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Phylogenetic Abnalysis of Malaysian Pineapples Cultivars Based on the DNA Sequence of the Internal Transcribed Spacer Region

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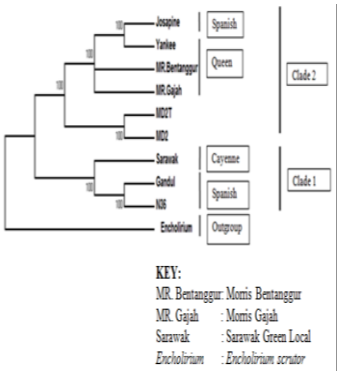
Graphical abstract



Abstract

The phylogenetic study was conducted to determine the phylogenetic status and evolutionary relationships among the nine commercial pineapple cultivars using sequences of the internal transcribed spacer (ITS) region. Genomic DNA was extracted, and the ITS region was amplified and sequenced. Parsimony analysis revealed that Malaysian cultivars could be classified into two major groups based on the ITS region. The first group comprised of the cultivars Sarawak Green Local, Gandul, and N36 whereas the second group consisted of the cultivars Josapine, Yankee, Morris Bentanggur, Morris Gajah, MD2 and MD2/T. Several combinations of synapomorphic characters (leaf and fruit) support this classification system, suggesting the ITS region has the ability to determine the phylogenetic status and relationships of pineapple cultivars. Since each group has its own similar genetic pattern and presumably certain specific biochemical properties, the relationships of pineapple cultivars revealed in the phylogenetic tree can be used as a basis for successful hybridizations to generate new pineapple cultivars.

Keywords: *Ananas comosus*; Bromeliaceae; ITS region; Malaysian pineapple cultivars; molecular phylogenetic

Abstrak

Kajian filogenetik telah dilakukan untuk mengetahui status dan hubungan evolusi antara sembilan kultivar nanas Malaysia komersial menggunakan urutan basa DNA 'internal transcribed spacer' (ITS). Genomik DNA diekstrak dan urutan nukleotida ITS diamplikasi dan di hantar untuk penjujukan. Analisis filogenetik menggunakan kaedah Parsimoni menunjukkan kultivar nanas Malaysia boleh dikategorikan kepada dua kelompok berdasarkan urutan nukleotida ITS. Kelompok pertama terdiri daripada Sarawak Green Local, Gandul dan N36. Kelompok kedua pula terdiri daripada kultivar Josapine, Yankee, Morris Bentanggur, Morris Gajah, MD2 dan MD2/T. Beberapa gabungan ciri sinapomorphik (daun dan buah) menyokong sistem klasifikasi ini, mencadangkan bahawa urutan nukleotida ITS mempunyai keupayaan untuk menentukan status filogenetik dan hubungan kekerabatan antara kultivar nanas. Oleh kerana setiap kelompok mempunyai keseragaman dalam corak genetik, maka hubungan ini boleh digunakan sebagai asas untuk hibridisasi dalam usaha untuk menghasilkan kultivar nanas yang baru.

Kata kunci: *Ananas comosus*; Bromeliaceae; urutan nukleotida ITS; kultivar nanas Malaysia; molekular filogenetik

1.0 INTRODUCTION

Pineapple (*Ananas comosus* (L.) Merr.) is the most economically important plant in the family Bromeliaceae.[1] The pineapple industry in Malaysia was first established in the late 1880s by a European in Singapore. The available data on the molecular phylogenetic diversity of pineapple is limited and is usually characterized using morphological characters.[2]

In Malaysia, many cultivars of pineapple are grown, such as Maspine, Sarawak, Morris, Josapine, MD2, Yankee, Gandul, and N36 (www.mpib.gov.my). Each cultivar has distinct economic importance. However, few studies have focused on the molecular phylogenetic relationships of Malaysian pineapple cultivars. Indeed, most of the work has focused on breeding, agronomy, physiology, pathology, entomology, and post-harvest management (www.mardi.my).

The popular approach to distinguish cultivars is based on morphological characteristics, which is not very suitable because of disagreements among morphologists who use different methods for phylogenetic analysis or for the interpretation of characteristics. This method is therefore time consuming and inconsistent.

Comparisons of the DNA sequences of various genes between different organisms can yield much information about the relationships between organisms that cannot otherwise be inferred from morphology. This is because, in contrast to morphological characteristics, DNA sequences are relatively consistent.[3]

Therefore, in the present study, phylogenetic analysis of 9 commercial Malaysian pineapple cultivars was conducted using sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA to determine the phylogenetic status and evolutionary relationships among these cultivars. The ITS have been widely used by plant systematists to investigate the relationship between closely related taxa, mainly because of its rapid evolutionary rate, small size, and highly conserved flanking regions.[4] The ITS region, which separates the 18S and 26S nrDNA and the coding sequence region of 5.8S nrDNA, has become widely characterized across interspecific and intergeneric level divergences. The entire ITS region ranges between 565 bp and 700 bp. [5] The high copy number of this region allows easy amplification from total DNA.[6] This study is important as it provides information on the evolutionary relationships of Malaysian pineapple cultivars. Furthermore, molecular phylogenetic analysis plays a crucial role in revealing basic knowledge on relationship patterns, based on which genetic sources can be improved by generating new cultivars.

2.0 EXPERIMENTAL

A total of nine pineapple cultivars that were used in this study were Morris Bentanggur, Morris Gajah, Sarawak Green Local, MD2, MD2 Tissue Culture (MD2T), Josapine, N36, Yankee and Gandul. All the specimens that were used are obtained from 'Lembaga Perindustrian Nanas Malaysia' (LPNM). The leaves were stored in the -20˚C freezer to maintain its freshness.

Total DNA was extracted from leaves of pineapples with QIAGEN DNeasy Mini Plant Kit following the manufacturer's instructions with slight modification. The ITS region amplification was performed using the primer pairs AB101 and AB102 (Figure 1). The polymerase chain reaction (PCR) profile consisted of an initial 2 min premelt at 94˚C and 30 cycles of 50 s of denaturation at 94˚C, 1 min annealing at 55˚C and 1 min extension at 72˚C, followed by a final extension of 7 min at 72˚C.

The PCR- amplified fragments were ligated with pGEM-T easy Vector System (Promega) and transformed into NEB-5α as recommended by the supplier. Plasmids were isolated using the Qiagen Plasmid purification Kit using manufactures' instruction. The restriction enzyme digestion was conducted using *EcoRI* to reconfirm whether the plasmid has the insert desired or not. Digestion mixture were added in 1.5 mL tube and mixed together by pipeting before adding *EcoRI*. The mixtures were gently mixed by pipeting and incubated for four hours at 37˚C. The cloning products were sent to 1st Base Laboratories for DNA sequencing. The sequencing was done in two way using the primer SP6 and T7.

The obtained DNA sequences were edited and assembled using CodonCode Aligner (http://www.codoncode.com/aligner/). Multiple alignments were conducted using ClustalX.[7] The total nine samples sequences with an outgroup sequences were subjected to phylogenetic analyses. The 10 aligned sequences were used to construct the phylogenetic tree using MEGA version 4 (www.megasoftware.net/). Phylogenetic analysis was done using the Parsimony method. The principle of this method is that the differences observed among the cultivar under the study were identified by minimization of character transformations of a tree.[8] The outgroup *Encholirium scrutor* (GenBank ID: JN016950) which the data is available in the Gene Bank was used because it has been recognized as sister group to the family Bromeliaceae.

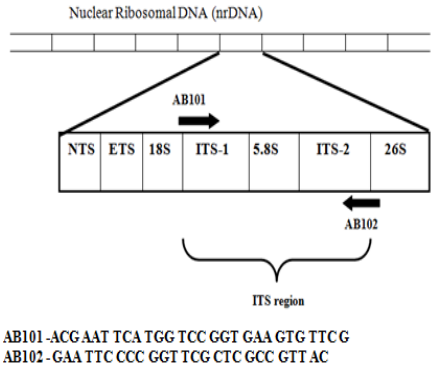


Figure 1 Organization of ITS region of nrDNA with location of primers used in this study. Information of primers is also provided

3.0 RESULTS AND DISCUSSION

The phylogenetic analysis was based on the maximum parsimony (MP) and bootstrap reliability tests with 1000 replicates. The phylogenetic tree was constructed using MEGA 4. Insertions and deletions were treated as missing data. The results show that the ITS region comprises 561 characters. Of these, 449 were potentially informative and 112 characters were constant. From the analysis, 3 phylogenetic trees were constructed, with a consistency index (CI) of 0.888 and a retention index (RI) of 0.842. The consensus tree based on these 3 constructed trees is shown in Figure 2. The size of the ITS region for *A. comosus* was determined through comparative analysis with sequences on GenBank. The results indicate that size of the ITS region is around 570 bp.

From Figure 2, it can be observed that the 9 cultivars are separated into 2 main clades. Sarawak Green Local, Gandul, and N36 are in the first clade, whereas Josapine, Yankee, Morris Bentanggur, Morris Gajah, MD2, and MD2 Tissue Culture (MD2T) form the second clade; both clades have 100% bootstrap support (BS).

The first clade shows that the cultivars Sarawak, Gandul, and N36 have a BS of 100%. The Gandul cultivar is united with N36 (100 % BS), and the Sarawak cultivar is grouped together with both of these cultivars (100 % BS) (Figure 2)

The second clade is further divided into two subclades, both of which have 100% BS. In the first subclade, the Josapine cultivar is united with the Yankee cultivar with 100% BS. Morris Bentanggur and Morris Gajah cultivars form a sister group to these cultivars. Meanwhile, the second subclade consists of MD2 and MD2T cultivars, with 100% BS (Figure 2).

The second clade has Josapine, Yankee, Morris Bentanggur, Morris Gajah, MD2, and MD2T grouped together to form a monophyletic group. In phylogenetic analysis, a group of organisms that have a high similarity in their characteristics are assumed to be closely related and to derive from a single ancestor, forming a monophyletic group.[10] In the first clade, all of the cultivars shared synapomorphic characteristics such as presence of spines on the leaves and a subulate leaf apex (Hidayat, unpublished data). This might be why these 6 cultivars are grouped together in a clade.

The leaf margin is important in distinguishing between cultivars. Six leaf margin types were originally described by Ref. [11] : spiny, spiny tip, scallop, smooth, piping, and sandpaper. The environment is known to influence the expression of spines in some genotypic backgrounds, and this, if not properly understood, can result in erroneous varietal descriptions. However, the leaf margin type remains an important descriptor in pineapple, and is one of the distinguishing characteristics of cultivars and botanical varieties. A spiny leaf margin can be considered the usual condition in pineapple. It is easily identifiable, as the entire leaf margin on all leaves is completely spiny. Spine shape, size, and density are highly variable among cultivars. [12]

The consensus tree in Figure 2 shows that the Josapine cultivar is a sister cultivar to Yankee although Yankee is from Queen group. In addition, there are some morphological similarities between these two cultivars. For example, these two cultivars have rough and long spines arranged along the margin. In addition both cultivars have linear shape and smooth surface of leaves. The leaf color for upper and beneath surface of both of these cultivars is brownish green and reddish green (Hidayat, unpublished data).

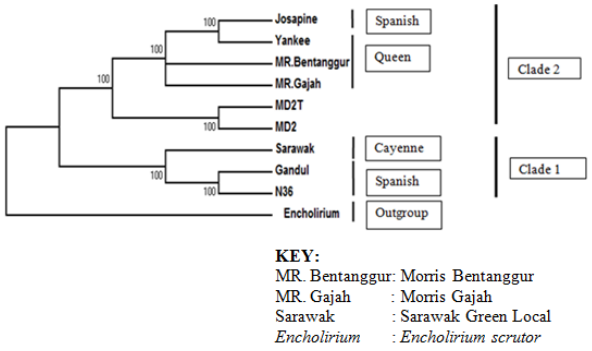


Figure 2 Strict consensus tree from the parsimony analysis of the ITS region (3 Most Parsimonious Trees, Length= 831 steps, CI=0.888, RI= 0. 842). The value in the branch shows the bootstrap support (BS)

However, there are still some morphological differences between these 2 cultivars. The spines in the Josapine and Yankee cultivars are distantly and closely arranged, respectively. The leaf colour is purplish green in Josapine and reddish green in Yankee. The fruit shapes are also different: Josapine bears cylindrical fruit, whereas Yankee bears tapered fruit. The eye shapes are projected in Josapine but flat in Yankee (Hidayat, unpublished data).

In the consensus tree as shown in Figure 2, indicated that the Morris Bentanggur and Morris Gajah cultivars are separated into 2 different groups, even though both of them are derived from the same group. The BS values for these 2 cultivars were low in the 3 phylogenetic trees generated (tree not shown). There are not many differences in their morphologies (Hidayat, unpublished data). This might be due to the number of cultivars used in this study may have been insufficient. In phylogenetic analysis, increasing the number of samples can help overcome this difficulty. Therefore, further phylogenetic analysis is needed after more extensive sampling.

From Figure 2, it can be observed that MD2 and MD2T form the second subclade in the second clade. The morphology of both of the cultivars is almost similar. A few differences remain in the morphology of MD2T and MD2 because tissue cultures have shown that cells in long-term cultures are genetically unstable. Genetic variations can also occur in the morphological traits of regenerated plants. Thus, tissue culture sources are direct sources of genetic variability.[13] Some variations are a result of specific genetic changes or mutations and are transmitted to the progeny. Such genetically controlled variability is known as somaclonal variation. This may hamper clonal propagation but at the same time generate desirable somaclonal variants that can be selected for the development of novel cell lines.[14] Furthermore, the source of explants has often been considered a critical variable for somaclonal variation. Pineapple plants raised from the callus of slip, crown, and axillary buds showed alterations only in spine characters, while those raised from the callus of the syncarp showed variations in leaf colour, spine wax, and foliage.[15]

The cultivar MD2 and MD2/T were found to be more closely related to the Queen and Spanish group than to the Smooth Cayenne. The history of MD2 cultivar showed that it has parentage that is somewhat a complicated mixture of ‘Smooth Cayenne’, ‘Smooth Guatamelan’, a ‘Spanish’ group, ‘Queen’ and ‘Pernambuco’. This cultivar has more than 50% of ‘Smooth Cayenne’ parentage.[16,17] The relatedness of MD2 and MD2/T to the Queen and Spanish group rather than to the Cayenne might be because the environment adaption of this plant. Such outcome agrees with [18], who described and reported that the differentiation between species of Ananas might be because of ecological adaptations.

The first clade shows that the cultivars Sarawak, Gandul, and N36 have a high BS. There no much morphological similarities that are shared by these three cultivars. But, both Gandul and N36 are united with Sarawak Green Local with 100% BS. The presence of these 3 cultivars in 1 clade might also be due to the close relationship between them, because the N36 cultivar is a hybrid selected from a cross between Gandul (Spanish) and Sarawak (Smooth Cayenne).[19] The N36 cultivar, which was developed by the Malaysian Agricultural research and Development Institute (MARDI), has special characteristics that render it suitable for export by sea. One of the special characteristics of this variety is its resistance to black heart disease. This is definitely beneficial for the industry, especially for the fresh fruit market. With an average of 12°–14° Brix and a pale yellow colour, this variety is also suitable for canning (www.mpib.gov.my).

Even though N36 is a hybrid between Gandul and Sarawak, N36 is much more closely related to Gandul with 100% BS. Figure 2 suggests that Gandul could be a sister group to N36, because both these cultivars have similar morphological characteristics on several parts of the leaves: both these cultivars have short and fine spines that are closely arranged, and the leaf apex is subulate in both cultivars (Hidayat, unpublished data). Furthermore, both these cultivars show similarities in the cylindrical fruit shape. The size of the fruit in both cultivars is medium and the weight is approximately 1.8 kg. Moreover, both of these cultivars have crispy pulp (http://www.mpib.gov.my/).

The N36 cultivar has similarities with the Sarawak cultivar in its pale yellow pulp colour, acidity content, which is around 0.5–0.9%, and sugar content (12°–16° Brix) [19]. This might be the reason why the cultivars Gandul and N36, which are from the Spanish group, are more closely related than N36 and the Sarawak Green Local.

From the phylogenetic study, we can predict which is the group evolved earlier compared to others. From the consensus tree (Figure 2), we can predict that the second clade is the clade that evolved earliest. Within the second clade, the first subclade is believed to have evolved first, followed by the second subclade. This might be because the Josapine cultivar was developed earlier than the other hybrids developed at the MARDI Integrated Peat Research Station in Pontian, Johor in

1984. The name 'Josapine' was derived from 'Johore' and 'Sarawak', the parent cultivars, and it was officially released by MARDI on 5 August 1996. Josapine was derived from a deliberate cross and is the first successful commercial pineapple hybrid in the world. Meanwhile, MD2 is a new cultivar in Malaysia and is believed to have been developed by Pineapple Research Institute of Hawaii.[20]

It is hypothesized that hybridization is possible among the first clade cultivars rather than between the 2 main clades. This is because hybridization results in the combination of 2 or more attributes from different cultivars. Therefore, the cultivars should have a similar genetic pattern and a tendency to follow the same evolutionary path. [20]In other words, plants that are hybridized should have a close evolutionary relationship.

In the context of phylogenetic analysis, plants should have a monophyletic relationship, i.e. same ancestry. If the evolutionary relationship of the plants to be hybridized is not monophyletic, hybridization is less or not feasible as they are not genetically matched, as is usually the case for most hybridizations.[21]

■4.0 CONCLUSION

From the topology of the phylogenetic tree, the nine pineapple cultivars were separated clearly into two main clades. From this research, it was shown that the cultivars that were derived from the same species (*A. comosus*), the subgroups form a monophyletic group, and the ITS region is believed to have the ability to determine the phylogenetic status and relationship among pineapple cultivars. From the phylogenetic tree constructed, it was revealed that in the first clade, Gandul cultivar is sister group to N36 cultivar and both of this cultivar together with the Sarawak cultivar suggested that these cultivars might have same evolutionary patterns. The second clade consisting of Josapine, Yankee, Morris Bentanggur, Morris Gajah, MD2, and MD2/T cultivar grouped together to form a monophyletic group. The results also showed that the cultivars that were grouped together have a similar genetic pattern, and therefore have a tendency to follow the same evolutionary path. This information can be used as basic knowledge for successful hybridization to generate new cultivars. Although the study was considered as preliminary, it provided new molecular data on the phylogenetic analysis of commercial Malaysian pineapple cultivars by using DNA sequences of the ITS region of nrDNA, as the existing molecular data on our Malaysian pineapple cultivars is inadequate.

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