



**UNIVERSITI PUTRA MALAYSIA**

***MOLECULAR AND MICROPROPAGATION APPROACHES FOR EX-SITU  
CONSERVATION OF *Boesenbergia stenophylla* R. M. SM.***

**AICHER JOSEPH TOYAT**

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**By**

**AICHER JOSEPH TOYAT**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in  
Fulfilment of the Requirements for the Degree of Master of Science**

**February 2017**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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**February 2017**

**Chairman : Nur Ashikin Psyquay Abdullah, PhD**  
**Faculty : Agriculture and Food Sciences (Bintulu)**

Jerangau Merah (*Boesenbergia stenophylla*) is highly endemic to the highland of Borneo. Due to its medicinal properties, it is highly sought which made their population declining. Rapid development and anthropogenic disturbance near the habitat may give negative impact on the Jerangau Merah population in their natural habitat. To date, there are no conservation strategies prepared for this species. Therefore a study was conducted to develop strategies to conserve this species which includes DNA profiling, DNA barcoding, micropropagation and nursery planting. A total of three natural locations of *B. stenophylla* were found in Bario, Ba'kelalan and Long Semadoh forest. Based on the DNA profiling with RAPD and SSRs markers there was no genetic variations found among sub populations from Bario. This finding implied that no variations may be caused to low hybridization because flowering is rare and propagation through rhizome is more dominant. DNA analysis using CpDNA and NrDNA markers showed that this species clades under the Borneo-*Boesenbergia* section. This proves further that this species is endemic and could not be found outside of Borneo. A micropropagation protocol was achieved for the *in vitro* cultures of *B. stenophylla*. It includes initial culturing using shoot tips in MS media containing 0.2 mg/L NAA for shoot induction followed by sub-culturing to MS media with 2 mg/L BAP + 0.4 mg/L NAA for rapid shoot elongation. *B. stenophylla* is highly endemic, having small populations, possibly lacks genetic variations and difficult to grow in *in vitro* cultures. The nursery studies show that the plant has its unique sympodial growth that proved that cannot be confined in the small polybag, so bedded planting was suggested for improving the growth in the nursery conditions. Therefore, this study suggests that their conservation should remain as *in situ*, seedlings production under optimum nursery conditions should be carried out near to their natural populations and further monitoring of new population occurrence for documentations.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**PENDEKATAN SECARA MOLEKULAR DAN MIKROPROPAGASI  
PEMULIHARAAN EX-SITU *Boesenbergia stenophylla* R. M. SM.**

Oleh

**AICHER JOSEPH TOYAT**

**Febuari 2017**

**Pengerusi : Nur Ashikin Psyquay Abdullah, PhD**  
**Fakulti : Sains Pertanian dan Makanan (Bintulu)**

Jerangau Merah (*Boesenbergia stenophylla*) adalah endemik kepada tanah tinggi Borneo. Oleh kerana nilai perubatannya, spesis ini telah di ambil secara banyak dan menyebabkan populasinya menurun. Pembangunan secara pesat dan gangguan antropogenik yang berdekatan dengan habitat telah memberikan kesan negatif ke atas populasi Jerangau Merah dihabitat semula jadinya. Sehubungan itu, tiada lagi strategi pemuliharaan disediakan untuk spesis ini. Justeru itu, kajian telah dilaksanakan untuk membangunkan strategi bagi spesis ini yang merangkumi memprofilokan DNA, membarkodkan DNA, mikropropogasi dan menanam di peringkat nurseri. Sejumlah tiga lokasi semulajadi ditemui didalam hutan Bario, Ba'Kelalan dan Long Semadoh. Berdasarkan profolio DNA dengan menggunakan penanda RAPD dan SSRs tiada variasi genetik di ditemui diantara sub populasi di dalam Bario. Hal ini sedemikian kerana kurangnya berlaku kacukan sesama spesis, pembungaan yang jarang berlaku dan spesis ini lebih dominan mempropogasi melalui bahagian vegetatifnya. Analisa DNA menggunakan penanda CpDNA and NrDNA menunjukkan spesis ini berklad dibawah seksyen Borneo-Boesenbergia. Menjelaskan serta membuktikan bahawa spesis ini adalah endemik dan tidak dapat dijumpai di tempat lain selain di Borneo. Selain itu juga, protokol untuk mikropropagasi *in vitro* *Boesenbergia stenophylla* telah berjaya dicapai. Dengan menggunakan pucuk yang dikulturkan didalam media MS yang mengandungi 0.2 mg/L NAA untuk mencetuskan percambahan pucuk baru dan diikuti dengan sub-kultur ke media MS dengan 2 mg/L BAP + 0.4 mg/L NAA bagi pemanjangan pucuk baru secara rapid. Oleh kerana, *Boesenbergia stenophylla* endemik, mempunyai populasi kecil, keberangkalian kurangnya variasi genetik serta kesusahan untuk tumbuh di dalam *in vitro* kultur. Kajian di nursery pula, menunjukkan bahawa keunikan pertumbuhan simpodial telah membuktikan bahawa pertumbuhan spesis ini akan terhad di dalam polibeg yang kecil dan cadangan untuk penambahbaikan pertumbuhan di nurseri, adalah dengan menanam di atas batas. Oleh itu, kajian mencadangkan untuk pemuliharaan patut dikekalkan *in situ*, produksi nurseri anak pokok patut di optimumkan berdekatan kawasan semulajadi dan meneruskan dokumentasi dalam pencarian populasi semula jadi yang lain.

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**Nur Ashikin Psyquay Abdullah, PhD**

Associate Professor  
Faculty of Agriculture and Food Science  
Universiti Putra Malaysia  
(Chairman)

**Rusea Go, PhD**

Professor  
Faculty of Science  
Universiti Putra Malaysia  
(Member)

**Tohirah Lee Abdullah@ Lee Chin Chin, PhD**

Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

---

**ROBIAH BINTI YUNUS, PhD**

Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:

## TABLE OF CONTENTS

|                              | Page |
|------------------------------|------|
| <b>ABSTRACT</b>              | i    |
| <b>ABSTRAK</b>               | ii   |
| <b>ACKNOWLEDGEMENTS</b>      | iii  |
| <b>APPROVAL</b>              | iv   |
| <b>DECLARATION</b>           | v    |
| <b>LIST OF TABLES</b>        | xi   |
| <b>LIST OF FIGURES</b>       | xiii |
| <b>LIST OF ABBREVIATIONS</b> | xvi  |

### CHAPTER

|          |   |    |
|----------|---|----|
| <b>1</b> | <b>INTRODUCTION</b>   | 1  |
|          | 1.1 Objectives of study   | 2  |
|          | 1.2 Hypothesis  | 2  |
| <b>2</b> | <b>LITERATURE REVIEW</b>  | 3  |
|          | 2.1 <i>Boesenbergia stenophylla</i>   | 3  |
|          | 2.1.1 Others <i>Boesenbergia</i> genus  | 5  |
|          | 2.1.2 Important Uses of <i>Boesenbergia stenophylla</i>   | 9  |
|          | 2.1.3 Traditional Uses of <i>Boesenbergia stenophylla</i>                                       | 10 |
|          | 2.1.4 Potential Development of <i>Boesenbergia stenophylla</i>                                  | 11 |
|          | 2.1.5 Economy Value of <i>Boesenbergia stenophylla</i>  | 11 |
|          | 2.2 Conservation of <i>Boesenbergia stenophylla</i>   | 12 |
|          | 2.3 Conservation Through Molecular Marker   | 14 |
|          | 2.3.1 Genetic Variation by using SSR  | 15 |
|          | 2.3.2 Genetic Variation by using RAPD   | 16 |
|          | 2.3.3 Genetic Diversity of <i>Boesenbergia</i> spp.   | 16 |
|          | 2.4 DNA Barcoding   | 17 |
|          | 2.5 Conservation Approach   | 18 |
|          | 2.6 Micropropagation  | 19 |
|          | 2.7 The Usage of 5 Aminolevulinic Acid (5-ALA)  | 20 |
|          | 2.8 Usage of Cocopeat in Nursery Planting   | 20 |
| <b>3</b> | <b>MATERIALS AND METHODS</b>  | 22 |
|          | 3.1 Materials   | 22 |
|          | 3.2 Data Collections for <i>Boesenbergia stenophylla</i> Natural Occurrence in Northern Sarawak | 22 |
|          | 3.3 Morphological Identifications   | 23 |
|          | 3.4 Preliminary Soil Nutrient Analysis  | 24 |
|          | 3.4.1 Location  | 24 |
|          | 3.4.2 Materials   | 24 |
|          | 3.4.3 Determination of Total N  | 24 |
|          | 3.4.4 Determination of Available P  | 25 |
|          | 3.4.5 Determination of K  | 26 |
|          | 3.5 Genetic analysis  | 26 |
|          | 3.5.1 DNA Extraction Method   | 26 |



|        |  |    |
|--------|--|----|
| 3.5.2  | Quantification of DNA  | 27 |
| 3.5.3  | Genetic Variations Studies using<br>Microsatellite Markers                               | 27 |
| 3.5.4  | Primer Screening for Specific Amplifications<br>Polymorphism                             | 27 |
| 3.5.5  | Screening of Population by SSR   | 28 |
| 3.5.6  | RANDOM Amplified Polymorphic DNA or<br>RAPD  | 29 |
| 3.5.7  | RAPD Amplification on PCR  | 29 |
| 3.5.8  | Population Screening by RAPD   | 29 |
| 3.5.9  | DNA Barcoding of ITS and cpDNA   | 30 |
| 3.5.10 | Purification of PCR Product of ITS and<br>cpDNA  | 31 |
| 3.5.11 | Sequencing Reactions   | 31 |
| 3.6    | Shoot Multiplication Through Micropropagation  | 31 |
| 3.6.1  | Location   | 31 |
| 3.6.2  | Materials  | 31 |
| 3.6.3  | Explant Sterilization  | 32 |
| 3.6.4  | Medium Preparation   | 33 |
|        | 3.6.4.1 Stock Solutions  | 33 |
|        | 3.6.4.2 Plant Growth Regulator   | 33 |
|        | 3.6.4.3 Preparation of Media   | 33 |
| 3.6.5  | Sterilization  | 33 |
|        | 3.6.5.1 Autoclave  | 33 |
|        | 3.6.5.2 Ultra Violet Light   | 34 |
|        | 3.6.5.3 Explants Preparation   | 34 |
|        | 3.6.5.4 Open Flame   | 34 |
| 3.7    | Culturing Procedures   | 34 |
|        | 3.7.1 Working Area   | 34 |
|        | 3.7.2 Culturing  | 34 |
|        | 3.7.3 Growth Chamber   | 34 |
| 3.8    | Treatments   | 35 |
|        | 3.8.1 Sub Culturing Explant  | 36 |
| 3.9    | Determination of Optimum Concentration of 5-ALA  | 36 |
| 3.10   | Hardening and Acclimatization for Plantlets  | 36 |
| 3.11   | Planting Media Mixture   | 36 |
|        | 3.11.1 Location  | 36 |
|        | 3.11.2 Materials   | 36 |
|        | 3.11.3 Planting Media Treatments   | 36 |
|        | 3.11.4 Parameter Collected   | 37 |
|        | 3.11.5 Preparation of Nursery  | 37 |
|        | 3.11.6 Statically Analysis   | 37 |
| 3.12   | Nutrient Analysis for Planting Media Mixture   | 38 |
|        | 3.12.1 Location  | 38 |
|        | 3.12.2 Materials   | 38 |
|        | 3.12.3 pH in Water   | 38 |
|        | 3.12.4 Loss of Ignition Method for Determines Total<br>Organic Carbon and Organic Matter | 38 |
|        | 3.12.5 Soil Electric Conductivity Measurement (EC)                                       | 38 |
|        | 3.12.6 Determination of Total N  | 39 |
|        | 3.12.7 Determination of Available P  | 39 |

|          |  |            |
|----------|--|------------|
| 3.12.8   | Determination of K   | 40         |
| 3.12.9   | Determination of Mg  | 41         |
| <b>4</b> | <b>RESULTS AND DISCUSSION</b>  | <b>42</b>  |
| 4.1      | Distributions of <i>Boesenbergia stenophylla</i>                               | 42         |
| 4.2      | Ecological Descriptions  | 51         |
| 4.2.1    | Bario  | 51         |
| 4.2.1.1  | Bario-Location 1   | 51         |
| 4.2.1.2  | Bario-Location 2   | 52         |
| 4.2.1.3  | Bario-Location 3   | 52         |
| 4.2.2    | Ba'kelalan   | 53         |
| 4.2.2.1  | Ba'Kelalan- Location 1   | 53         |
| 4.2.2.2  | Ba'Kelalan- Location 2   | 54         |
| 4.2.3    | Long Semadoh   | 54         |
| 4.2.3.1  | Long Semadoh-Location 1  | 54         |
| 4.3      | DNA Extraction   | 55         |
| 4.4      | Genetic Variation using SSR and RAPD Markers                                   | 56         |
| 4.4.1    | Development of SSR Markers for <i>Boesenbergia stenophylla</i>                 | 56         |
| 4.4.2    | SSR Analysis   | 57         |
| 4.4.3    | RAPD Analysis  | 59         |
| 4.5      | DNA Barcoding  | 63         |
| 4.5.1    | Maximum Likelihood Analysis  | 64         |
| 4.6      | Surface Sterilization Explant  | 67         |
| 4.7      | In vitro Propagation   | 68         |
| 4.7.1    | Results on 16 Week of Inoculations   | 72         |
| 4.7.2    | Sub Culture After Explants   | 73         |
| 4.7.3    | Effects of 5-ALA on Explants Growth  | 74         |
| 4.8      | Determination of Suitable Planting Media for <i>B. stenophylla</i> Propagation | 75         |
| 4.8.1    | General Condition in Nursery House   | 75         |
| 4.8.2    | Effects of Different Planting Media on Growth of <i>B. stenophylla</i>         | 76         |
| 4.9      | Morphological Description of New Emerged Rhizome                               | 82         |
| <b>5</b> | <b>SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH</b>             | <b>84</b>  |
|          | <b>REFERENCES</b>  | <b>86</b>  |
|          | <b>APPENDICES</b>  | <b>97</b>  |
|          | <b>BIODATA OF STUDENT</b>  | <b>107</b> |
|          | <b>LIST OF PUBLICATIONS</b>  | <b>108</b> |

## LIST OF TABLES

| Table |   | Page |
|-------|---|------|
| 2.1   | The Phylogeny and Classification of <i>B. stenophylla</i>   | 3    |
| 2.2   | List of Species in which Inflorescence Structure was examined. Anther Dehiscence Patterns are classified into Slit (anther thecae dehiscing throughout length), Short Slit (thecae dehiscing by slits much shorter than thecae) and Pore (dehiscing by pores) | 7    |
| 2.3   | Chemical Constituents of the Essential Oils of <i>B. stenophylla</i>  | 9    |
| 2.4   | Previous Location Documentation for Biodiversity of <i>Boesenbergia stenophylla</i>   | 13   |
| 2.5   | Merits and Demerits of Different Marker Systems   | 15   |
| 3.1   | Collection Site and Date for Studies of <i>Boesenbergia stenophylla</i>   | 22   |
| 3.2   | Equipment Used for <i>In Situ</i> Equipment for General Ecology Data Collections  | 23   |
| 3.3   | Morphological Characteristic of <i>Boesenbergia stenophylla</i>   | 23   |
| 3.4   | Component of PCR Amplification SSR  | 28   |
| 3.5   | PCR Amplification Profile   | 28   |
| 3.6   | Component of PCR Amplification RAPD   | 29   |
| 3.7   | Sequences of Primers Used for the Amplification of <i>B. stenophylla</i> DNA  | 30   |
| 3.8   | PCR Amplification Profile   | 30   |
| 3.9   | Method for Three Different Surface Sterilization Techniques   | 33   |
| 3.10  | Plant Growth Regulators of BAP and NAA Treatments Used on <i>B. Stenophylla</i> Shoot Tip Explants  | 35   |
| 3.11  | Composite of Planting Media Mixture for Treatments Volume per Volume  | 37   |
| 3.12  | Device Used to Measure Environmental Data at Nursery  | 37   |

|      |  |    |
|------|--|----|
| 4.1  | New Occurrences and Previous Records   | 43 |
| 4.2  | Nutrient Analysis of Soil Samples from the Natural Population  | 45 |
| 4.3  | General Ecology Data for Sub Population Natural Occurrences and Domesticated <i>B. stenophylla</i> at Northern Sarawak   | 50 |
| 4.4  | Twenty Primer Pairs Developed to Determine the Genetic Variations of <i>B. Stenophylla</i> . Selections were based on the High GC and Zero 3' (Self Complementarity) Content and Their Annealing Temperature | 57 |
| 4.5  | Selected primer for Population Genetic Variation with the Scoring and Alleles Size   | 58 |
| 4.6  | RAPD Scoring and Alleles Size  | 60 |
| 4.7  | The Percentage of Contamination after 4 Weeks of Culture. Three Methods were Compared  | 68 |
| 4.8  | Results of the number of shoots, roots and leaves after 12 weeks of culture on MS media with different concentrations of 5-ALA   | 71 |
| 4.9  | General Reading Condition for Indoor and Outdoor Environment of Nursery House  | 75 |
| 4.10 | Chemical Composition in 7 Treatments Media Before Planting (in Grey Shade) and after 9 Months of Planting (without shade)  | 81 |
| 4.11 | Parameter Collected After 9 Month of Planting  | 84 |

## LIST OF FIGURES

| Figure |   | Page |
|--------|---|------|
| 2.1.   | Protologue of <i>Boesenbergia stenophylla</i> .(A) habit $\times \frac{2}{3}$ ;(B) apical part of lamina $\times \frac{2}{3}$ ; (C) bract X 1;(D) bracteole $\times 1$ ; (E) calyx $\times 1$ ; (F) L.S of corolla showing lateral staminodes, stamen and dorsal petal $\times 1$ ; (G) L.S of corolla showing labellum and lateral petal $\times 1$ ; (H) stamen (style and stigma removed) $\times 2$ ; (I) ovary in T.S. $\times 3$ . (A, B from dried material of Hansen 1081, C- I from living material of Argent 18/85. (Source: Smith, 1987) | 4    |
| 2.2    | The Flower of <i>B. stenophylla</i> R.M. sm. in Natural Habitat. (Source:Poulsen, 2006)   | 5    |
| 2.3    | A hypothetical model showing the complementary use of different conservation technologies.(Source: <a href="http://www.biodiversityinternational.org">http://www.biodiversityinternational.org</a> )  | 18   |
| 3.1    | (a) Shoot tips used as explant and (b) Shoot tip with outer leaf layer removed.   | 32   |
| 4.1.   | Maps Showing the Three sites for <i>B.stenophylla</i> , Bario (B), Ba'kelalan (BK) and Long Semadoh (LS).   | 42   |
| 4.2    | <i>B. stenophylla</i> Growing in Soil Rich with Leaf Litter and Organic Matter, the Source of Nutrients.  | 44   |
| 4.3    | <i>B. stenophylla</i> Collected from Long Semadoh Adult Plants with Rhizome and White Flower. Petals Resemble Orchid Flower with Yellow Streak on the Lips. Scales: 10cm black bar, 1cm white bar and 0.5cm yellow bar.   | 46   |
| 4.4    | Variations of the Stoloniferous Rhizome of <i>B. stenophylla</i> (A) Stoloniferous Rhizome with Curly and Straight (10cm bar) (B) <i>B. stenophylla</i> Sympodial Growth (30cm bar).  | 47   |
| 4.5    | The Morphological Characteristics of <i>B.stenophylla</i> (A) s: Shoot with Maroon Sheath, r: Root (B) l: Narrow Leaf Blade, p: Petiole Longer Than Leaf Blade, m: Maroon Abaxial (C) Erected Rhizome (n) Node (i) Internode (e) Eye Scale Bud (D) Flower of <i>B. stenophylla</i> . Black Scale Bar (5cm) and White Scale Bar (1cm).   | 48   |
| 4.6    | Natural <i>B. stenophylla</i> Habitats in Bario.  | 51   |

|      |   |    |
|------|---|----|
| 4.7  | <i>B. stenophylla</i> Sampled Originally from the Forest of Romudu, Bario but Planted within the Vicinity of Houses.  | 52 |
| 4.8  | <i>Boesenbergia stenophylla</i> Habitats in Ba'Kelalan.   | 53 |
| 4.9  | <i>B. stenophylla</i> Habitats Found in Long Semadoh.   | 54 |
| 4.10 | Forest Cover an Condition in Malaysia Borneo and Brunei in 2009. Intact Forest, Degraded Forest, Mangroves, Plantations and Regrowth are Shown.(Source:Byran <i>et al.</i> , 2013). | 55 |
| 4.11 | DNA Amplification of <i>B. Stenophylla</i> On 3% Metaphor Agarose Gels Compared With 100bp DNA Ladder.  | 57 |
| 4.12 | DNA Amplification of the SSR Regions using 8 Primers on Sub-Populations Collected from (1) Bukit Besar, (2) Arur Dalan (3) Ramudu and (4) Glasshouse.                               | 58 |
| 4.13 | An Example of SSR DNA Amplification using Primer BS109 on 20 Individuals from Arur Dalan Showing No Variations among Accessions.  | 59 |
| 4.14 | Amplified of Screening Primer (M) 100bp Ladder (A) OPA 1-20, (B) OPB 1-20.  | 61 |
| 4.15 | RAPD Primer OPA 1 Amplified on 3 Sub Population with No Variation among Sub Populations. (M) 100bp Ladder, (1-20) Individual DNA from Each Sub-populations.                         | 62 |
| 4.16 | RAPD Primer OPA 15 Amplified on 3 Sub Population with No Variation among Sub Populations (M) 100bp Ladder, (1-20) Individual DNA from Each Sub-populations                          | 62 |
| 4.17 | Selected Primer for Screening Population Show No Variation between Population. (M) 100bp ladder, (1) Arur Dalan, (2) Bukit Besar, (3) Ramudu.                                       | 63 |
| 4.18 | The Inferences of Evolutionary History based on the ITS DNA Region.   | 64 |
| 4.19 | Molecular Phylogenetic Analysis of <i>matK</i> Region using Maximum Likelihood method.  | 65 |

|      |  |    |
|------|--|----|
| 4.20 | Molecular Phylogenetic Analysis of <i>rbcL</i> Region using Maximum Likelihood method.   | 66 |
| 4.21 | Source of Contamination: (A) Bacteria and (B) Fungus on MS Media with <i>B. stenophylla</i> Shoot Tip Cultures.  | 68 |
| 4.22 | Responds of an Explant with the Treatments after 12 Weeks.(a) T5, 0 mg/L BAP and 0.2 mg/L NAA (b) T12, 2 mg/L BAP and 0.4 mg/L NAA (c) T14, 4 mg/L BAP and 0.4 mg/L NAA + 10mg/L 5ALA (d) T17, 2 mg/L BAP and 0.5 mg/L NAA + 5 mg/L 5ALA (e) T18, 3 mg/L BAP and 0.5 mg/L NAA + 5 mg/L 5ALA (f) T23, 3 mg/L BAP and 0.6 mg/L NAA. All of the Explant was Cultured on MS Media. | 69 |
| 4.23 | Respond of Explant after 16 Weeks Culture on Media Treatments.   | 72 |
| 4.24 | Sub-culturing of Five Explants from Different Treatments (A,C,E,G) into Media Containing MS without Any Hormone (B,D,F,H). The Multiplication of Shoots After One Month of Cultures (B,D,F,H).   | 73 |
| 4.25 | Treatment (A) 0 mg/l (B) 10 mg/l (C) 20 mg/l (D) 30 mg/l (E) 40 mg/l (F) 50 mg/l of 5 ALA in MS media with 2 mg/l BAP and 0.4 mg/l NAA.  | 74 |
| 4.26 | <i>Boesenbergia stenophylla</i> Plant Growth by Monthly on Mixture Media of Cocopeat and Control Nursery Media (A) Zero Months (B) 3 <sup>rd</sup> Months (C) 6 <sup>th</sup> Months (D) 9 <sup>th</sup> Months of Planting.   | 77 |
| 4.27 | (a) Rhizomes of <i>B. stenophylla</i> with Very Short Internodes. Black Scale 5cm  | 78 |
| 4.28 | The Number of Seedlings Grown in Different Ratio Planting Media After 9 Months of Planting.  | 78 |
| 4.29 | Growth of <i>B. stenophllya</i> for 9 Months in Different Media Treatments.  | 79 |
| 4.30 | New Rhizomes after 9 Month Planting (Black bar, 5cm).  | 82 |

## LIST OF ABBREVIATIONS

|                                      |  |
|--------------------------------------|--|
| °C                                   | Celsius                                |
| μl                                   | Microlitre                             |
| μM                                   | Micromolar                             |
| μmol m <sup>-2</sup> s <sup>-1</sup> | Micromoles per square meter per second |
| μS/cm                                | Microsiemens per centimetre            |
| 5-ALA                                | 5-aminolevulinic acid                  |
| AAS                                  | Atomic Absorption Spectroscopy         |
| B                                    | Bario                                  |
| BAP                                  | Benzylaminopurine                      |
| BK                                   | Ba'Kelalan                             |
| BOLD                                 | Barcode of Life Database               |
| CBOL                                 | Consortium for the Barcode of Life     |
| CpDNA                                | Chloroplasts DNA                       |
| DNA                                  | Deoxyribonucleic acid                  |
| EC                                   | Electric Conductivity                  |
| g/l                                  | Gram per litre                         |
| ITS                                  | Internal transcribed spacer            |
| K                                    | Potassium                              |
| Km                                   | Kilometre                              |
| KN                                   | Kinetin                                |
| LS                                   | Long Semadoh                           |
| LUX                                  | Luminous flux                          |
| M                                    | Meter                                  |
| <i>matK</i>                          | Maturase K                             |
| Mg                                   | Magnesium                              |



|             |   |
|-------------|---|
| mg/l        | Milligram per litre                     |
| Min         | Minute                                  |
| mM          | Millimolar                              |
| MS          | Murashige and Skoog                     |
| mS/cm       | Milisiemens per centimetre              |
| N           | Nitrogen                                |
| NAA         | 1-Naphthaleneacetic acid                |
| Ng          | Nanogram                                |
| Nm          | Nanometre                               |
| NrDNA       | Nuclear ribosomal DNA                   |
| OM          | Organic Matter                          |
| P           | Phosphorus                              |
| p.s.i       | Pounds per square inch                  |
| pH          | Potential hydrogen                      |
| Pmol        | Picomole                                |
| RAPD        | Random Amplification of Polymorphic DNA |
| <i>rbcL</i> | Ribulose-bisphosphate carboxylase       |
| <i>rDNA</i> | Ribosomal DNA                           |
| Rpm         | Revolutions per minute                  |
| SSRs        | Simple Sequences Repeats                |
| TOC         | Total Organic Carbon                    |
| UV          | Ultraviolet                             |
| Uv/Vis      | Ultraviolet-visible spectrophotometry   |
| v/v         | Volume per volume                       |
| w/v         | Weight per volume                       |

## CHAPTER 1

### INTRODUCTION

The species of *Boesenbergia stenophylla* R. M. Sm. is a member of the Zingiberaceae family and was recorded as highly endemic to the Borneo Highlands. It bears few local names depending on locality and recognition by ethnic group. In general it is mainly known as Jerangau Merah.

It was first documented and discovered in Long Pasia, Sabah. This species was named for having a very narrow leaf as its unique characteristics. The plant produced very unique single flower with different colours and shape that are used as key description to differentiate it from other species. Taxonomic studies recorded that this species is highly endemic to the northern region of the highland in Borneo. This species could only be found in its natural habitat near to riverine or humid area which grown well under total shaded in undisturbed forest of Tropical Heath Forest and Mixed Dipterocarp Forest in the highland of Bario.

The most valuable part of *Boesenbergia* is the rhizome which contained valuable medicinal properties. A sister species to *B. stenophylla* is *Boesenbergia rotunda* and is considered as an important crop because of its unique medicinal properties with a promising anti-cancer remedy where it has been domesticated and cultivated as one of a major herbs in Thailand.

Indigenous communities in different part of Borneo reported various medicinal use of this species such as to treat headaches, backaches, stomach-ache disorders, antidote and rheumatic pain. There is also myth belief it is use to evict bad ghost spirits and as protecting charm from ghost for baby's. Apart from that, there were also some folklore beliefs that by chewing the dried rhizome of *B. stenophylla*, a person will not get drunk after consuming alcohol and this belief is still practised by some people until today. Although there were no scientific evidences on the effectiveness of its rhizome against alcohol intoxication, users of the Lun Bawang and Kelabit communities claimed that it was able to do so.

The rhizomes of *B. stenophylla* were harvested and were dried before sold. The chemical properties from the rhizome were never extracted and they were usually sold as small cuttings and demand for it were drastically increasing. This led to overharvesting of *B. stenophylla* causing depletion of its abundance in natural habitat. Besides overharvesting, excessive deforestation also decrease the population of *B. stenophylla*. These will potentially lead towards the extinction of this species in the future.

Pollination biology for this species was not known and the genetic diversity was never reported. To date, there are no natural varieties or form that suggest that this

species is undergoing speciation and the distinction in term of their flower morphology from other species is still considered wide. Without the reproductive parts of the flower, it is not easy to identify among the *Boesenbergia* species. As flowering is considered as an expensive physiological process and with limited resources, in which what this species is experiencing, the alternative technique to continue with regeneration of new seedling will be more favourable towards rhizome development. Even when *B. stenophylla* are flowering the chances for it to be pollinated is very little because the blooming period is short. Nevertheless, since the rhizome is the main economic part, the success of this mode of reproduction becomes an advantage for its cultivation.

However, there were no reports found on the conservation practices for *B. stenophylla*. Through ex situ conservation it will assist to sustain the population in the nature and producing new method for propagation through application of biotechnology that will give the awareness to the villagers nearby the populations and reduced the dependence of harvesting from the wild. This will indirectly help to conserve their occurrence in the wild. A study was carried out to find ways to sustain the population in the wild by carrying out the fundamental studies of the *B. stenophylla*. This fundamental study included genetic variation studies and DNA barcoding for its conservation, propagation studies under nursery conditions and micropropagation to produce seedlings. This was done with an aim to produce uniform seedlings which can be used to produce rhizomes and also seedlings for the purpose of reintroduction into their natural population for conservation.

### **1.1 Objectives of Study**

Objectives of this study are:

1. To elucidate the genetic variations among three populations based on SSR markers, RAPD markers and to obtain DNA sequences of *Boesenbergia stenophylla* in *matK*, ITS and *rbcL* regions for the purpose of DNA barcoding by using molecular marker.
2. To determine the optimum *in vitro* culture conditions and the effects of 5-ALA on shoot tip cultures.
3. To determine the optimum planting media for *Boesenbergia stenophylla* cultivation under nursery conditions.

### **1.2 Hypothesis**

1. Conservation of *Boesenbergia stenophylla* will be achieved through in vitro culture, phylogenetic and propagation

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