, Article#15323.

R. Vemireddy, L., Archak, S., & Nagaraju, J. (2007). Capillary electrophoresis is essential for microsatellite marker based detection and quantification of adulteration of Basmati rice (*Oryza sativa*). *Journal of Agricultural and Food Chemistry*, *55*(20), 8112-8117.

Rahim, F. H. A., Hawari, N. N., & Abidin, N. Z. (2017). Supply and demand of rice in Malaysia: A system dynamics approach. *International Journal of Supply Chain Management*, *6*, 1-7.

Ramtekey, V., Cherukuri, S., Modha, K. G., Kumar, A., Kethineni, U. B., Pal, G., … Kumar, S. (2021). Extraction, characterization, quantification, and application of volatile aromatic compounds from Asian rice cultivars. *Reviews in Analytical Chemistry*, *40*(1), 272-292.

Raza, A., Razzaq, A., Mehmood, S. S., Zou, X., Zhang, X., Lv, Y., & Xu, J. (2019). Impact of climate change on crops adaptation and strategies to tackle its outcome: A review. *Plants*, *8*(2), Article#34.

Rhowell Jr., N. T., Fernie, A. R., & Sreenivasulu, N. (2021). Meeting human dietary vitamin requirements in the staple rice via strategies of biofortification and post-harvest fortification. *Trends in Food Science & Technology*, *109*, 65-82.

Universiti Malaya

Rogayah, S., Rohaiza, A., Nazrul, H., Amin, A., Nora'ini, A., Sew, Y., & Nurain, I. (2017). Towards the production of genetically modified rice with resistance to sheath blight disease. *Transactions of the Malaysian Society of Plant Physiology*,*24*(1), 112-116.

Rohman, A., Helmiyati, S., Hapsari, M., & Setyaningrum, D. L. (2014). Rice in health and nutrition. *International Food Research Journal*, *21*(1), Article#13.

Roma-Burgos, N., San Sudo, M. P., Olsen, K. M., Werle, I., & Song, B.-K. (2021). Weedy rice (*Oryza* spp.): what's in a name? *Weed Science*, *69*(5), 505-513.

Ronald, P. C., Albano, B., Tabien, R., Abenes, L., Wu, K.-S., McCouch, S., & Tanksley, S. D. (1992). Genetic and physical analysis of the rice bacterial blight disease resistance locus, Xa21. *Molecular and General Genetics MGG*, *236*(1), 113-120.

Roy, S. C. (2015). DNA barcoding for wild rice [*Oryza rufipogon* Griff.] of NBU campus based on *matK* gene and assessment of genetic variation using *DREB* and *BAD2* gene sequences. *Plant Gene and Trait*, *6*.

Ruzmi, R., Ahmad-Hamdani, M. S., Abidin, M. Z. Z., & Roma-Burgos, N. (2021). Evolution of imidazolinone-resistant weedy rice in Malaysia: The current status. *Weed Science*, *69*(5), 598-608.

Sabar, M., Bibi, T., Farooq, H. U., Haider, Z., Naseem, I., Mahmood, A., & Akhter, M. (2016). Molecular screening of rice (*Oryza sativa* L.) germplasm for *Xa4*, *xa5* and *Xa21* bacterial leaf blight (BLB) resistant genes using linked marker approach. *African Journal of Biotechnology*, *15*(41), 2317-2324.

Sabi, S. C. (2021). Poverty, Compromised Dietary Intake and Health Implications among South Africa's Sub-Populations: A Conceptual Analysis. In *Lifestyle and Epidemiology-The Double Burden of Poverty and Cardiovascular Diseases in African Populations*. IntechOpen.

Sahila, T., Suganya, T., Mani, A. P., & Nivitha, G. (2022). Blast disease in rice and its management. *Biotica Research Today*, *4*(3), 203-205.

Salgotra, R. K., Millwood, R. J., Agarwal, S., & Stewart Jr, C. N. (2011). High-throughput functional marker assay for detection of *Xa/xa* and *fgr* genes in rice (*Oryza sativa* L.). *Electrophoresis*, *32*(16), 2216-2222.

Salleh, A. M., Harun, N. Z., & Halim, S. A. (2020). Urban Agriculture as a Community Resilience Strategy against Urban Food Insecurity. *Environment-Behaviour Proceedings Journal*, *5*(13), 369-376.

Sarkozy, D., & Guttman, A. (2022). Capillary Sodium Dodecyl Sulfate Agarose Gel Electrophoresis of Proteins. *Gels*, *8*(2), Article#67.

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, *3*, 430-439.

Universiti Malaya

Schneider, P., & Asch, F. (2020). Rice production and food security in Asian Mega deltas—A review on characteristics, vulnerabilities and agricultural adaptation options to cope with climate change. *Journal of Agronomy and Crop Science*, *206*(4), 491-503.

Searchinger, T., Waite, R., Hanson, C., Ranganathan, J., Dumas, P., Matthews, E., & Klirs, C. (2019). *Creating a sustainable food future: A menu of solutions to feed nearly 10 billion people by 2050.* Washington, DC: World Resources Institute.

Sethuraman, G., Mohd Zain, N. A., Yusoff, S., Ng, Y. M., Baisakh, N., & Cheng, A. (2021). Revamping ecosystem services through agroecology—The case of cereals. *Agriculture*, *11*(3), Article#204.

Shahriar, S. A., Imtiaz, A. A., Hossain, M. B., Husna, A., & Eaty, M. N. K. (2020). Rice Blast Disease. *Annual Research & Review in Biology*, *35*(1), 50-64.

Shahzad, A., Ullah, S., Dar, A. A., Sardar, M. F., Mehmood, T., Tufail, M. A., … Haris, M. (2021). Nexus on climate change: Agriculture and possible solution to cope future climate change stresses. *Environmental Science and Pollution Research*, *28*(12), 14211-14232.

Shakri, A. A., Kasim, K., & Rukunudin, I. (2021). Chemical Compositions and Physical Properties of Selected Malaysian Rice: A Review. IOP Conference Series: Earth and Environmental Science, 765(1), Article#012024.

Shamim, M., Sharma, D., Bisht, D., Hussain, R., Khan, N., Pandey, P., … Singh, K. (2017). Molecular tools for controlling of sheath blight disease of rice and its management. In *Biotic Stress Management in Rice* (Vol. 1st edition, pp. 109). Apple Academic Press.

Sharma, A., McClung, A. M., Pinson, S. R., Kepiro, J. L., Shank, A. R., Tabien, R. E., & Fjellstrom, R. (2009). Genetic mapping of sheath blight resistance QTLs within tropical japonica rice cultivars. *Crop Science*, *49*(1), 256-264.

Sheehy, J. E., & Mitchell, P. (2011). Rice and global food security: The race between scientific discovery and catastrophe. In *ACCESS NOT EXCESS—The search for better nutrition.* (pp. 81-90). Smith-Gordon.

Sherwood, S., & Uphoff, N. (2000). Soil health: Research, practice and policy for a more regenerative agriculture. *Applied Soil Ecology*, *15*(1), 85-97.

Shikari, A. B., Shafi, A., Najeeb, S., Sakina, A., Mir, S., Noor, N., … Ashraf, M. (2019). Towards the understanding about distribution and architecture of R-genes underlying resistance to rice blast. *SKUAST Journal of Research*, *21*(1), 1-14.

Shiming, L., & Gliessman, S. R. (2017). *Agroecology in China: Science, practice, and sustainable management.* CRC Press.

Shrestha, S., Sharma, G., Stallworth, S., Redona, E. D., & Tseng, T. M. (2022). Exploring the genetic diversity among weedy rice accessions differing in herbicide tolerance and allelopathic potential. *Diversity*, *14*(1), Article#44.

Universiti Malaya

Shu, X., Wang, A., Jiang, B., Jiang, Y., Xiang, X., Yi, X., … Zhu, J. (2021). Genome-wide association study and transcriptome analysis discover new genes for bacterial leaf blight resistance in rice (*Oryza sativa* L.). *BMC Plant Biology*, *21*(1), 1-13.

Siebrecht, N. (2020). Sustainable agriculture and its implementation gap—Overcoming obstacles to implementation. *Sustainability*, *12*(9), Article#3853.

Simkhada, K., & Thapa, R. (2022). Rice Blast, A Major Threat to the Rice Production and its Various Management Techniques. *Turkish Journal of Agriculture-Food Science and Technology*, *10*(2), 147-157.

Singh, A., Nayak, R., & Singh, P. (2015). Identification of bacterial leaf blight resistance genes in rice (*Oryza sativa* L.). *International Journal of Science and Nature*, *6*(2),283-287.

Singh, K., Kumar, V., Saharawat, Y., Gathala, M., Ladha, J., & Chauhan, B. (2013). Weedy rice: An emerging threat for direct-seeded rice production systems in India. *Rice Research*, *1*(1).

Sombunjitt, S., Sriwongchai, T., Kuleung, C., & Hongtrakul, V. (2017). Searching for and analysis of bacterial blight resistance genes from Thailand rice germplasm. *Agriculture and Natural Resources*, *51*(5), 365-375.

Somerville, C., & Koornneef, M. (2002). A fortunate choice: The history of *Arabidopsis* as a model plant. *Nature Reviews Genetics*, *3*(11), Article#883.

Song, B. K., Chuah, T. S., Tam, S. M., & Olsen, K. M. (2014). Malaysian weedy rice shows its true stripes: wild Oryza and elite rice cultivars shape agricultural weed evolution in Southeast Asia. *Molecular Ecology*, *23*(20), 5003-5017.

Song, W.-Y., Wang, G.-L., Chen, L.-L., Kim, H.-S., Pi, L.-Y., Holsten, T., … Zhu, L.-H. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science*, *270*(5243), 1804- 1806.

Sood, B., & Siddiq, E. (1978). A rapid technique for scent determination in rice [India]. *Indian Journal of Genetics and Plant Breeding*, *38*.

Sreenivasulu, N., Zhang, C., Tiozon Jr, R. N., & Liu, Q. (2022). Post-genomics revolution in the design of premium quality rice in a high-yielding background to meet consumer demands in the 21st century. *Plant Communications*, *3*(3), Article#100271.

Stellwagen, N. C. (2009). Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis*, *30*(S1), S188-S195.

Stewart, S., Wickramasinghe, D., Dorrance, A. E., & Robertson, A. E. (2011). Comparison of three microsatellite analysis methods for detecting genetic diversity in *Phytophthora sojae* (Stramenopila: Oomycete). *Biotechnology Letters*, *33*(11), 2217-2223.

Stuart, A. M., Kong, P., Then, R., Flor, R. J., & Sathya, K. (2020). Tailor-made solutions to tackle rodent pests of rice through community-based management approaches in Cambodia. *Crop Protection*, *135*, Article#104717.

Universiti Malaya

Sudianto, E., Neik, T.-X., Tam, S. M., Chuah, T.-S., Idris, A. A., Olsen, K. M., & Song, B.-K. (2016). Morphology of Malaysian weedy rice (*Oryza sativa*): Diversity, origin and implications for weed management. *Weed Science*, *64*(3), 501-512.

Suh, H. (2003). Characterization of weedy rice germplasm. *Wild Crop Germplasm Bank, Yeungnam University*, 1007.

Sultana, S., Faruque, M., & Islam, M. R. (2022). Rice grain quality parameters and determination tools: A review on the current developments and future prospects. *International Journal of Food Properties*, *25*(1), 1063-1078.

Sun, J., Qian, Q., Ma, D. R., Xu, Z. J., Liu, D., Du, H. B., & Chen, W. F. (2013). Introgression and selection shaping the genome and adaptive loci of weedy rice in northern China. *New Phytologist*, *197*(1), 290-299.

Sutrisno, Susanto, F. A., Wijayanti, P., Retnoningrum, M. D., Nuringtyas, T. R., Joko, T., & Purwestri, Y. A. (2018). Screening of resistant Indonesian black rice cultivars against bacterial leaf blight. *Euphytica*, *214*(19).

Syme, J. (1970). A high-yielding Mexican semi-dwarf wheat and the relationship of yield to harvest index and other varietal characteristics. *Australian Journal of Experimental Agriculture*, *10*(44), 350-353.

Tahamtan, A., & Ardebili, A. (2020). Real-time RT-PCR in COVID-19 detection: Issues affecting the results. *Expert Review Of Molecular Diagnostics*, *20(5)*(5), 453-454.

Tanchotikul, U., & Hsieh, T. C. (1991). An improved method for quantification of 2-acetyl-1-pyrroline, a" popcorn"-like aroma, in aromatic rice by high-resolution gas chromatography/mass spectrometry/selected ion monitoring. *Journal of Agricultural and Food Chemistry*, *39*(5), 944-947.

Tanweer, F. A., Rafii, M. Y., Sijam, K., Rahim, H. A., Ahmed, F., Ashkani, S., & Latif, M. A. (2015). Introgression of blast resistance genes (putative *Pi-b* and *Pi-kh*) into elite rice cultivar MR219 through marker-assisted selection. *Frontiers in Plant Science*, *6*, Article#1002.

Tester, M., & Langridge, P. (2010). Breeding technologies to increase crop production in a changing world. *Science*, *327*(5967), 818-822.

Theriault, V., Smale, M., & Haider, H. (2018). Economic incentives to use fertilizer on maize under differing agro-ecological conditions in Burkina Faso. *Food Security*, *10*(5), 1263-1277.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., & Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature*, *418*(6898), Article#671.

Toriyama, K., Arimoto, Y., Uchimiya, H., & Hinata, K. (1988). Transgenic rice plants after direct gene transfer into protoplasts. *Nature Biotechnology*, *6*(9), 1072-1074.

Universiti Malaya

Tu, J., Zhang, G., Datta, K., Xu, C., He, Y., Zhang, Q., … Datta, S. K. (2000). Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin. *Nature Biotechnology*, *18*(10), Article#1101.

Tuan, T., Duong, V. X., & Khanh, T. D. (2020). Evaluation of genetic diversity of rice blast fungus (*Magnaporthe oryzae* Barr) isolates collected from South Central coast areas of Viet Nam. *Chiang Mai Journal of Science*, *2*, Article#3.

Uchimiya, H., Iwata, M., Nojiri, C., Samarajeewa, P. K., Takamatsu, S., Ooba, S., … Toki, S. (1993). Bialaphos treatment of transgenic rice plants expressing a bar gene prevents infection by the sheath blightpathogen (*Rhizoctonia solani*). *Nature Biotechnology*, *11*(7), 835-836.

United Nations Department of Economic and Social Affairs. (2017). *World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100.*

Unnevehr, L., Duff, B., & Juliano, B. O. (1992). *Consumer demand for rice grain quality: Terminal report of IDRC projects, National grain quality (Asia), and International grain quality economics (Asia)*. International Rice Research Institute.

Usatov, A., Kostylev, P., Azarin, K., Markin, N., Makarenko, M., Khachumova, V., & Bibov, M. Y. (2016). Introgression of the rice blast resistance genes *Pi1*, *Pi2* and *Pi33* into Russian rice varieties by marker-assisted selection. *Indian Journal of Genetics and Plant Breeding*, *76*(1), 18-23.

Vân Bạch, L. (2021). *Pseudomonas and Bacillus spp. associated with rice grown in acid sulfate soils in Vietnam: Taxonomy and potential for biocontrol and bistimulation* [PhD Thesis, Ghent University].

Van, T. (1967). Diseases of rice in West Malaysia and the breeding of resistant varieties with particular reference to blast and Penyakit Merah. In *Rice Diseases and Their Control by Growing Resistant Varieties and Other Measures* (pp. 113-122).

Varshney, R. K., Singh, V. K., Kumar, A., Powell, W., & Sorrells, M. E. (2018). Can genomics deliver climate-change ready crops? *Current Opinion in Plant Biology, 45,* 205-211.

Verma, D. K., & Srivastav, P. P. (2022). Extraction, identification and quantification methods of rice aroma compounds with emphasis on 2-acetyl-1-pyrroline (2-AP) and its relationship with rice quality: A comprehensive review. *Food Reviews International*, *38*(2), 111-162.

Verma, H., Pathak, K., Rathi, S., & Sarma, R. (2015). Genetic arechetecture for grain quality parameters in *indica* rice. *The EcoScan*, *3&4*, 963-967.

Vidotto, F., & Ferrero, A. (2000). Germination behaviour of red rice (*Oryza sativa* L.) seeds in field and laboratory conditions. *Agronomie*, *20*(4), 375-382.

Vidya, V., & Ramalingam, J. (2018). Marker assisted selection for sheath blight, blast and bacterial blight resistance in two popular rice varieties. *Madras Agricultural Journal*, *105*(4-6), 127-134.

Universiti Malaya

Wahab, A., & Suhaimi, O. (1991). Padi angin, adverse effects and methods of eradication. *Teknologi Padi*, *7*, 27-31.

Wang, C., Guo, L., Li, Y., & Wang, Z. (2012). Systematic comparison of C3 and C4 plants based on metabolic network analysis. Proceedings of the 23rd International Conference on Genome Informatics (GIW 2012), Taiwan.

Wang, P., Vlad, D., & Langdale, J. A. (2016). Finding the genes to build C4 rice. *Current Opinion in Plant Biology*, *31*, 44-50.

Wang, X., Guo, X., Ma, X., Luo, L., Fang, Y., Zhao, N., ... Qin, B. (2021). Development of new rice (*Oryza sativa* L.) breeding lines through marker-assisted introgression and pyramiding of brown planthopper, blast, bacterial leaf blight resistance, and aroma genes. *Agronomy*, *11*(12), Article#2525.

Wang, X., Lee, S., Wang, J., Ma, J., Bianco, T., & Jia, Y. (2014). Current advances on genetic resistance to rice blast disease. In *Rice-Germplasm, Genetics and Improvement* (pp. 195-217). InTech.

Wang, Z. Y., Zheng, F. Q., Shen, G. Z., Gao, J. P., Snustad, D. P., Li, M. G., … Hong, M. M. (1995). The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. *The Plant Journal*, *7*(4), 613-622.

Weerasinghe, W., Udawela, K. S., Heiyantuduwa, U., & Wickramasinghe, H. (2017). Molecular marker validation for bacterial leaf blight resistant genes Xa21 and Xa4 in Ld 99-12-38/IRBB 60 genetic background. *Tropical Agricultural Research*, *28*(4), Article#402.

Welch, V. A. (1983). Semi-dwarf maize and method. In: Google Patents.

Williams, V. R., Wu, W.-T., Tsai, H. Y., & Bates, H. G. (1958). Rice starch, varietal differences in amylose content of rice starch. *Journal of Agricultural and Food Chemistry*, *6*(1), 47-48.

World Food Summit. (1996). Rome declaration on world food security and world food summit plan of action. In World Food Summit Plan of Action. Rome, Italy. FAO.

Wu, Y., Chen, Y., Pan, C., Xiao, N., Yu, L., Li, Y., … Liang, C. (2017). Development and evaluation of near-isogenic lines with different blast resistance alleles at the *Piz* locus in japonica rice from the lower region of the Yangtze River, China. *Plant Disease*, *101*(7), 1283-1291.

Xia, H. B., Wang, W., Xia, H., Zhao, W., & Lu, B. R. (2011). Conspecific crop-weed introgression influences evolution of weedy rice (*Oryza sativa* f. *spontanea*) across a geographical range. *PLoS One*, 6(1), Article#e16189.

Yadav, M. K., Aravindan, S., Raghu, S., Prabhukarthikeyan, S., Keerthana, U., Ngangkham, U., … Kar, M. K. (2019). Assessment of genetic diversity and population structure of *Magnaporthe oryzae* causing rice blast disease using SSR markers. *Physiological and Molecular PlantPathology*, *106*, 157-165.

Universiti Malaya

Yadav, S., Anuradha, G., Kumar, R. R., Vemireddy, L. R., Sudhakar, R., Donempudi, K., … Marathi, B. (2015). Identification of QTLs and possible candidate genes conferring sheath blight resistance in rice (*Oryza sativa* L.). *SpringerPlus*, *4*(1), Article#175.

Yahya, F., Fryer, P. J., & Bakalis, S. (2011). The absorption of 2-acetyl-1-pyrroline during cooking of rice (*Oryza sativa* L.) with Pandan (*Pandanus amaryllifolius* Roxb.) leaves. *Procedia Food Science*, *1*, 722-728.

Yajima, I., Yanai, T., Nakamura, M., Sakakibara, H., & Hayashi, K. (1979). Volatile flavor components of cooked kaorimai (scented rice, *O. sativa japonic*a). *Agricultural and Biological Chemistry*, *43*(12), 2425-2429.

Yang, Y.-Y., Mei, F., Zhang, W., Shen, Z., & Fang, J. (2014). Creation of Bt rice expressing a fusion protein of Cry1Ac and Cry1I-like using a green tissue-specific promoter. *Journal of Economic Entomology*, *107*(4), 1674-1679.

Yano, K., Yamamoto, E., Aya, K., Takeuchi, H., Lo, P.-C., Hu, L., … Hirano, K. (2016). Genome-wide association study using whole-genome sequencing rapidly identifies new genes influencing agronomic traits in rice. *Nature Genetics*, *48*(8), 927-934.

Yap, R., Hsu, Y. C., Wu, Y. P., Lin, Y. R., & Kuo, C. W. (2016). Multiplex PCR genotyping for five bacterial blight resistance genes applied to marker-assisted selection in rice (*Oryza sativa*). *Plant Breeding*, *135*(3), 309-317.

Yasuda, N., Mitsunaga, T., Hayashi, K., Koizumi, S., & Fujita, Y. (2015). Effects of pyramiding quantitative resistance genes *pi21*, *Pi34*, and Pi35 *on ric*e leaf blast disease. *Plant Disease*, *99*(7), 904-909.

Yu, H., Du, Q., Campbell, M., Yu, B., Walia, H., & Zhang, C. (2021). Genome-wide discovery of natural variation in pre-mRNA splicing and prioritising causal alternative splicing to salt stress response in rice. *New Phytologist*, *230*(3), 1273-1287.

Yu, S., Ali, J., Zhang, C., Li, Z., & Zhang, Q. (2020). Genomic breeding of green super rice varieties and their deployment in Asia and Africa. *Theoretical and Applied Genetics*, *133*(5), 1427-1442.

Yu, Y.-Y., Jiang, C.-H., Wang, C., Chen, L.-J., Li, H.-Y., Xu, Q., & Guo, J.-H. (2017). An improved strategy for stable biocontrol agents selecting to control rice sheath blight caused by *Rhizoctonia solani*. *Microbiological Research*, *203*, 1-9.

Zhang, H., Yang, H., Rech, E., Golds, T., Davis, A., Mulligan, B., … & Davey, M. (1988). Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. *Plant Cell Reports*, *7*(6), 379-384.

Zhang, Q. (2007). Strategies for developing green super rice. *Proceedings of the National Academy of Sciences*, 104(42), 16402-16409.

Zhang, S.-l., Da-hu, N., Cheng-xin, Y., Li, L., Xiu-feng, W., Zong-yang, W., & Jian-bo, Y. (2005). Lowering grain amylose content in backcross offsprings of indica rice variety 057 by molecular marker-assisted selection. *Rice Science*, *12*(3), Article#157.

University Malaya

Zhang, Y., Fang, J., Wu, X., & Dong, L. (2018). Na+/K+ balance and transport regulatory mechanisms in weedy and cultivated rice (*Oryza sativa* L.) under salt stress. *BMC Plant Biology*, *18*(1), Article#375.

Zheng, A., Lin, R., Zhang, D., Qin, P., Xu, L., Ai, P., … Liu, Y. (2013). The evolution and pathogenic mechanisms of the rice sheath blight pathogen. *Nature Communications*, *4*(1), 1-10.

Zhou, H., Xia, D., & He, Y. (2020). Rice grain quality—traditional traits for high quality rice and health-plus substances. *Molecular Breeding*, *40*(1), 1-17.

Zhu, Y.-Q., Fang, J., Wang, Y., Pang, L.-H., & Lu, B.-R. (2021). Key roles of de-domestication and novel mutation in origin and diversification of global weedy rice. *Biology*, *10*(9), Article#828.

Ziska, L. H., Gealy, D. R., Tomecek, M. B., Jackson, A. K., & Black, H. L. J. P. O. (2012). Recent and projected increases in atmospheric CO_2 concentration can enhance gene flow between wild and genetically altered rice (*Oryza sativa*). *PLOS One*, *7*(5), Article#e37522.

Ziska, L. H., Gealy, D. R., Burgos, N., Caicedo, A. L., Gressel, J., Lawton-Rauh, A. L., … Merotto Jr, A. (2015). Weedy (red) rice: an emerging constraint to global rice production. *Advances in Agronomy*, 129, 181-228.

Universiti Malaya