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GENETIC VARIATION, PHYLOGENETICS AND FRUIT QUALITY CHARACTERISTICS OF Physalis minima L. IN PENINSULAR MALAYSIA

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By

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

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GENETIC VARIATION, PHYLOGENETICS AND FRUIT QUALITY CHARACTERISTICS OF *Physalis minima* L. IN PENINSULAR MALAYSIA

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August 2017

Chairman : Associate Professor Nur Ashikin Psyquay Binti Abdullah, PhD
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Physalis is a member of the Solanaceae family and it is abundantly found growing as weeds in Malaysia. This plant reported to have various antioxidants, antimicrobials and anticancer compounds such as physalin B and F which have great potential for tumors treatment. However, due to its classification as weed, little attention is given. There is a lack of information on genetic variation and phylogenetic relationship including genetics distance to the other member of Solanaceae family. A germplasm collection consisted of 130 samples representing 19 accessions from 11 states of Peninsular Malaysia was established. *Physalis minima* was found growing under various environmental conditions including at farm, plantation area (especially of oil palm (*Elaeis guineensis*)), thus proving its high adaptability to a wide variety of ecological niches in Peninsular Malaysia.

The genetic diversity of the germplasm collection was estimated using 42 qualitative and quantitative morphological characteristics and eight ISSR molecular markers. Results indicate that high morphological and molecular variations existed between the 19 accessions of *P. minima* collected. The 19 accessions of *P. minima* shared similar qualitative characteristics. Results of analysis of variance revealed that there were significant differences among the accessions for all the quantitative characteristics measured. The 19 accessions collected were grouped into five diverse clusters based on their morphological characteristics using UPGMA clustering method. The dendrogram revealed that accessions 14 (B – 01) ,15 (B – 02) and 16 (B – 03) distinctly detached from other accessions.

ISSRs were found to be informative molecular markers for investigating genetic diversity among the *P. minima* populations as indicated by the high Nei's gene diversity coefficient and Shannon's information index (0.28 and 0.31, respectively). Results showed that AG and CA microsatellite repeats exhibited high polymorphism. The relatively low coefficient of genetic differentiation obtained from the accessions (0.398) revealed that this plant is cross-pollinating plants.

Accessions 14 (B – 01), 15 (B – 02) and 16 (B – 03) were found to be distinctly separated from all other accessions studied. The results were similar to those revealed by the cluster and PCA analyses based on morphological characteristics. Although similarity coefficients among the accessions studied obtained from morphological characteristics and molecular markers were found not to be correlated with each other, both morphological and molecular characterizations revealed that accessions 14 (B – 01) ,15 (B – 02) and 16 (B – 03) were distinctly different from the other accessions. This indicates major differences in morphology and genome composition between these populations and the other populations studied.

Phylogenetic analysis was done for 13 samples from the 19 accessions by using 4 regions of cpDNA and inter transcribed spacer (ITS) region. Result indicated that, it is easier to identify *P. minima* from similar family member by using ITS region since cpDNA is maternally inherited and less variation occurred between sequences. However, the region *rbc*L on cpDNA region was able to separate *P. minima* from *P. peruviana* and other members of Solanaceae family. Phylogenetic study of *P. minima* by using ITS and combined ITS and cpDNA regions showed that B – 02 and B – 03, which come from accessions 15 (B – 02) and 16 (B – 03) were different from other accessions with 0.03 number of nucleotide changes with 99 to 100% of bootstrap value. Therefore, it can be concluded that these two accessions has some mutation in genomes, make them able to produce better agronomic performance. Accessions of B – 02 which has high performance and superior characteristics was further study for phytochemical profiling and effects of storage on its fruits quality. n-Hexadecanoic acid ($C_{16}H_{32}O_2$) and 2- Furancarboxaldehyde, 5- (hydroxymethyl)- ($C_6H_6O_3$) were major compounds that had been identified in the fruits, leaves and roots ethanolic extract with percentage of quality more than 90. Results indicated that 98% of *Physalis* weight loss and firmness were affected by storage duration (r^2 =0.98) which were indicator of senescence. Discoloration of the fruit from greenish yellow to yellow orange occurred during storage where the value of C* and h° decreased. Storage duration longer than 6 days will increase the level of soluble solids concentration. However, the ascorbic acids, titratable acidity and pH level will decrease. Result indicated that accessions 15 (B – 02) has high level of beneficial phytocomponents and the fresh fruits can be stored up to 3 days to obtain optimum postharvest quality characteristics.

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

VARIASI GENETIK, FILOGENETIK DAN CIRI-CIRI KUALITI BUAH *Physalis minima* L. DI SEMENANJUNG MALAYSIA

Oleh

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Physalis adalah salah satu ahli dalam famili Solanaceae dan banyak dijumpai sebagai rumpai di Malaysia. Tumbuhan ini dilaporkan mempunyai pelbagai komposisi antioksidan, antimikrobial dan antikanser seperti physalin B dan F yang mana berpotensi sebagai rawatan tumor. Walaubagaimanapun, disebabkan pengkelasannya sebagai rumpai, ia tidak diberi perhatian. Maklumat berkaitan perbezaan genetik, hubungan filogenetik termasuk jarak genetik dengan ahli family Solanaceae yang lain amat sedikit. Kajian terhadap kesan tempoh penyimpanan ke atas buah *Physalis minima* dan juga pengenalpastian komposisi fitokimia tidak pernah dijalankan sedangkan ia amat penting sebagai nilai komersialnya. Koleksi gerplasma yang mengandungi 130 sampel meliputi 13 aksesi daripada 11 negeri daripada seluruh Semenanjung Malaysia dilakukan. *Physalis minima* ditemui hidup didalam pelbagai persekitaran, termasuk diladang, kawasan perladangan (terutama sekali kelapa sawit (*Elaeis guineensis*)), menunjukkan ia berupaya hidup didalam pelbagai pelbagai ekologi nich di Semenanjung Malaysia.

Kepelbagaian diversiti koleksi gerplasma diuji dengan menggunakan 42 kualitatif dan kuantitatif karakter morfologi dan juga lapan penanda molekular ISSR. Keputusan menunjukkan terdapat perbezaan morfologi dan molekular diantara 19 aksesi *P. minima* yang dihimpunkan. Kesemua 19 aksesi *P. minima* mempunyai karakter morfologi kualitatif yang sama dimana jenis bunga adalah lengkap dengan bentuk kampanulat. Jenis kalik pula adalah gamasepal bewarna hijau dan jenis corolla adalah gamapetal dengan warna kuning. Jenis buah adalah beri dengan bentuk bulat dan bewarna kuning. Semua populasi yang dikaji mempunyai daun berbentuk ovate dengan bahagian apeks, akuminat dan obtus dibahagian dorsal. Keputusan analisis variant menunjukkan terdapat berbezaan signifikan diantara

negeri bagi semua karakter morfologi kauntitatif. Kesemua 19 aksesi yang dihimpun dibahagikan kepada lima kluster yang berbeza berdasarkan karakter morfologi dengan menggunakan kaedah pengklusteran UPGMA. Dendrogram menunjukkan aksesi 14 (B – 01),15 (B – 02) and 16 (B – 03) terpisah daripada semua populasi yang lain.

ISSR adalah penanda molekular berinformasi untuk mengkaji diversiti genetik dikalangan populasi *P. minima* seperti yang tunjukkan oleh ketinggian nilai pekali Nei diversiti gen dan indek informasi Shannon (0.28 dan 0.31, masing-masing). Keputusan menunjukkan pengulangan mikrosatelit AG dan CA mempunyai polimorfisma yang tinggi. Aksesi yang dikutip dari Negeri Sembilan (Aksesi N – 01 dan N – 02) didapati mempunyai nombor band yang tinggi berbanding yang lain. Pekali perbezaan genetik yang diperoleh agak rendah (0.398) menunjukkan bahawa pokok ini adalah pokok yang membiak secara pendebungaan silang.

Aksesi 14 (B – 01) ,15 (B – 02) dan 16 (B – 03) didapati terpisah daripada populasi yang lain. Walaupun pekali persamaaan dikalangan populasi daripada kedua-dua morfologi dan molekular tidak korelasi, keduanya menunjukkan aksesi 14 (B – 01), 15 (B – 02) dan 16 (B – 03) terpisah daripada yang lain. Ini menunjukkan terdapat perbezaan morfologi dan komposisi genom antara aksesi ini dengan yang lain.

Analisis filogenetik dijalankan untuk 13 sampel daripada 19 aksesi dengan menggunakan 4 bahagian cpDNA dan inter transcribed spacer (ITS). Keputusan menunjukkan terdapat satu bahagian daripada cpDNA dan ITS yang boleh digunakan sebagai pemisah spesis. Keputusan mendapati lebih mudah untuk membezakan *P. minima* daripada ahli keluarga yang lain dengan menggunakan bahagian ITS kerana cpDNA adalah diwarisi daripada ibu dan kurang perbezaan diantara sekuen. Bahagian *rbc*L dalam cpDNA mampu memisahkan *P. minima* daripada *P. peruviana* dan ahli keluarga Solanaceae. Kajian filogenetik *P. minima* menggunakan ITS dan kombinasi ITS dan cpDNA menunjukkan aksesi B – 02 dan B – 03, daripada aksesi 15 (B – 02) dan 16 (B – 03) adalah berbeza daripada aksesi yang lain dengan perbezaan nukleotid sebanyak 0.03 dengan 99 hingga 100% nilai bootstrap. Kesimpulannya, kedua aksesi ini mempunyai mutasi didalam genom yang menyebabkan mereka boleh menghasilkan mutu agronomi yang lebih baik.

Aksesi B – 02 yang memiliki sifat yang terbaik dan berkualiti digunakan untuk kajian profil fotokimia dan kesan penyimpanan terhadap kualiti buah. n-Hexadecanoic acid ($C_{16}H_{32}O_2$) dan 2- Furancarboxaldehyde, 5- (hydroxymethyl)- ($C_6H_6O_3$) adalah komposisi-komposisi yang dijumpai dalam daun, akar dan buah. Octadecanoic acid ($CH_3(CH_2)_{16}CO_2H$), Octadec-9-enoic acid ($CH_3(CH_2)_7CH=CH(CH_2)_7COOH$), 9,12-Octadecadienoic acid (Z,Z)- ($C_{18}H_{32}O_2$) and 9,12,15-Octadecatrienoic acid, ($C_{18}H_{30}O_2$) adalah asid fatik yang dijumpai didalam ekstrak daun dan buah, sementara Phytol ($C_{20}H_{40}O$) dijumpai didalam ekstrak etanol daun.

Keputusan menunjukkan kelembapan, kerapuhan, warna, kepekatan pepejal terlarut, asid askorbik, keasidan tertitrat dan pH berbeza secara signifikan mengikut hari penyimpanan pada tahap signifikan 0.05. Kehilangan kelembapan meningkat sementara kerapuhan buah berkurangan sebanyak 24% selepas penyimpanan selama sembilan hari. Perubahan warna hijau kekuningan kepada kuning oren berlaku didalam tempoh penyimpanan dimana nilai C* dan h° berkurangan. Askorbik asid dan keasidan tertitrat berkurangan dengan pertambahan tempoh penyimpanan. Kepekatan pepejal terlarut meningkat sebanyak 6% secara beransur-ansur sementara nilai pH berkurangan selepas penyimpanan selama sembilan hari.

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Norhanizan binti Usaizan

I certify that a Thesis Examination Committee has met on 9 August 2017 to conduct the final examination of Norhanizan binti Usaizan on her thesis entitled "Genetic Variation Phylogenetics and Fruit Quality Characteristics of *Physalis minima* L. in Peninsular Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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AA	Ascorbic acid
CI	Consistency index
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic acid
GC-MS	Gas chromatography-mass spectra
ISSR	Inter simple sequence repeats
ITS	Internal transcribed spacer region
ML	Maximum likelihood
MP	Maximum parsimony
nrDNA	Nuclear ribosomal DNA
R^2	Coefficient determination
RAPD	Random amplified polymorphic DNA
RI	Retention index
SAS	Statistical analysis system
SSC	Soluble solids concentration
TA	Titratable acidity
UPGMA	Unweighted pair-group method with arithmetic average

CHAPTER 1

1INTRODUCTION

1.1 General introduction

Physalis is one of the largest genera in the Solanaceae family, with 80 to 100 species that can be found around the world (D'Arcy, 1991; Ofelia *et al.*, 2010). *Physalis minima* L. is one of the popular species which is native to Colombia but commonly found as weeds in Malaysia. This herb is commonly known as Cape gooseberry, bladder cherry, pygmy ground cherry and 'pokok letup-letup'. Colombia and some African countries such as Egypt, Zimbabwe and South Africa are the main producers, consumers and exporters of *Physalis* with Colombia stand out as the leader (Whitson and Manos, 2005).

Among the unexploited tropical fruits, *Physalis* has proven to be very promising. *Physalis* has already been popular in some international markets. Recently, the economic importance of *Physalis* is rising due to high acceptance for local consumption, achieving a great success in Arabic and European markets (Muniz *et al.*, 2015). This exotic fruit can be enjoyed in many ways, such as an interesting ingredient in salads, cooked dishes, dessert and jam and natural snacks. Fruits can be stored in a sealed container and can be frozen or kept in a dry atmosphere for several months.

P. minima is annual or- short lived perennial plants with less hairy as compared to few other species. The berry like fruit is almost round in shape and entirely hidden in calyx (Parmar and Kaushal, 1982). The fruiting calyx is the distinct characters of *Physalis* and differentiates it from other genera in Solanaceae family (Whitson and Manos, 2005). This interesting plant grown very well in most of soil types but do well on sandy to gravelly loam under full of sun exposure. *P. minima* is tolerant to drought seasons and can grow up to 1.5 meter tall. It has broad leaves and grows rapidly on disturbed soil which makes it difficult to control by farmers. The entire plant, from root to the shoot has been reported to be safe as traditional medicine except for the calyx (Azlan *et al.*, 2002). The chemical compound known as physalin, extracted from this plant is reported to have some anticancer activity (Shweta and Poonam, 2014).

Currently, some others *Physalis* such as *Physalis peruviana* have become major crop planted in California, South Africa, India, New Zealand, Australia and Great Britain but it is still considered as backyard fruits in some area (Muniz *et al.*, 2015). In Malaysia, *Physalis* are not cultivated but are considered as weeds and the numbers of species or ecotypes are relatively unknown. The genetic relationships and genetics distance to the other member of Solanaceae genera are also unknown. To determine the genetic relationships and genetic variations, molecular and morphological markers are often employed.

The Inter sequence simple repeats (ISSR) are the regions which consisted of mono, di-, tri or tetra- nucleotides repeats and located between the flanking regions of the microsatellites DNA (Gupta *et al.,* 1994). Variation occurs according to the size and sequence of ISSR resulting from mutation or slippage. The utilization of microsatellites as DNA markers are often the choice of researchers as it is co-dominant, can be used to assess across a wide genome of the same genus and highly reproducible (Reddy *et al.,* 2002). However, developments of specific primers are labourious and costly.

ISSR have the same advantages of microsatellites and primers produced multiple banding patterns. It is nearly identical to random amplified polymorphic DNA (RAPD) technique except that ISSR primers sequences are designed from microsatellites regions and the annealing temperature are higher than those used for RAPD markers (Zietkiewicz *et al.,* 1994). ISSR is simple and does not require previous knowledge of the sequence of the genome being tested. Thus, it is extremely useful data for estimating genetic diversity, phylogenetic analysis and the data can be used to study inter and intra specific relationship among plants.

Although DNA provides accurate genetic and inheritance information, morphological markers are as important in identifying and selection of plants with desirable traits. Often, molecular markers analysis is accompanied by morphological characteristics as it is important to observe the physical traits (Kaur *et al.,* 2016). Quantitative and qualitative traits of the vegetative and reproductive organs are the importance data to identify the distinct characteristics which are able to delimitate accession into clusters or most desirable traits are identified (Jesus *et al.,* 2013). However, when wild plants or weeds are collected for the intention of cultivation, organs which are destined as products with commercial value are considered as desired traits (Canter *et al.,* 2005). This often includes fruits, flowers or tubers. Leaf is considered as valuable when it contained secondary metabolite which is important in the pharmaceutical industries (Aqil *et al.,* 2006). In cases like these, the variations in their chemical content are of interest.

Phylogenetic is a study of evolutionary relationships of a group of organism which can be discovered through molecular sequences and morphological data matrices (Smaal *et al.,* 2004). Internal transcribed spacer region (ITS), chloroplast DNA and mitochondrial DNA are often employed to elucidate the phylogenetic relationships of a group of taxon (Soltis *et al.,* 2000; Olmstead *et al.,* 2008). Rate of mutation and mode of inheritance are the means that differentiated the both DNA marker in providing the best evolutionary history. Internal transcribed spacer regions (ITS 1 and ITS 2) are non-coding region of nuclear ribosomal DNA (nrDNA) that separates the 18S, 5.8S and 25S (White, 1990). The nucleotide sequence variation found in each of the ITS sequences were often best suited for comparing species and closely related genera (Alvarez and Wendel, 2003). The internal transcribed spacer region and intergenic spacer of the nuclear rRNA repeat units evolve fastest and may vary among species within a genus or among populations. Furthermore, primers are

universal and they are more suitable for comparison of closely related taxa (Hillis *et al.,* 1996).

Chloroplast DNA (cpDNA) is highly conserved region which recently used in phylogenetic field. The coding and non-coding regions of chloroplast DNA (cpDNA) are reported to be informative and useful to discriminate plant above the genus level (Shaw *et al.,* 2007). The regions included *trn*L, *ndh*F, *rbc*L and *mat*K which are successfully resolve the phylogenetic relationships of varieties of plant taxa. Mitochondrial DNA particularly *CO1* provide a very powerful marker in elucidating the phylogenetic relationships of animals, but work contrary in plant (Small *et al.,* 2004). This is due to the lack of mutation rate in mitochondrial DNA sequences as compared to the nuclear genome and chloroplast genome. DNA mutations occurred by base substitutions and insertion or deletion (indel), where these two characterisations are informative to infer phylogenetic relationships in plant taxon.

In the context of potential use of this species as an alternative production, the postharvest management should be taken as one of importance part to be studied. Despite the increasing demand for these berries, there is very little information about postharvest management including the storage of the fruit and also the phytochemical compound of the plant. *Physalis* is climacteric fruit where the productions of ethylene vary according to its maturity stages. Studies on post-harvest storage of *Physalis* fruits are very important in order to maintain the quality and extending the shelf life of the fruits. Fruit colour, ascorbic acid contents, soluble solids concentrations, fruit firmness, weight loss and titratable acidity are the major indicators of fruit quality.

Products including fruit are continuing the biochemical process including respiration and transpirations after detach from mother plant and deteriorations has commenced (Wills *et al.,* 2008). Fruit weight loss indicated the loss of water from cell due to transpiration process. As water loss, the ascorbic acid content will decreased because vitamin C is water soluble and it loss with water (Wills *et al.,* 2008). The breakage of carbohydrate into sugar (glucose, fructose and sucrose) will increase the soluble solids concentration of the fruit but the firmness of the fruit texture will decrease due to cell wall brake (Venkatesan and Tamilmani, 2014). Most of the fruits start to show the decreasing in quality after five percent water loss and the fruits are no longer in good quality for consumption (Kvikliene *et al.,* 2006).

Phytochemical compounds are secondary metabolites produced by plant to act as protector against several of microorganisms, insects and higher herbivorous predators' infections (Nathiya and Dorcus, 2012). The phytochemical compounds of plants have been used as therapeutic agent, new synthetic compound for drug formulations and as taxonomic markers for discovery of new compounds. Extraction of *Physalis minima* plants particularly from India and China has reported to have numerous of antioxidants and anticancer activities (Pietro *et al.,* 2000; Gandhiappan

and Regasamy, 2012; Shweta and Poonam, 2014). With this information, an attempt was made to study the different phytochemical compounds that presence in leaves, roots and fruits of *P. minima* L. from Malaysia.

This study was conducted to determine any differentiation among *P. minima* populations that had been collected from Peninsular Malaysia by looking at their genetic constituents. Two major approaches were used. Firstly, the level of genetic diversity among population was investigated by using morphological characteristics and molecular markers, ISSR. Secondly, the phylogenetic relationships between populations were inferred by using nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA). Population with desirable characteristics such as high fruit numbers was chosen to be planted. The fruit quality characteristics were determined by employed storage duration experiment and phytochemical analysis by using GC-MS.

1.2 Objectives

The objectives of this study were:

1)To identify genetic variation of *P. minima* accessions from Peninsular Malaysia using morphological and ISSR marker
2)To determine the phylogenetic relationships among populations of *P. minima* using chloroplast DNA (cpDNA) and inter transcribe spacer region (ITS)
3)To study the different phytochemical compounds present in the leaves, roots and fruits of the B – 02 accession
4)To determine the effects of storage duration on the physico-chemical changes of fruits of a superior accession assessed by morphological and genetic variation

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