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SYSTEMATICS, DNA BARCODING AND CONSERVATION OF *VANILLA* SPECIES FROM PENINSULAR MALAYSIA

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SYSTEMATICS, DNA BARCODING AND CONSERVATION OF *VANILLA* SPECIES FROM PENINSULAR MALAYSIA

By

MOHD AKMAL BIN MOHD RAFFI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2018

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

SYSTEMATICS, DNA BARCODING AND CONSERVATION OF *VANILLA* SPECIES FROM PENINSULAR MALAYSIA

By

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December 2018

Chair : Rusea Go, PhD
Faculty : Institute of Bioscience

Among the 25 000 orchid species in Orchidaceae family, *Vanilla* is the most appreciated as it is the only genus with species of high commercial agriculture value for its vanillin production. The species from Peninsular Malaysia which represents the secondary gene pool of the cultivated *Vanilla planifolia* serves as valuable resources for genetic improvement in *Vanilla*. However, the potential of each species still remain in secrecy as the genus is not comprehensively studied yet. This study was conducted to assess the native species through systematics and molecular investigations. The data obtained will be used in their conservation management. Therefore, efforts in locating, collecting and documenting local *Vanilla* were initiated. The field samplings were conducted from 2011 to 2017 in the states of Kedah, Perak, Pahang, Kelantan, Terengganu, Selangor, Negeri Sembilan and Johor. In general, wild *Vanilla* populations in Peninsular Malaysia were difficult to locate because they were found in many habitats. The diversity in the genus was updated with the discovery of two new species to science, *Vanilla norashikiniana* R. Go et A. Raffi and *Vanilla sanguineovenosa* R. Go et A. Raffi which made up the total count from this region to be represented by seven confirmed species. *Vanilla* spp. most likely could tolerate the cardinal temperature of 17 to 27 ^{o}C, relative humidity of 72 to 92% and 64 mm to 733 mm of monthly precipitation. Five important growth events and their interactions with different environmental cues were presented with the emphasized on the flowering stages. The fluctuations of temperature and high light intensity were seen as the natural flowering promoters. Anthesis in *Vanilla* spp. were ideal to be categorized into five important phases: floral evocation, floral bud emergence, ovary development, size increment in bud and ovary and anthesis. Ephemeral flowering sequences of both newly described taxa were photographed where the anthesis and senescence processes were completed within 24 hours. Investigations at the histological level on accessions from Perak revealed the taxonomic value of the

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marginal outlines from leaf transverse sections. Somatic chromosome counts (2n) among selected accessions including two new species were found to be different in their most frequent number but shared a common denominator of 2n= 24. Species discrimination inferred by the phylogenetic trees generated from nuclear ribosomal internal transcribed spacer (*nrITS*) region had classified the accessions into their respective genotypes with two unidentified genotypes suggesting presence of additional species in this region. Barcoding gap was also reported in genetic distance analysis with the speciation threshold recommended to be at 1.5% divergence. Finally, provisional conservation action plan was proposed based on the identified threats of which natural disasters and over collection from the wild were noted as the major threats. Five approaches were evaluated to be pragmatic and should be incorporated in the genus conservation action plan which were species mapping based on the documented localities, local community involvement of Orang Asli, propagation via stem cuttings using BAP, DNA barcodes deposition in BOLD and GenBank and also public education via MyBIS. This project as a whole has successfully documented important findings in the botany and natural history of the genus *Vanilla* from Peninsular Malaysia and these data are beneficial to the species strategic management planning.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SISTEMATIK, BAR KOD DNA DAN KONSERVASI SPESIS VANILLA DARI SEMENANJUNG MALAYSIA

Oleh

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Vanilla merupakan satu-satunya genus yang mempunyai spesis bernilai tinggi dalam pertanian di kalangan 25 000 spesis dalam famili Orchidaceae kerana keupayaannya menghasilkan vanillin. Spesis dari Semenanjung Malaysia yang juga merupakan kelompok gen sokongan kepada tanaman *Vanilla* planifolia menjadi sumber penting dalam penambahbaikkan genetik *Vanilla*. Walau bagaimanapun, potensi setiap spesis masih tidak diketahui memandangkan genus ini tidak dikaji secara menyeluruh. Kajian ini dijalankan untuk menilai spesis tempatan melalui sistematik dan kajian pada peringkat molekular. Data yang diperolehi akan digunakan dalam konservasinya. Oleh itu, usaha untuk mengenalpasti lokasi, mengumpul dan mendokumentasi *Vanilla* tempatan telah dimulakan. Secara amnya, populasi liar *Vanilla* di Semenanjung Malaysia adalah sukar untuk dikenalpasti dan terdapat dalam banyak habitat. Kepelbagaian dalam genus ini telah dikemaskini dengan penemuan dua spesis baharu, *Vanilla norashikiniana* R. Go et A. Raffi and *Vanilla sanguineovenosa* R. Go et A. Raffi seterusnya menjadikan keseluruhan bilangan spesis tempatan diwakili dengan tujuh spesis yang dikenalpasti. *Vanilla* spp. boleh toleran dengan suhu kardinal dari 17 sehingga 27 ^{o}C, kelembapan relatif dari 72 sehingga 92% dan jumlah hujan bulanan dari 64 mm sehingga 733 mm. Lima peringkat pertumbuhan utama dan interaksi terhadap pelbagai keadaan persekitaran telah ditunjukkan dengan tumpuan kepada peringkat pembungaan. Perubahan suhu dan keamatan cahaya yang tinggi telah dikenalpasti sebagai penyebab pembungaan semulajadi. Antesis *Vanilla* spp. boleh dikategorikan dalam lima fasa penting: evokasi bunga, penjelmaan kudup bunga, pembentukan ovari, penambahan saiz dalam kudup dan ovari serta antesis. Pengembangan bunga yang singkat telah dirakamkan ke atas

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kedua-dua taksa yang baharu di mana proses dari antesis ke senesens dapat dilengkapkan dalam masa 24 jam. Kajian pada peringkat histologi ke atas aksesi dari Perak mendedahkan nilai taksonomi pada bentuk luaran pinggiran dari potongan melintang daun. Pengiraan kromosomom soma (2n) di kalangan aksesi terpilih termasuk dua spesis yang baharu menunjukkan kiraan paling kerap yang berbeza tetapi berkongsi kiraan 2n = 24 yang sama. Diskriminasi spesis yang disimpulkan oleh pohon filogenetik daripada rantau penjarak transkripsi dalaman nuklear ribosom (*nrITS*) telah mengklasifikasikan aksesi kepada genotip masing-masing dengan dua genotip yang tidak dikenalpasti menandakan wujudnya spesis tambahan dalam rantau ini. Jurang barkod turut dilaporkan dalam analisis jarak genetik dengan mengesyorkan ambang spesiasi pada pencapahan 1.5 %. Pelan tindakan konservasi semasa telah diusulkan berdasarkan ancaman yang dikenalpasti seperti kemusnahan semulajadi dan pengambilan secara berlebihan dari habitat asalnya. Lima tindakan telah dikaji sebagai pragmatik dan sepatutnya dimasukkan ke dalam pelan tindakan konservasi genus yang terdiri daripada pemetaan spesis berdasarkan lokaliti yang telah didokumentasi, pembabitan komuniti tempatan Orang Asli, pembiakan melalui keratan menggunakan BAP, mendepositkan barkod DNA ke dalam BOLD dan GenBank serta pendidikan awam melalui MyBIS. Projek ini secara keseluruhannya telah berjaya mendokumenkan penemuan-penemuan penting dalam botani dan sejarah semulajadi genus *Vanilla* dari Semenanjung Malaysia dan data-data ini adalah bernilai kepada perancangan pengurusan spesis yang strategik.

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LIST OF ABBREVIATIONS

2n	Somatic chromosome numbers
ANOVA	Analysis of variance
a.s.l	Above sea level
BAP	6-Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data Systems
BRIS	Beach Ridges Interspersed with Swales
CG	Cytosine-Guanine
CS	Conserved sites
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FRIM	Forest Research Institute Malaysia
GPS	Global Positioning System
IUCN	International Union for Conservation of Nature
K	Royal Botanic Gardens, Kew
KEP	Forest Research Institute Malaysia, Kepong
L	Naturalis, Leiden
ML	Maximum likelihood
Mybis	Malaysia Biodiversiti Infromation System
n	Number of sample
nov.	Novo
NCBI	National Center for Biotechnology Information
NPBD	National Policy on Biological Diversity

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nrITS	Nuclear internal transcribed spacer
PIS	Parsimony informative sites
SAR	Department of Forestry, Sarawak
SING	Singapore Botanic Gardens, Singapore
SNP	Sabah Parks, Kota Kinabalu
SS	Singleton sites
VS	Variable sites

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CHAPTER 1

INTRODUCTION

1.1 Background of study

The name vanilla is derived from the orchid genus by the same name, *Vanilla*. Among the 25 000 orchid species in Orchidaceae family, *Vanilla* is the most appreciated as it is the only genus with species of high commercial agriculture value which is also known to be the oldest spices used by the Aztecs (Ramachandra-Rao & Ravishankar, 2000). As an orchid, vanilla is cultivated not for the flower but the capsules or pods. That part is responsible for vanillin production, the distinguishing natural aromatic compounds that gives *Vanilla* its desirable characteristics when subjected to a lengthy curing process. The curing process requires remarkably long man hours and costs, thus made *Vanilla* the world's second most expensive spice after saffron (Anuradha, Shyamala, & Naidu, 2012). The flavour has a special bond with human as it is used directly in culinary as well as extracts in perfume and pharmaceutical products.

The genus of *Vanilla* which comprises a large number, more than 100 species nevertheless has only three commercialized species which are *Vanilla planifolia* (*V. planifolia*) and *V. tahitensis* and *V. pompona.* Among the three species, *V. planifolia* is the most cultivated vanilla as it yields the highest amount of vanillin making it the most valued one. The high value of vanilla has attracted the interest among growers in many countries including Malaysia. *Vanilla* cultivation in Malaysia began in 1992 when *Vanilla* cuttings from Indonesia were imported and their compatibility as an integrated crop for coconut plantation was observed and studied (Abd.-Jalil, 1999). Since then, many farms were established in many states including Pahang, Perak, Negeri Sembilan, Johor, Selangor, Sabah and Sarawak.

However, the yields were not promising as the cuttings failed to produce flowers, most likely due to the requirement of optimum temperature and light intensity and other unknown environmental factor that causes this introduced species inability to adapt. In addition, long juvenile phase which is more than three years and asynchronous flowering makes the local vanilla cultivation more difficult. Therefore, it is important to develop hybrids with the capability to adapt to the local environment and to overcome the problem of floral development in *Vanilla*. With that, there is a must to manage their genetic resources to protect the species and to ensure the sustainability of vanilla production will always meets the world's demand.

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1.2 Problem statements and justification

The potential of each native species still remain in secrecy as the genus is not comprehensively studied yet. However, some of the species are known to have several desirable traits such as autogamy and ability to produce many inflorescences and pods in the same vine. With that, native taxa which represents the secondary gene pool of the cultivated vanilla serves as valuable resources for genetic improvement in *Vanilla*. Furthermore, Peninsular Malaysian *Vanilla* species populations are feared to be depleted in the wild as a result of disruption of the habitat caused by ill-managed development processes. The natural habitats are vanishing rapidly and the forest coverage in this region is expected to be less than 40% in the upcoming years (Saw, 2010). Therefore, the first and crucial step for the utilization of native *Vanilla* species is to conserve them which will ultimately lead to a sustainable cultivation system. The conservation measures should include the comprehensive diversity study including systematics revisions through morphological, physiological and cytological assessments and also genetic elucidation that serve as important data in the species management of the genus. The conservation measures which incorporate with pragmatic approaches involved by both legislative body and the public will protect these valuable genetic resources from devastation.

1.3 Objectives of study

This study was proposed to provide insights into the understudied native *Vanilla* species from Peninsular Malaysia focusing on determination of their diversity and conservation strategies.

The specific objectives of this study were:

1. To collect samples and update the species diversity of the genus *Vanilla* from Peninsular Malaysia.
2. To document the growth stages and the leaf and root histology of selected *Vanilla* accessions.
3. To identify the collected species using nuclear internal transcribed spacer (*nrITS*).
4. To survey the conservation status and propose provisional conservation action plan of the genus from Peninsular Malaysia.

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