ASSESSMENT OF GENETIC RELATEDNESS AMONG *Hydrilla verticillata* (L. F.) ROYLE ACCESSIONS IN MALAYSIA USING RAPD AND AFLP MARKERS.

BY

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ABSTRAK

Hubungan genetik antara 119 aksesi *Hydrilla verticillata* dari 28 lokasi di Malaysia ditentukan dengan menggunakan kaedah DNA Polimorfik Jalur Teramplifikasi (RAPD) dan 82 aksesi *H. verticillata* dari 26 lokasi dikaji melalui kaedah Polimorfisme Panjang Fragmen Teramplifikasi (AFLP). Sejumlah sembilan pencetus RAPD menghasilkan 143 jalur teramplifikasi dengan 105 daripadanya adalah jalur polimorfik. Data RAPD menghasilkan pohon Neighbour-Joining serta Analisis Koordinat Prinsipal (PCO) yang mengelompokkan aksesi-aksesi kajian ke dalam empat kumpulan yang tidak berteraskan kawasan geografi. Sejumlah 326 jalur polimorfik telah dijana melalui kaedah AFLP dengan menggunakan kombinasi dua pencetus. Pohon Neighbour-Joining yang terhasil dari data AFLP membahagikan aksesi ini ke dalam 4 kumpulan yang hampir mirip dengan kaedah RAPD. Namun analisis PCO pula berbeza daripada Neighbour-Joining dengan 3 kumpulan yang jelas dapat dikenalpasti. Keseluruhannya aras kepelbagaian genetic antara aksesi dalam satu-satu lokasi berbeza. Ini menunjukkan pentingnya pemahaman tentang pembiakan spesies ini yang berlaku di sesebuah lokasi.

ASSESSMENT OF GENETIC RELATEDNESS AMONG *Hydrilla verticillata* (L. F.) ROYLE ACCESSIONS IN MALAYSIA USING RAPD AND AFLP MARKERS.

ABSTARCT

Genetic relationships among 119 accessions of *Hydrilla verticillata* from 28 locations in Malaysia were determined using Random Amplified Polymorphic DNA (RAPD) and 82 accessions of *H. verticillata* from 26 locations were studied using the Amplified Fragment Length Polymorphism (AFLP) technique. A total of nine RAPD primers produced 143 amplified fragments with 105 of them were polymorphic. RAPD data used to generate a Neighbour-Joining tree and Principal Coordinate Analysis (PCO) clustered these accessions into four groups that did not separate them according to geographical areas. A total of 326 polymorphic bands were generated using the AFLP technique with two primers combination. The Neighbour-Joining tree produced from the AFLP data divided the accessions into four groups that closely resemble those from RAPD analysis. However, the PCO results is dissimilar with Neighbour-Joining in which only three groups were recognized. Overall the level of genetic variation among accessions within a location is lower as compared to between accessions from different locations. This shows that it is important to understand the reproduction of the species within a location.

CHAPTER ONE
INTRODUCTION

1.1 GENERAL INTRODUCTION

Hydrilla verticillata (L.f.) Royle is a submerged aquatic plant, macrophyte and identified as the perfect aquatic weed due to its extensive adaptation in a variety of shallow aquatic environments (Langeland, 1996). It can grow in various kinds of water such as lakes, irrigation canals, ponds, rivers and ditches. The plant has a major detrimental impact on water use. Most drainage and irrigation canals are gradually being blocked and subsequently it will impede the whole aquatic ecosystem. Particularly in the irrigated rice growing areas, the weed populations greatly reduced the overall efficiency of water supply and cause serious losses annually (Mansor & Azinuddin, 1991; Mansor, 1999). According to Hasmah (1999), *H. verticillata* is categorized as the most dominant and noxious submerged weed in Malaysia.

Many studies on the biology, ecology and physiology *of H. verticillata* have been carried out. However, study on the level and patterns of genetic variation in *H. verticillata* are few and focusing on specific areas or on global comparisons (Ryan *et al*., 1995; Madeira *et al*., 1997, 1999; Hofstra *et al*., 2000). The diverse distribution of plants and its route of spread in Malaysia are important for facilitating the development and implementation of appropriate management after weed. Up until now, there is no genetic diversity study on *H. verticillata* in Malaysia have been conducted. Population studies using molecular markers are needed to collect information on the level and structure of genetic diversity.

Initially, morphological observations and measurements are the easiest and the only available ways to assess biodiversity. However, morphological traits may be influenced by environmental conditions and growth practices (Khan *et al*., 2000). Direct assessment of the DNA-based data to the genetic variation will be more consistent and accurate.

One of the earliest PCR-based DNA fingerprinting techniques is Random Amplified Polymorphic DNA, RAPD (Kresovich *et al*., 1992, Stiles *et al*., 1993, Yang & Quiros, 1993, Koller *et al*., 1993, Comincini *et al*., 1996). RAPD analysis has been widely used to describe population structure and genetic polymorphism in many plant species, such as *Theobroma*

cacao L. (Russell *et al*., 1993), *Vaccinium macrocarpon* (Stewart & Excoffier, 1996), *Thalassia testudinum* (Kirsten *et al*., 1998), *Halodule wrightii* (Angel, 2002), *Aldrovanda vesiculosa* (Martin *et al*., 2003), *Salvinia minima* (Madeira *et al*., 2003), *Cocos nucifera* L. (Upadhyay *et al*., 2004), *Potamogeton lucens* (Uehara *et al*., 2006), *Prunus africana* (Muchugi *et al*., 2006) and *Sinocalycanthus chinensis* (Li & Jin, 2006). To complement RAPD analysis, another molecular marker namely AFLP (Amplified Fragment Length Polymorphism) was employed to study the genetic diversity of *H. verticillata* in Peninsular Malaysia.

Population structure was assessed using statistical methods specifically designed to overcome the shortcomings inherent in dominant marker data such as RAPD and AFLP. Therefore, the RAPD and AFLP dataset were subjected to analyses to check on genetic relationship – similarity and dissimilarity was calculated using variety of algorithm and then used as a starting point for statistical procedures such as clustering analysis and principal coordinate analysis.

The objectives of this research were:

- To evaluate the level and patterns of genetic divergence of *Hydrilla verticillata* among the accessions in Malaysia, in order to have a better understanding of the distribution pattern.
- To compare the differences arising from separate analyses from these two molecular markers and the impact on the ordination of populations relatedness.

CHAPTER TWO
LITERATURE REVIEW

2.1 PLANT MATERIALS – *Hydrilla verticillata* (L. f) Royle

Hydrilla verticillata is an invasive, submerged aquatic weed that is native to the warmer areas of Asia. It is mostly perennial but sometimes annual (Cook, 1996). *Hydrilla verticillata* has several physiological, morphological and reproduction characteristics that allow this plant to be well-adapted to live in diverse terrestrial freshwater environments. It forms dense stands from the bottom to the top of the water, sprawling across the surface and spreading rapidly. Their abundant growth causes economic hardship once it has infested an area (Langeland, 1996). *Hydrilla verticillata* is highly polymorphic and its appearance can vary substantially. This could be explained by the fact that its geographical range is extremely wide and might imply differences in survival strategy (Verkleij *et al*., 1983).

2.1.1 Taxonomy and Identification

Hydrilla verticillata grows rooted to the bottom, against submerged obstacles, such as fallen trees (Hofstra *et al*., 1999), and sometimes can be found as detached floating mats. They have adventitious roots which are usually glossy white. When the plant grows in deep water, stem elongate sparsely until the plant grows near to the water surface and then branching become profuse. *Hydrilla verticillata* spreads via underground stems (rhizomes) and aboveground stems (stolons). It also forms hibernacula, turions in leaf axils and tubers (subterranean turions) terminally on rhizomes. Turions are compact buds produced in the leaf axils and they break off the parent plant when mature. They are 5 – 8 mm long, dark green and spiny. Tubers are underground turions, which form at the end of rhizomes and can be found 30 cm deep in the sediment. They are 5 – 10 mm long and are white or yellowish (Langeland, 1996; Cook, 1996).

The leaves of *Hydrilla verticillata* are small, 2 – 4 mm wide, 6 – 20 mm long and occur in whorls of 3 – 8 (Figure 2.1). They generally have 11- 39 sharp teeth per cm along the leaf margin. They also often have spines or glands along the lower midrib of the leaf and are often

reddish in colour. *Hydrilla verticillata* can be monoecious (both male and female flowers on the same plant) (Figure 2.2) or dioecious (male and female flowers on different plants) (Figure 2.3). Each male and female has its own unique growth characteristics. Male flowers have three whitish red or brown sepals that are 3 mm long by 2 mm wide, and three whitish or reddish linear petals about 2 mm long. They have three stamens, which forms in the leaf axils until they break loose, at maturity and float to the surface where they free-float. Female flowers consist of three translucent petals 10 – 50 mm long by 4 – 8 mm wide and three whitish sepals. They grow attached to the leaf axils and float on the water surface (Langeland, 1996).

The list below shows the taxonomic classification of *Hydrilla verticillata* (L.f.) Royle (Cook, 1996):

Kingdom	:	Plantae (Plants)
Subkingdom	:	Tracheobionta (Vascular plants)
Superdivision	:	Spermatophyta (Seed plants)
Division	:	Magnoliophyta (Flowering plants)
Class	:	Liliopsida (Monocotyledons)
Subclass	:	Alismatidae
Order	:	Hydrocharitales
Family	:	Hydrocharitaceae (Tape grass family)
Genus	:	*Hydrilla*
Species :	*Hydrilla verticillata* (L. f.) Royle	

Hydrilla verticillata closely resembles other members of its family such as *Elodea canadensis* and *Egeria densa*. In 1995, Bowmer *et al*. distinguished *H. verticillata* from *E. canadensis* by the number of leaves in the whorls, usually three in *Elodea* and four to six in *Hydrilla*. While *E. densa* is distinguished by its larger leaves which is usually in whorls of four or five. *Hydrilla verticillata* has been called Florida elodea, water thyme, lelumut (Leones *et al*., 1991) and ‘same’ tree (Mansor, 1994).

2.1.2 Geographical Distribution and Origin

Hydrilla verticillata is reported to be a native of Southeast Asia and Australia by Madsen and Owens in 1998 and apparently originated from Central Africa (Leones *et al*., 1991). This was later confirmed by Cronk and Fuller in 2001 that the regions of origin for *H. verticillata* are Australasia (Northeast Australia), Africa (East Africa), East Asia and Southeast Asia. It later invaded North America, Central America (Panama), Pacific (Fiji), Australasia (New Zealand), the greater portion of India, China and Europe (Cronk & Fuller, 2001; Allen, 1976).

Although the weed is cosmopolitan in distribution, it occurs more abundantly in the tropics (annual temperature more than 24 ºC) and sub-tropics (annual temperature between 18 to 24 ºC) (Varghese & Singh, 1976). Malaysia lies at the latitude 0º 60' to 6º 40'N and longitude 99º 35' to 119º 25'E. The country enjoys a warm and sunny equatorial climate (approximately 30 ºC) (Mansor, 1996). So, it is not surprising that *Hydrilla* can be found in most parts of the country (Varghese & Singh, 1976).

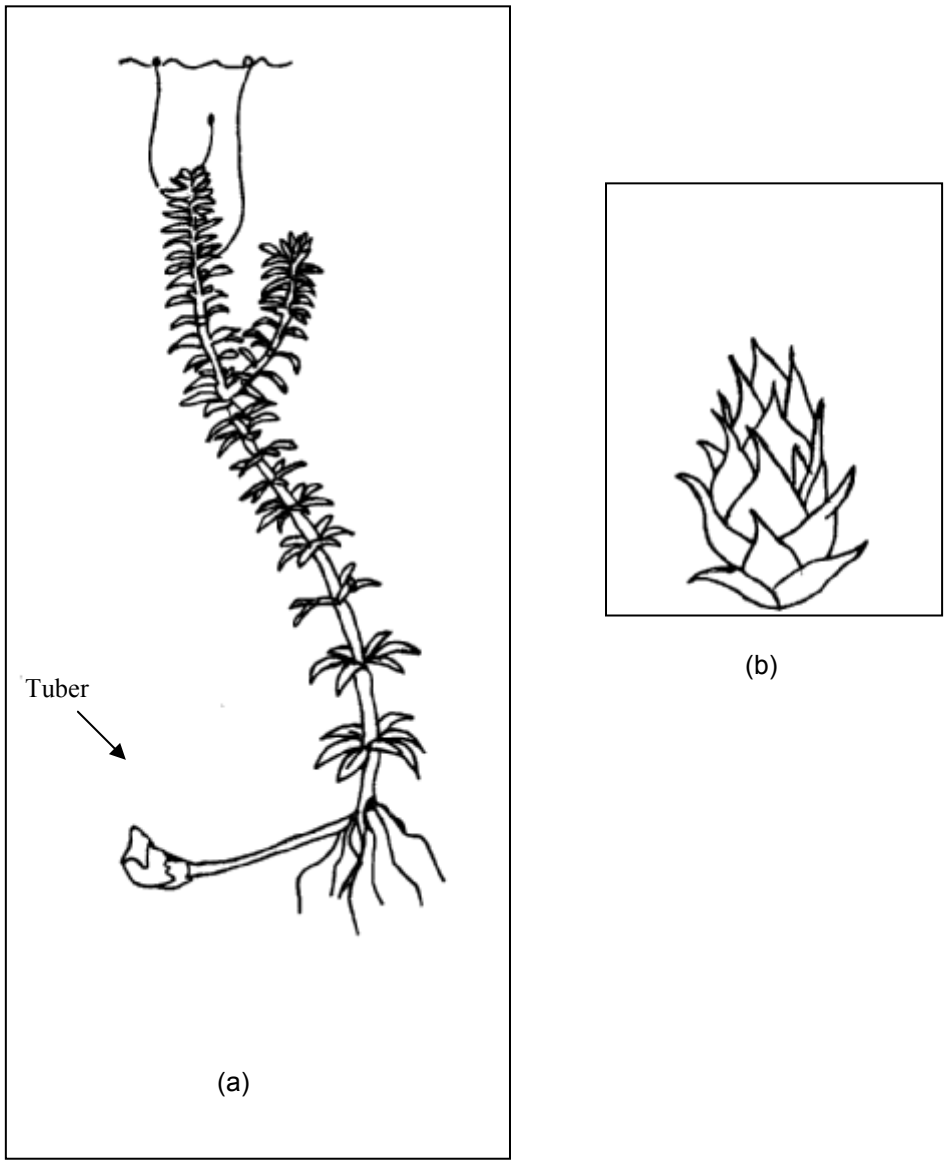

Figure 2.1 The morphology of *H. verticillata,* a) With tuber at the edge of rhizomes b) Turions broken off from the parent plant. (Aquatic, Wetland and Invasive Plant Particulars and Photographs, 2005)

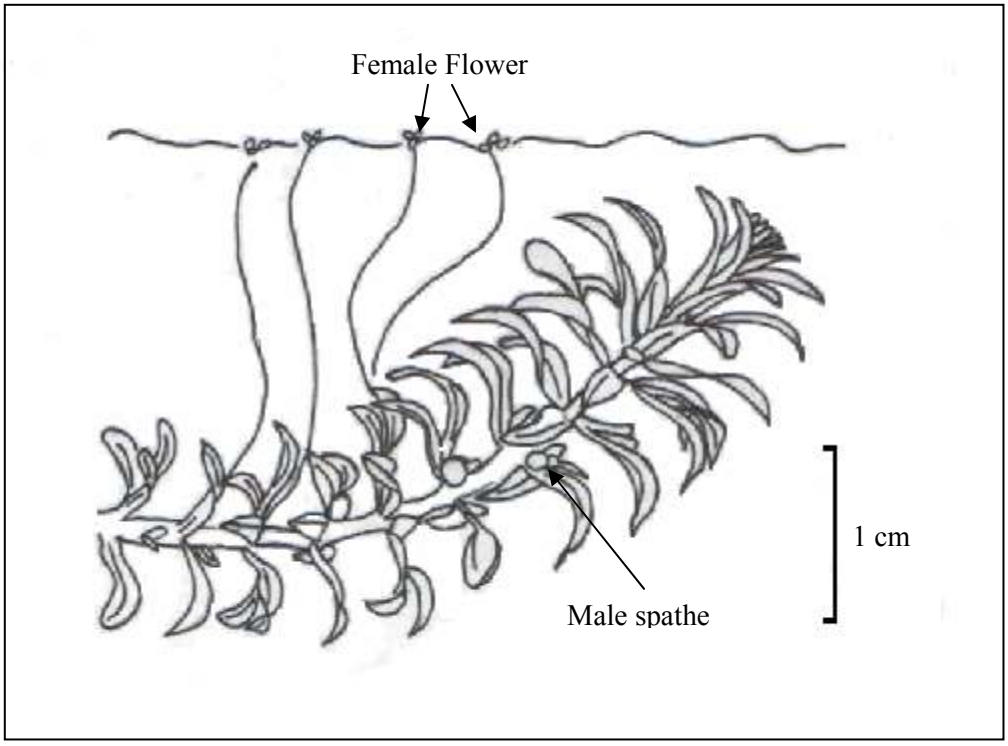

Figure 2.2 Monoecious *H. verticillata* with both male spathe and female flower on the same plant. (Natural Resources, Environment and the Arts, 2006)

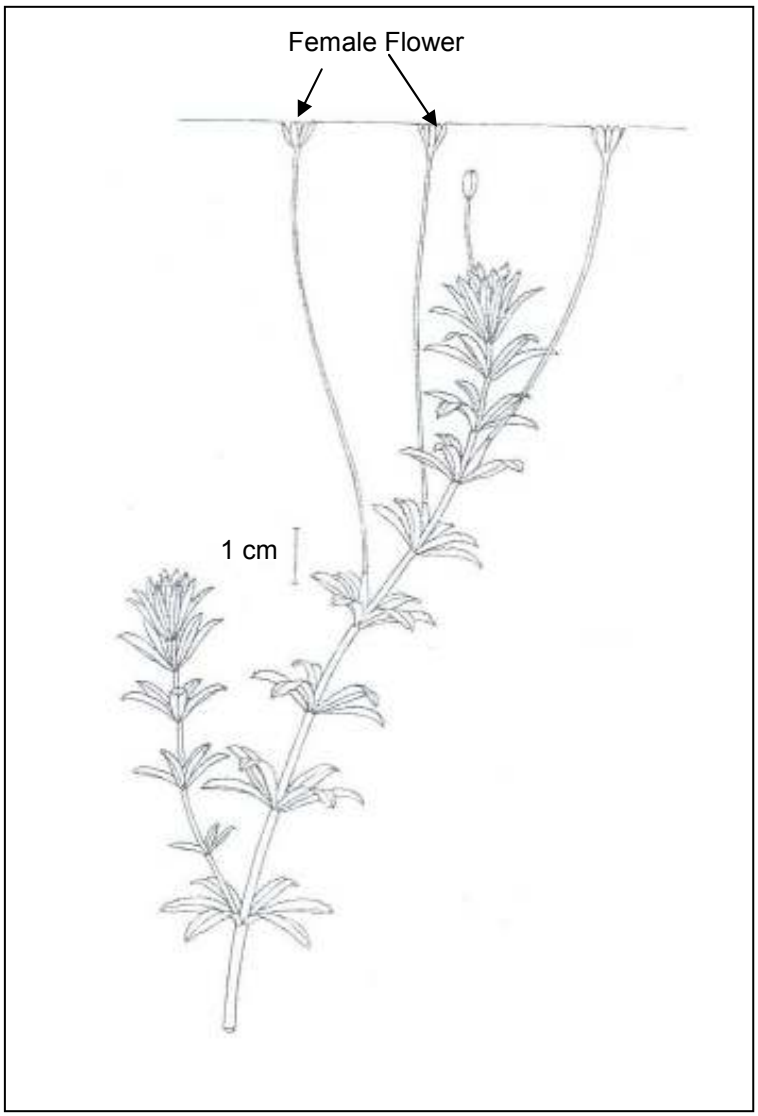

Figure 2.3 Dioecious *H. verticillata* with female flowering shoot. (Aquatic and Wetland Plants of India, 1996)

2.1.3 Biology and Physiology

Hydrilla verticillata has several physiological and morphological adaptations which allow it to outcompete other aquatic plants. The ability of *H. verticillata* to grow under direct sunlight at the water surface, as well as under canopy light, may be the factor that leads to its success. A tolerance of low light conditions may enable *H. verticillata* to colonize deeper water than other species (Hofstra *et al*., 1999), and this capability also enables *H. verticillata* to start photosynthesis earlier in the morning. Comparison among three aquatic plant species (*H. verticillata*, *Vallisneria spiralis* L. and *Potamogeton pectinatus* L.) proved that *H. verticillata* showed minimum dark and photorespiration and maximum apparent photosynthesis with vigorous growth (Jana and Choudhuri, 1979).

The availability of inorganic carbon for photosynthesis differs considerably in water and air. This is because of slower diffusion rate of carbon dioxide in water than air (Casati *et al*., 2000). So, aquatic autotrophs have to develop various ways to cope with limited carbon dioxide and as well as high oxygen source (Magnin *et al*., 1997). *Hydrilla verticillata* is able to switch from the C_3 type mechanism to the C_4 type carbon metabolism (fixing inorganic carbon into malate and asparate) when the percentage of carbon dioxide is low (Rao *et al*., 2002).

According to Kahara and Vermaat (2003), *H. verticillata* can tolerate a wide range of pH with the maximal pH tolerated being approximately pH 10.5. It can tolerate up to 7% salinity of seawater and survive under limited nutrients such as carbon, nitrogen and phosphorus (Langeland, 1996). *Hydrilla verticillata* can also store extra phosphorus for later use if conditions are unfavorable. In short, the plant can survive in a wide range of environmental regimes, whether in oligotropic (low nutrient) or eutropic (high nutrient) conditions.

Hydrilla verticillata has many effective means of propagation. There are four ways (fragmentation, tubers, turions, seed production) of reproduction. *Hydrilla verticillata* reproduces mainly by fragmentations. It can sprout new plants from stem fragments containing as few as two nodes or whorls of leaves. Under unfavorable conditions, the plants propagate itself asexually through stolons, tubers and turions (Leones *et al*., 1991). *Hydrilla verticillata* is difficult to control because it produces axillary turions and tubers for regrowth after control of

the parent plants. Turions of monoecious *H. verticillata* can survive for 1 year under tank conditions, but tubers can survive for a period of over four years (Sutton, 1996). The sexual reproduction appears to be less important as compared to vegetative propagation. *Hydrilla verticillata* is probably diploid (2n = 2x = 16) and sometimes endopolyploid, with triploid (2n = 3x = 24) and tetraploid (2n = 4x = 32) cells observed in the same populations (Langeland *et al*., 1992).

2.1.4 Importance

As a problematic plant, *H. verticillata* always causes economic hardship wherever it occurs. Examples in drainage and irrigation canals, the weed reduces the water flow and results in higher water levels, greater loss of water by evaporation and inadequate supply of irrigation water to rice fields (Mansor and Azinuddin, 1991). In addition, *H. verticillata* also interferes with boating and navigation through the canals for both recreational and commercial purposes. While the control of the weed will cost up to millions of ringgit, clearing task may also alter the water quality and dissolved oxygen level. Although the changes of water chemistry is not too destructive, but the effect on the habitat is quite pronounced (Mansor and Azinuddin, 1991).

Conversely, coupled with all the negative impact, *H. verticillata* also play an important role in the fish populations. Ali (1988) suggested that, besides competing for nutrients and blocking sunlight for photosynthesis, *H. verticillata* also able to inhibit zooplankton production. However, these weeds also modify the predator-prey relationship among various zooplankton groups as well as between zooplankton and fish (fish larvae, frys and adult fish) by providing refuges for prey species to hide from predation. So the greater density of zooplankton observed as weed biomass increased. The impact of this factor on fish population is quite significant and extremely important to the success of fish population. This is because the year class strength of fish population depends entirely on the initial survival of fish larvae and frys.

2.1.5 Management

There are a few control methods for *H. verticillata*, different type of water bodies have to be treated differently and it depends on the end results required. Management or controlling methods available are mechanical control, chemical control and biological control. In some cases, physical control (manual removal) is favorable, but by using this method the weed is never eradicated completely because it involves cutting only the top parts of the plants and not the underground roots and buds.

There are harvester machines specially designed for mechanically removing *H. verticillata*. Although this method is effective but the cost of the machine is high and can only be used in large water bodies.

For chemical control, there are four commonly used active ingredients namely fluridone, endothall, copper and diquat (Langeland, 1996). Different chemicals or combinations of chemicals can be used to control *Hydrilla verticillata* at different levels. Fluridone significantly inhibits the growth of *H. verticillata*, but the disadvantages using fluridone include its high cost, slow-action, and non-selectivity toward other macrophyte species (Fox *et al*., 1996; Hofstra and Clayton, 2001).

Endothall, a fast-acting contact herbicide, is used when immediate control of vegetation is needed. Copper is used for its algicidal properties when heavy periphytic growth on the *H. verticillata* may interfere with herbicide intake. Usually, to increase the efficiency of the management system, dipotassium salt of endothall was often used in combination with diquat (6,7-dihydrodipyrido (1,2-a : 2', 1'-c) pyrazinediium dibromide, chelated copper or the mono(N, Ndimethylalkalamine) salt of endothall. Pennington *et al*. (2001) proved that herbicides applied in combination, specifically dipotassium salt of endothall/copper and dipotassium salt of endothall/diquat, can provide excellent control of *Hydrilla* using lower application rates. Apart from the four chemicals named, acetic acid has proven to be effective in reducing survival of monoecious *Hydrilla verticillata* tubers (Spencer and Ksander, 1999). Bensulfuron methyl also greatly inhibits the tuber formation of *H. verticillata* (Bowmer *et al*., 1995).

Biological control is a technique that utilizes insects, microbial pathogens, or the combination of them to manage the growth of *H. verticillata*. *Hydrellia pakistanae* is one of the widely established biological control agent for *H. verticillata* (Wheeler and Center, 2001), and its establishment is sufficient to impact the long-term growth of *Hydrilla* (Doyle *et al*., 2002). Other potential biocontrol agents are *H. verticillata* leaf-mining fly, (*Hydrellia balciunasi* Bock) and the *H. verticillata* stem boring weevil (*Bagous hydrilla* O'Brien) (Bowmer *et al*., 1995). *Paraponyx diminutalis* (Lepidoptera: Pyralidae), an aquatic moth may also cause some damage on *H. verticillata* but does not result in full control (Cronk and Fuller, 2001). Combination of chemical fluridone with fungal pathogen *(Mycoleptodiscus terrestris*) greatly enhanced control, reduced exposure requirement and increased the susceptibility of *H. verticillata* to fluridone (Netherland and Shearer, 1996).

In 1998, Shearer once again tested the efficacy of *Mycoleptodiscus terrestris* against dioecious *H. verticillata* and successfully found a biocarrier for this fungus. Another promising biocontrol is triploid grass carp (*Ctenopharyngodon idella* Valenciennes). Killcore *et al*. (1998) proved that grass carp effectively controlled *Hydrilla* but did not create any detectable negative effects on the littoral fish assemblage. In search of the natural enemies of *H. verticillata,* two insects were identified, *Aphis* sp. and *Nymphula* sp. Malaysia is believed to be the natural habitat for this two insects (Varghese and Singh, 1976).

2.2 DNA MARKERS IN POPULATION GENETIC STUDY

2.2.1 Molecular Markers

In recent years, classical methods to evaluate genetic variation have been complemented by molecular techniques. Allozyme markers were the first molecular markers that were used for studying genetic variation within a number of species (Hoey *et al*., 1996; Sonnante *et al*., 1997). Generally, allozyme markers which are co-dominant reveal polymorphism at the protein level ie. heterozygotes can be distinguished from the homozygotes. But allozyme only detects variations in protein coding loci, so only a portion of variation are estimated and this may not

reveal the true situation for the studied individual or population. In addition, the development stage of a plant is one of the critical factors that are accounted for in this analysis.

Direct assessment at the DNA level is more sensitive and reliable. There are a whole range of different DNA-based markers, that can be categorized into three aspects:

a. Non-PCR based approaches – Restriction fragment length polymorphism (RFLP), Minisatellites, Variable Numbers of Tandem Repeats (VNTRs).
b. PCR based analysis – Microsatellites, RAPD, Inter-Simple-Sequence-Repeats (ISSR), Inter-Retrotransposon Amplified Polymorphism (IRAP), AFLP.
c. DNA-sequence polymorphism – Single Nucleotide Polymorphism (SNP), DNA-sequencing.

2.2.2 Random Amplified Polymorphic DNA

Random Amplified Polymorphic DNA (RAPD) developed by Williams *et al*. in 1990, is one of the earliest DNA fingerprinting techniques that makes use of PCR. It is also known as arbitrarily primed PCR. It gives a very quick and less expensive way to screen genetic diversity in germplasms. RAPD became very popular because of its simplicity and wide applicability. It is based on the amplification of random DNA segments using single primers of arbitrary nucleotide sequence. Primers with ten nucleotides and a GC content of at least 50% are generally used. Primers with lower GC content usually do not yield amplification products. Because a G-C bond consists of three hydrogen brands and the A-T bond of only two, a primer-DNA hybrid with less than 50% GC will probably not withstand the temperature at which polymerization takes place (72^{o}C). Therefore, the DNA-primer hybrid will have melted before the polymerase has started polymerization. The amplification products are separated on an agarose gel and detected by staining with ethidium bromide.

The enormous attraction of RAPD is due to several factors:

i. Only 25 nanogram of total genomic DNA are required as a starting material for the analysis. This is particularly a good option if limited materials are available, for example herbarium .

ii. Since the nucleotide order within the 10-mer primers is arbitrary, no preliminary work, such as isolation of cloned DNA probes, preparation of filters for hybridizations or prior knowledge of DNA sequences is needed. A large number of potential markers can be generated using commercially available primers (Mailer *et al*., 1994).

iii. RAPD assay are relatively quick, simple and less expensive. Only a thermocycling machine and agarose gel apparatus are required in a laboratory. It is a method of choice for analyses that requires bulking of genomic DNA or less advanced laboratories (Paran *et al*., 1991; Upadhyay *et al*., 2004).

However, there are several difficulties and limitations with the approach:

i. The first concern is the nature of the data generated. RAPD are dominant markers, the absence of a band represents the homozygous recessive, but the presence of band cannot be differentiated between homozygous dominant or heterozygotes. Therefore, in heterozygotes, the differences may appear only as differences in band intensity, which is usually not reliable.

ii. The assumption that co-migrating bands represent homologous loci is commonly made. A research by Rieseberg (1996) found out that 90% of the bands that co-migrated on the same length in the agarose gel were truly homologous when further examined with Southern hybridization or restriction digest. To achieve the same degree of statistical power, on the order of 2 to 10 times more individuals need to be sampled per locus when dominant markers

are relied upon, as compared to co-dominant markers (Lynch and Milligan, 1994).

iii. Another general problem of RAPD is concerning the robustness of the data generated. There are many factors that may influence its reproducibility, such as size of primer, annealing temperature, template DNA quality and thermal cycler used. Ramser *et al*. (1996) found that the main critical factor for reproducibility turned out to be the quality of template DNA. According to Skroch and Nienhuis (1995), reproducibility expressed as the percentage of RAPD bands scored that are also scored in replicate data, was 76%. Although statistical and physical differences may exist, they were not significant enough to result in differences in genetic distance estimates greater than expected by chance. After all, careful standardization and optimization in identical amplification profiles proved to be reliable and highly reproducible (Heun and Helentjaris, 1993; Lacerda *et al*., 2001; Shrestha *et al*., 2002).

The resolution of RAPD offers several potential applications in biological systems:

i. Assessment of genetic variation in populations and species. Similarity values were generated and subjected in cluster analysis and analysis of molecular variation (AMOVA). For example, Madeira *et al*. (2003) used RAPD analysis to detect differences among geographically referenced samples of *Salvinia minima* within and outside of Florida. The results showed that there is evidence from PCO and AMOVA analysis that all the sampled populations can somewhat group together according to chronologically infestation.

ii. To study relationships among species and subspecies. The determination of taxonomic relatedness is only valid between taxa for which the diagnostic RAPD fingerprint patterns have been established. Similarly, fragments polymorphic at the species level will operationally identify members of a given

species if the fragment is constant among all members of that species (Hadrys *et al*., 1992). Khan *et al*. (2000) reported that it is possible to differentiate very closely related subspecies such as *Gossypium herbaceum* and *Gossypium herbaceum* Africanum by using RAPD. Furthermore, most of the species can be individually characterized with species-specific RAPD markers and these results suggest that the RAPD technique produces reliable results with which to construct the phylogenetic history of the genus *Gossypium*. Similarly, Ontivero *et al*. (2000) showed that RAPD analysis provide useful data to recognize wild strawberries and its related species in northwestern Argentina.

iii. To construct and understand genetic linkage maps, gene tagging, and identification of cultivars. According to Ranade *et al*. (2001), gene tagging using RAPD markers has at least three major advantages over other methods. A universal set of primers can be used and screened in a short period, isolation of cloned DNA probes or preparation of hybridization filters is not required and only small quantity of genomic DNA is needed for each analysis. Gene tagging and marker-assisted selection is an essential component of molecular breeding and is based on saturation mapping of the genomes.

iv. Assignment of parentage. For example, Besse et al. (2004) used RAPD marker to analyze the intergeneric hybrid of the genus Vanila Swartz. Morphologically, *Vanilla tahitensis* is very similar to *V. planifolia*, but it resembles *V. pompona* in its aromatic characteristics and the absece of fruit dehiscence at maturity. Therefore, it was suggested that *V. tahitensis* could be the result of a hybridization event between *V. planifolia* and *V. pompona*. This hypothesis was confirmed to be wrong when RAPD results revealed that *V. tahitensis* is probably not a species of direct hybrid origin (*V. planifolia* x *V. pompona*) but rather a species

2.2.3 Amplified Fragment Length Polymorphism

In 1995, Keygene Inc. developed a novel DNA fingerprinting technique termed Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995). This procedure is essentially a combination of RFLP and RAPD. It is based on the detection of genomic restriction fragments and followed by PCR amplification. The technique involves three steps: (1) restriction of the genomic DNA and ligation of oligonucleotide adaptors (2) selective amplification of a subset of all the fragments in the total digest and (3) gel-based analysis of the amplified fragments.

The first step requires genomic DNA to be digested by two restriction endonucleases, one a rare cutter and the other a frequent cutter. A frequent cutter will generate small sizes of DNA fragments, which are excellent for amplification and the optimal size range for separation on denaturing gel. While a rare cutter is to reduce the number of DNA fragments to be amplified. Too many fragments for resolution by gel electrophoresis will create indistinguishable bands. Double stranded adaptors are then added to the ends of the digested DNA fragments to provide known sequences for PCR amplification.

Although the numbers of fragments have been reduced, there are thousands of adapted fragments, depending on the genome size. The number of fragments obtained increased as the genome size increased. A stricter selection is required to achieve the ideal number of fragments within the range that can be resolved on a single gel. Thus, primers are designed to incorporate the known adaptor sequence plus one to three arbitrarily chosen base pairs. Fewer fragments will be amplified with more additional nucleotides. Normally PCR will be carried out twice. The first time, pre-selective; only one selective nucleotide is used. The second time, selective PCR, the same selective nucleotide plus one or two additional ones are used.

There is a variety of techniques that can be used to visualize the amplified products. Ranging from the simplest way i.e. agarose gel electrophoresis to automated genotyping. Polyacrylamide gel electrophoresis (manual or automated) is sufficient to separate the fragments even to a single base pair difference. An automation of AFLP genotyping that allows the use of

an internal lane size standard will ensure superior sizing accuracy with no lane-to-lane or gel-to-gel variation.

Two different types of polymorphism can be detected with AFLP: (1) point mutation in the restriction sites, or in the selective nucleotides of the primers which results in a signal in one case and absence of a band in the other, and (2) small insertion or deletions within the restriction fragment which results in different sized bands.

AFLP markers offer the following advantages:

i. It markers can be applied to any organism and no prior knowledge about the genomic makeup of the organism needed.
ii. It can be generated at great speed.
iii. AFLP analysis requires minimal amounts of DNA and is insensitive to the template DNA concentration. Therefore, DNA quantities that vary between individual samples will not affect the end results of AFLP analysis (Vos *et al*., 1995).
iv. The AFLP method is less problematic than RAPD technique, AFLP amplification is performed under conditions of high stringency, thus eliminating nonspecific binding and artefactual variation (Parsons and Shaw, 2001).
v. This technique is highly reproducible and provides nearly unlimited number of informative markers that spread over the entire nuclear genome. As it is, AFLP markers will be located in variable regions and even low genetic variability can be detected within any given group of organism (Hodkinson *et al*., 2000; Hedren *et al*., 2001; Després *et al*., 2003).

The high reliability of AFLP and unparalleled sensitivity to minor genetic differences make them valuable in diversity studies. AFLP markers have been proved useful for: (1) systematics and biodiversity surveys, (2) population and conservation genetics, (3) kinship and parentage analysis and (4) QTL mapping (Quantitative Trait Loci).

2.2.4 The reasons for using RAPD and AFLP

Genetic diversity in *H. verticillata* accession from most part of the world has been studied using isoenzyme markers (Madeira *et al*., 1997; Madeira *et al*., 1999; Hofstra *et al*., 2000). However, isoenzymes are less sensitive to genomic changes and it is limited by the functional enzymes in the coding region which is a relatively small number of loci (Angel, 2002).

By the year 1997, Madeira *et al*. have successfully studied the relationships among 44 accessions of *H. verticillata* from places around the world using RAPD. From their experiments, it was proven that RAPD is a useful tool for examining the inter-relationships of *Hydrilla* accessions. In the year 2000, isozyme together with RAPD was used to assess the genetic structure within and between four populations of *Hydrilla* in New Zealand. The RAPD results were congruent with the isozyme results. It shows that there was no variation within and between samples from four different populations in New Zealand and this is because the genetic diversity among populations is very low (Hofstra *et al*., 2000).

In the present study, RAPD technique was employed, as it has been successfully utilized in *H. verticillata*. In addition AFLP technique was also used since it has been suggested to perform well for plants that showed low genetic variability using RAPD. AFLP is used to complement the RAPD technique. Both RAPD and AFLP have advantages and disadvantages for assessing genetic diversity, and many studies have demonstrated the high potential of the two markers for population studies (Madeira *et al*., 1997; Kristen *et al*., 1998; Madeira *et al*., 1999; Hofstra, 2000; Angel, 2002; Madeira *et al*., 2003; Martin *et al*., 2003; Li and Jin, 2006). It is hoped that the use of these two markers will provide a better resolution of genetic diversity of *H. verticillata* in Malaysia.

CHAPTER THREE
MATERIALS & METHODS

3.1 PLANT SAMPLES

3.1.1 Sample Collection

Hydrilla verticillata was collected from all types of water bodies in Malaysia. Individual rooted shoots, totaling three to ten, were collected randomly and removed carefully from the substrate. After collection, samples were kept in plastic bags with its native water before transferring to the laboratory. The epiphytes were washed with water and blotted on paper towel. Samples were then placed into zipper bags containing silica gel desiccant. The desiccant was changed regularly to make sure the samples were totally dried. They were then kept in the freezer at temperature of –20 ˚C until removed for DNA extraction.

3.1.2 Sampling Sites

A total of 125 accessions of *H. verticillata* were collected from 31 locations around Peninsular Malaysia and Sarawak (Figure 3.1). Only 28 locations (1-8 accessions per location) were subjected for RAPD analysis and 26 locations (1-8 accessions per location) were subjected for AFLP analysis. Samplings have been done for two different period of time for each analysis. No sample were collected for the second time of sampling for a few locations (Sungai Tarum-Y, Sungai Gopeng-T, Jemaluang-J, Parit Raja-M and Alor Lintang-A). *Hydrilla* verticillata plants were discovered and collected in three new sampling sites namely Merapoh (AH), Sungai Sarawak (O) and Batu Lima (P) for AFLP analysis.

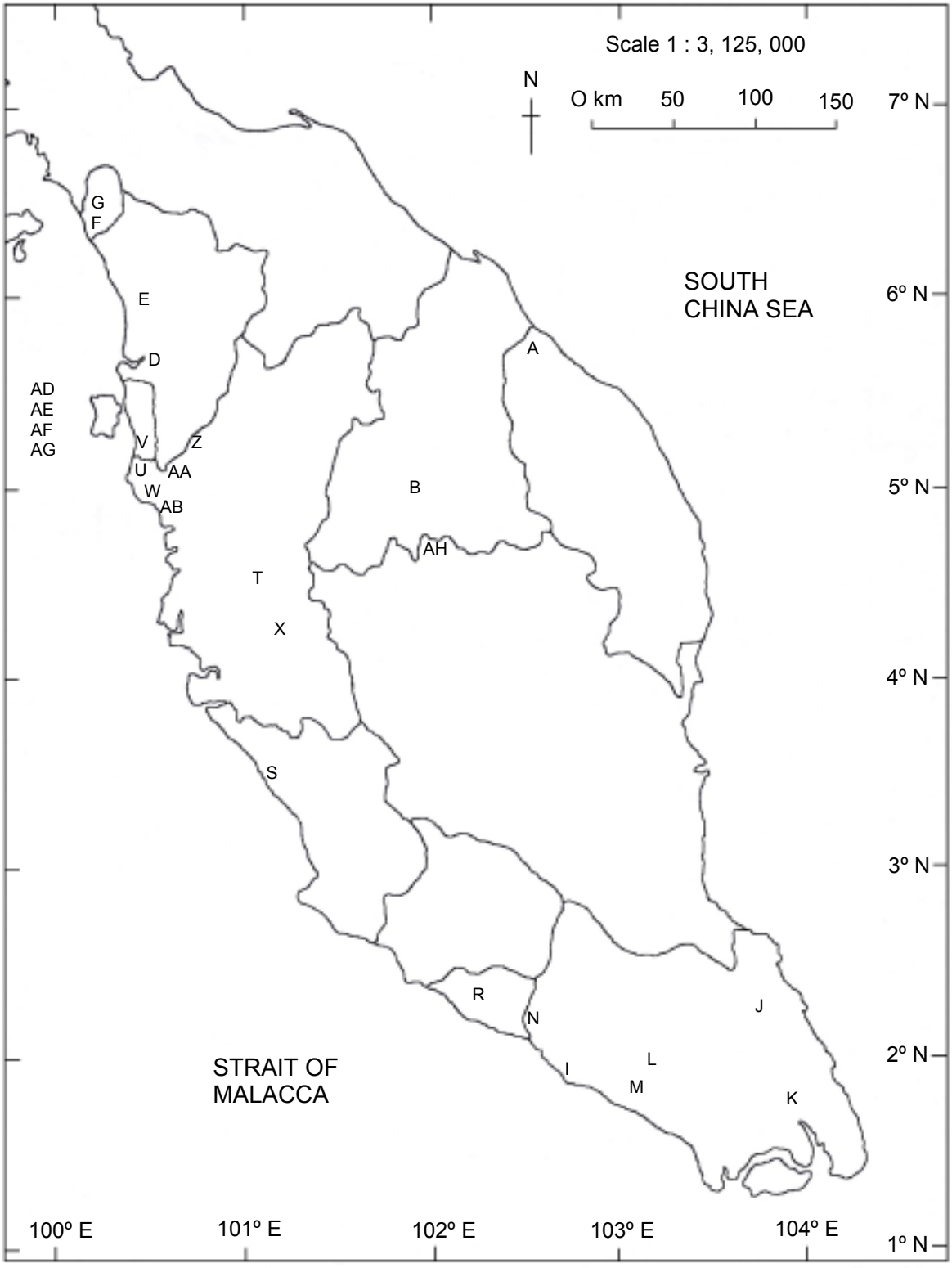

Figure 3.1 The 28 sampling sites of *Hydrilla verticillata* around Peninsular Malaysia. Three sampling sites from Sarawak (Bintawa-Q, Sungai Sarawak-O and Batu Lima-P) were not shown in the map. Code and number assigned in the map is described in Table 3.1 and Table 3.3.

3.1.3 DNA Extraction

DNA was extracted using the CTAB (cetyltrimethylammonium bromide) standard extraction procedure as described by Doyle and Doyle (1987) with minor modifications. 0.2 – 0.4g of silica gel-dried leaf sample was quickly frozen in liquid nitrogen and ground to fine powder using a mortar and pestle. 4 ml of preheated (55˚C) 2X CTAB extraction buffer (1.4M NaCl, 20mM EDTA at pH 8.0, 2% CTAB, 1% PVP, 0.1M Tris at pH 8.0 and 1 % β-mercaptoethonol) was added to the fine powder and transferred to a 10 ml clean tube. The suspension was incubated at 55˚C water bath for 30 minutes with occasional shaking to produce an homogenous solution.

The tube was cooled 5 minutes at room temperature. The mixture was emulsified by adding 2 ml of chloroform: isoamyl alcohol (24:1, v/v). Tube was spun at 4000 rpm for 10 minutes. The top aqueous layer was transferred to a clean tube then 2 ml of chloroform: isoamyl alcohol (24:1, v/v) and one ten volume of 10% CTAB was added into the supernatant. The tube was shaken vigorously before centrifuged at 4000 rpm for 10 minutes. Only 600 µl of the upper layer were transferred to each of four new 1.5 ml microcentrifuge tubes which contained two third volumes of cold isopropanol. The mixture was mixed by several inversions of the tubes.

Precipitated DNA can be observed at the bottom of the tubes as a transparent pellet after centrifugation at 10 000 rpm for 30 minutes. 600 µl of 76% ethanol/0.2M NaAc was added to the DNA pellet and it was left to stand at room temperature for 50 minutes. The supernatant was discarded and 300 µl of 76% ethanol/10 mM NH4Ac was added to wash the DNA pellet. Ethanol was poured out and the DNA pellet was left to air dry by inverting the tubes on a paper towel. DNA was dissolved in 50 µl sterile distilled water at 4˚C overnight.

3.1.4 DNA Repurification

Extracted DNA was purified with ammonium acetate. 50 µl aliquot of the same DNA sample from four tubes were combined into a single 1.5 ml microcentrifuge tube resulting in a total volume of 200 µl. 100 µl of cold 7.5M NH4Ac was later added to the tube. The tube was incubated at -4˚C for 15-20 minutes and centrifuged at 10 000 rpm for 15 minutes. The supernatant was removed using a sterile pipette tip and transferred into a new 1.5 ml microcentrifuge tube. DNA was precipitated using 2 volume of cold 95% ethanol, kept in -4˚C for 30 minutes and centrifuged again at 10 000 rpm for 15 minutes. The resulting pellet was washed with 350 µl cold 70% ethanol and centrifuged at 10 000 rpm for 15 minutes. The pelleted DNA was air dried and resuspened in 100 µl sterile distilled water at 4˚C overnight.

3.1.5 Quantification of DNA Samples

DNA yield and quality was assessed by gel electrophoresis and measured spectrophotometrically (Eppendorf Biophotometer 6131).

a) Evaluating DNA Quality by Gel Electrophoresis

The quality of the DNA was evaluated by electrophoresis in 0.8% agarose gel. 1 µl of 6X loading buffer (Fermentas) was applied to each 5 µl of DNA and loaded into each well. The electrophoresis was allowed to run at 80V for 1 hour in 0.5X TBE buffer and stained with ethidium bromide (1 µg/ml). The gel was visualized under a UV light transilluminator. Only samples yielding predominantly high quality DNA were included in the study.

b) Determination of DNA Concentration

The optical density for the extracted DNA samples was determined with spectrophotometer. The absorbance of the samples was read at wavelength 260 nm, 280 nm and 320 nm with Eppendorf Biophotometer 6131. Good quality DNA has a ratio value of OD_{260}/OD_{280} between 1.8 to 2.0. A ratio of less than 1.8 is indicative of contamination by protein and a value of more than 2.0 shows RNA contamination.

3.2 RAPD ANALYSIS PROTOCOL

3.2.1 Materials

A total of 119 accessions (1-8 accessions per populations) from 28 populations were subjected to RAPD analysis. Code and number assigned for each accession of every location are given in Table 3.1 and is referred in text and figure for comparisons.

3.2.2 Primer Screening

One hundred and twenty of random primers from four primer sets namely OPA, OPE, OPF, and OPH (MWG Biotech AG) (Appendix A) while two primer sets OPB and OPG (Operon Technologies) (Appendix B) were being screened. Two samples from the same location and one sample from a distant location were chosen to carry out the initial screening of primers. Primers were chosen based on the clarity and reproducibility of banding patterns in preliminary screening and were used for further analysis.

Table 3.1 Plant materials for RAPD analysis.

Populations	Sample size	Code and number assigned
Northern Peninsular		
D - Sungai Petani, Kedah	2	D1, D2
E - Simpang Tiga, Kedah	1	E1
F - Sungai Kok Mak (Padang Besar), Perlis	6	F1, F2, F3, F4, F5, F6
G - Sungai Korok (Padang Besar), Perlis	5	G1, G3, G4, G7, G9
Y - Sungai Tarum (Langkawi), Kedah	5	Y1, Y2, Y3, Y4, Y5
AD - Sungai Burung (Balik Pulau), Pulau Pinang	6	AD1, AD2, AD3, AD4, AD5, AD6
AE - Sungai Bayan Lepas (Bayan Lepas), Pulau Pinang	6	AE1, AE2, AE3, AE4, AE5, AE6
AF - Sungai Kampung Masjid (Bayan Lepas), Pulau Pinang	6	AF1, AF2, AF3, AF4, AF5, AF6
AG - Sungai Buaya (Balik Pulau), Pulau Pinang	4	AG1, AG3, AG4, AG6
West Coast		
V - Nibong Tebal, Pulau Pinang	4	V1, V2, V3, V4
AA - Bukit Panchor, Perak	3	AA1, AA2, AA4
Z -Selama, Perak	5	Z2, Z3, Z4, Z5, Z6
U - Simpang Lima, Perak	1	U1
W - Bagan Serai, Perak	5	W2, W3, W4, W5, W6
AB - Semanggol, Perak	7	AB1, AB2, AB3, AB4, AB5, AB6, AB7
T - Sungai Gopeng, Perak	3	T1, T2, T3
X – Temoh, Perak	7	X1, X2, X3, X4, X5, X6, X7
S - Tanjung Karang, Selangor	8	S1, S2, S3, S4, S5, S6, S7, S8
R - Pantai Belimbing, Melaka	4	R1, R2, R4, R5
Southern Peninsular		
I - Parit Besar, Johor	3	I1, I2, I3
J – Jemaluang, Johor	2	J1, J2
K - Sungai Bang (Kota Tinggi), Johor	3	K1, K2, K3
L - Ayer Hitam, Johor	5	L1, L2, L3, L5, L6
M - Parit Raja (Batu Pahat), Johor	5	M1, M2, M3, M4, M5
N - Sungai Sialang (Tangkak), Johor	5	N1, N3, N5, N6, N8
East Coast		
A - Alor Lintang, Terengganu	4	A1, A2, A3, A6
B - Sungai Terah (Gua Musang), Kelantan	3	B2, B3, B4
Sarawak		
Q - Bintawa (Kuching), Sarawak	1	Q1