VARIATION IN MYCORRHIZAL SPECIFICITY IN TIGER ORCHIDS (GRAMMATOPHYLLUM SPP.)

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MASTER OF SCIENCE
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Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Masters of Science.

VARIATION IN MYCORRHIZAL SPECIFICITY IN TIGER ORCHIDS (*GRAMMATOPHYLLUM* SPP.)

By

Salifah Hasanah Binti Ahmad Bedawi

January 2012

Chairperson: Assoc. Prof. Muskhazli Mustafa, PhD

Faculty: Science

Grammatophyllum seeds are minute and lack endosperm. As their other orchid's counterpart, the seeds are dependent on mychorrhizal fungi for seed germination in nature. Their nutrients uptake from substrate was assisted by preferable fungal symbionts. Introducing a compatible mycorrhiza into a site may facilitate the establishment of self sustaining populations. However this can only be performed if the orchid is present at the target localities or only when the seedlings that were cultured symbiotically were reintroduced as they can serve as both plant material and a source of mycorrhiza inoculums. To determine these fungal symbionts, fungal isolation was conducted on three species of host plant namely *G. speciosum*, *G. scriptum* and *G. stapeliiflorum*, all native to Malaysia. A total of 59 isolates of different species of fungus were isolated from the host plants. Molecular identification using ITS1 and ITS4 as the primers was made to these isolates. Results of the identification showed that the roots of mature *Grammatophyllum* spp. were the habitat of various fungal taxa consisting of both common orchid mycorrhizas and ubiquitous

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fungal taxa. Diversity index showed that *G. speciosum* harbored the most fungal taxa compared to *G. scriptum* and *G. stapeliiflorum* with value of 3.218 indicating the fungal preferences. Seed of *G. speciosum* and *G. stapeliiflorum* were used to determine the specificity of fungus relationship using fungal isolated from roots of *G. speciosum*, *G. stapeliiflorum* and *G. scriptum*. The result obtained from the test demonstrated that seeds of G. *speciosum* showed the highest volume increment when co-cultured with *Aspergillus niger, Paecilomyces lilacinus A. fumigatus, Trichoderma asperellum.* and an unidentified fungal endophyte. This result shows that specificity of *G. speciosum* towards mycorrhizas preferences was quite broad considering not all of these fungi were isolated from *G. speciosum* roots. An even wider specificity was exhibited by *G. stapeliiflorum.* Having performed the same germination test as *G. speciosum* seeds, the seeds of *G. stapeliiflorum* developed a symbiotic relationship with more numbers of mycorrhizas. The initial development of *G. stapeliiflorum* seed was assisted by 29 of 59 mycorrhizas isolated from different hosts. Based on germination tests, the *P. lilacinus* and *A. fumigatus,* were the most effective orchid mycorrhizas to promote seed germination of tropical orchid, *G. speciosum* and *G. stapeliiflorum*. Nevertheless, the difference between volume of symbiotic and asymbiotic (control) seed germination was not high, and the final protocorm development stage was not sufficient enough for propagation and conservation

The phylogeny tree showed that all these symbiotic mycorrhizas shared a common class namely Eurotiomycetes, including the unidentified fungi. It can be concluded that *Grammatphyllum* spp. were specialist toward Class of Eurotiomycetes but are generalist toward the members of the class. The specificity in this broadly associating orchid most likely due to the tendency for *Grammatophyllum* spp. to be colonized by multiple mycorrhizal fungi.

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

KEPELBAGAIAN PENGKHUSUSAN MIKORIZA DI KALANGAN ORKID HARIMAU (*GRAMMATOPHYLLUM* SPP.)

Oleh

Salifah Hasanah Binti Ahmad Bedawi

Januari 2012

Pengerusi: Prof. Madya Muskhazli Mustafa, PhD

Fakulti: Sains

Biji benih orkid *Grammatophyllum* adalah halus serta mempunyai kandungan endosperma yang sangat sedikit. Seperti mana biji orkid spesies lain, biji benih *Grammatophyllum* bergantung kepada kulat mikoriza bagi bercambah secara semulajadi. Kebolehan biji ini menyerap nutrien daripada substrat dibantu oleh kulat terpilih yang bersimbiosis dengannya. Dengan memperkenalkan mikoriza yang serasi kepada sesuatu habitat, akan memudahkan pembentukan populasi orkid yang mampu hidup sendiri. Walaubagaimanapun ia hanya boleh dilakukan sekiranya orkid tersebut wujud di lokasi yang disasarkan atau apabila anak pokok yang dihasilkan secara simbiosis dengan kulat diperkenalkan di lokasi tesebut, kerana mereka boleh memainkan peranan sebagai sumber pokok dan sumber mikoriza. Bagi menentukan kulat yang bersimbiosis seperti ini, pemencilan kulat telah dilakukan ke atas akar tiga

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pokok hos iaitu *G. speciosum*, *G. scriptum* dan *G. stapeliiflorum*, semua berasal dari Malaysia. Sejumlah 59 kultur tulen kulat telah dipencilkan daripada tumbuhan hos tersebut. Pengenalpastian identiti terhadap kulat telah dilakukan menggunakan primer ITS1 dan ITS4. Hasil pengenalpastian ini menunjukkan akar tumbuhan *Grammatophyllum* dewasa merupakan habitat kepada pelbagai taxa kulat merangkumi mikoriza orkid biasa malahan kulat yang tersebar luas. Indeks kepelbagaian menunjukkan *G. speciosum* mempunyai paling banyak penghuni kulat dibandingkan dengan *G. scriptum* dan *G. stapeliiflorum* dengan nilai 3.218. Biji orkid *G. speciosum* dan *G. stapeliiflorum* telah digunakan untuk menentukan spesifikasi hubungan kulat menggunakan semua kulat yang telah dipencilkan dari akar pokok *G. speciosum*, *G. stapeliiflorum* dan *G. scriptum*. Hasil yang diperolehi daripada ujian ini menunjukkan biji benih *G. speciosum* paling banyak bertambah isipadunya apabila dikultur bersama *Aspergillus niger, Paecilomyces lilacinus, A. fumigatus, Trichoderma asperellum.* dan satu spesies kulat endofit yang tak dapat dikenalpasti. Keputusan ini menunjukkan pengkhususan *G. speciosum* terhadap mikoriza adalah agak meluas memandangkan bukan semua kulat ini berasal daripada akar *G. speciosum*. Pengkhususan yang lebih meluas ditunjukkan oleh *G. stapeliiflorum*. Benihnya telah melalui ujian yang sama dengan benih *G. speciosum*, namun biji *G. stapeliiflorum* mewujudkan hubungan simbiosis dengan lebih banyak kultur kulat daripada *G. speciosum*. Percambahan awal bijinya telah dibantu oleh 29 daripada 59 kulat yang dipencilkan daripada tiga hos pada awal eksperimen. Berdasarkan keputusan eksperimen, kulat *P. lilacinus* dan *A. fumigatus* adalah yang paling efektif dalam membantu percambahan kedua-dua benih orkid tropika, *G. speciosum* dan *G. stapeliiflorum*. Namun, perbezaan jisim antara benih simbiosis dan asimbiosis (kawalan) pada akhir eksperimen tidak tinggi, dan pembentukan protokom tidak cukup maju untuk usaha propagasi dan pemeliharaan.

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Pokok filogeni pula menunjukkan kesemua kulat yang dipencilkan berkongsi satu persamaan iaitu semua adalah ahli Kelas Eurotiomycete, termasuklah beberapa kulat yang tak dapat dikenali identitinya. Dapat disimpulkan bahawa spesies orkid *Grammatophyllum* adalah mengkhusus kepada Kelas Eurotiomycete tetapi mengumum kepada ahli kelas itu. Pengkhususan orkid yang boleh berhubung luas ini mungkin disebabkan oleh kecenderungan orkid *Grammatophyllum* untuk dikoloni oleh pelbagai spesies kulat.

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I certify that an examination committee has met on 2011 to conduct the final examination of Salifah Hasanah Binti Ahmad Bedawi on her Master of Science thesis entitled "Variation in Mycorrhizal Specificity in Tiger Orchids *(Grammatophyllum* spp.)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Putra Malaysia (Higher Degree) Regulation 1981. The committee recommend that the student be awarded the relevant degree. Members of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The member of the Supervisory Committee were as follows.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, or concurrently, submitted for any other degree at Universiti Putra Malaysia or any other institutions.

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SALIFAH HASANAH BINTI AHMAD BEDAWI

Date: 18 January 2012

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LIST OF ABBREVIATION/SYMBOLS

BLAST	Basic local alignment search tool
bp	Base pair
cm	Centimeter
CITES	Convention on International Trades in Endangered Species
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
h	Hour
ITS	Internal transcribed spacer
ITS-rDNA	Internal transcribed spacer-ribosomal deoxyribonucleic acid
mg	Milligram
min	Minutes
ml	Milliliter
mm	Millimeter
mmol	Millimole
NaCl	Natrium chloride
OMA	Oat meal agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rpm	Revolutions per minute
s	Seconds
SDS	Sodium dodecyl sulfate

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SEA	South East Asia
sp.	Species (singular)
spp.	Species (plural)
TE	Tris-EDTA
TrisHCl	Tris hydrochloric
TTC	Triphenyl tetrazolium chloride
V	Volt
°C	Degree Celcius
%	Percents
µl	Microliter
µM	Micromolar
µg/ml	Microgram per milliliter

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Orchidaceae is the most species rich family of flowering plant. Members of this diverse family grow in a wide range of habitats and have a substantial variety of life strategies ranging from epiphytic to terrestrial, and from evergreen to achlorophyllous species (McCormick *et al.*, 2004). They can be found throughout any habitat and geographical zone except in poles and extremely dry desert but reach their sanctuary at the tropics (Holttum, 1964; Hawkes, 1965; Tremblay, *et al*., 2005). At present orchids are a million dollar industry in several countries like Thailand, Australia, Singapore, Malaysia and several others (Chugh *et al*., 2009).

The genus *Grammatophyllum* is an epiphyte from the family Orchidaceae; the largest flowering family, subfamily Epidendroideae and the tribe Cymbidiae. It is closely allied to genus *Cymbidium* and consists of 12 species confined only to dense rainforest of Indo-China, to Indonesia, Malaysia, the Philippines, New Guinea, and the Southwest Pacific islands. Two of the species are native to Malaysia; *G. speciosum* and *G. stapeliiflorum* (Holttum 1964). Convention on International Trade in Endangered Species (CITES) has placed *Grammatophyllum* in Appendix II since 1975, which means they are not necessarily now threatened with extinction but they may become so unless trade is closely controlled (http://www.cites.org/eng/resources/species.html). Malaysia fortunately still is good shelter for this genus. The neighboring country implemented several conservation

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efforts to propagate and reintroduce species that have special horticultural or educational value. Singapore for example successfully reintroduces locally the once locally extinct *G. speciosum* and four other native species into its locality with varying survival rate to up to 95 % (Yam *et al.* 2010).

In Malaysia, horticulturist simply called the genus *Grammatophyllum* as '*Gram'*. There are two types of growth form in the genus. One has very long pseudobulb which is really fleshy stems, bearing many leaves. The other has rather short proportionately thick pseudobulb which is not covered by leaves base, with few leaves at the apex. The flowers in the both are essentially the same. Both have the erect branched white roots. The plant at the first type is represented by *G. speciosum*, which is believed to be the largest orchid plant in existence and is the only species with this life form. The plants at the second type similar to *Cymbidium* in habit; the genera *Cymbidium* and *Grammatophyllum* are in fact very nearly allied. The most notable difference is in the pollinia, which are seated directly on the disc in *Cymbidium* and on separate upgrowth in *Grammatophyllum*.

Grammatophyllum speciosum in Malaysia can be found in the lowland throughout the country including east Malaysia and still is common nowadays if one know where to look to. They usually flower on July but all plants do not flower every year (Holttum 1964). *Grammatophyllum stapeliiflorum* distribution in Malaysia however is rare and has been recorded as early as 1899 but only found in Taiping Hills. More recent collection was in in Genting Highland when the research group joined by the author stumble upon a clump of *G. stapeliiflorum* in bloom on a dead tree trunk in an exposed area by a stream in Gunung Bunga Buah in 26 September 2005. A herbarium

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sample, voucher number km022 and a live specimen was made out of the sample and deposited in Herbarium of Biology Department, Faculty of Science, Universiti Putra Malaysia (UPM) and UPM greenhouse respectively.

1.2 Statement of the problem

Malaysia has a great potential to become world's major orchid flower exporter, but only less than 30 % of the flower export are orchids and the majestic native orchid *Grammatophyllum* do not even make it to the favorite list (Lim *et al.*, 1998). The fact that the plant is not considered decorative when flowerless, the slow growing nature and its fleshy pseudobulb that susceptible to infection by fungus and bacteria of all sorts could be responsible to the lack of interest. Secondly, it is a great importance in understanding the mycorrhizal symbionts of *Grammatophyllum* spp., as availability of the fungal symbionts may play a key role in determining orchid distribution and diversity. Most study on orchid mycorrhizas have concentrated on terrestrial orchids from temperate region such as *Goodyera pubescens, Tipularis discolor* and *Liparis lilifolia* (McCormick *et al.*, 2004) whereas the majority of orchid species are epiphytes in tropical region (Otero *et al.*, 2002). To date, apart from the study of general distribution and micropropagation of this genus, there is no published research regarding the orchid-fungus relationship of *Grammatophyllum* spp. in Malaysia. There are little efforts of identifying the mycorrhiza of Malaysian epiphytic orchids (Hadley and Williamson, 1971 & 1972) but no published report or known efforts to detect the mycorrhizal fungi of *Grammatophyllum* spp. and their specificities.

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1.3 Objectives of the study

The overall aim of this study is to select the isolate(s) that are best adapted as the mycorrhizal symbionts towards *Grammatophyllum* spp. to produce feasible fungal inoculants. To achieve this goal, the experiment was divided into three parts to achieve 3 objectives:

1. To isolate and to determine the identity of fungi isolated from roots of adult *G. speciosum, G. stapeliiflorum* and *G. scriptum* using both traditional and molecular methods.
2. To determine the specificities of *G. speciosum* and *G. stapeliiflorum* towards fungi isolated from roots of adult *Grammatophyllum* spp. by means of symbiotic seed germination and,
3. To generate a representative, molecular phylogeny tree from all *Grammatophyllum* spp. for all fungi isolated from *Grammatophyllum* spp. thus mapping the specificity of mycorrhiza on their host plant in the phylogeny tree.

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