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Genetic variations for 'Nam Hom' coconut (*Cocos nucifera* L.) grown in the western region of Thailand using AFLP markers

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Abstract

Using 10 selected Amplified fragment length polymorphism (AFLP) primers, to investigate the genetic diversity and relationships among 'Nam Hom' coconut grown in the western region of Thailand, resulting in amplification of 169 reproducible polymorphic fragment products out of 416 bands scored. The percentages of polymorphic markers for AFLP ranged from 70.00% for E-ACC/M-CAT to 24.14 % for E-ACC/M-CAA primers. The phylogenetic tree dendrogram showed that the 55 coconut accessions grown in western region of Thailand could be classified into four groups at 0.88 of similarity coefficients. This grouping observation is consistent with existing morphological classification of coconut tree. The first group to the third group in this study contains tall coconut group and mutant coconut from dwarf coconut group. While, the forth group contains all coconut which belongs to the dwarf coconut group including 'Nam Hom' ('Kon Chip' and 'Kon Klom' strains) and 'Nam Wan' coconut from all production area with 0.895-1.00 of similarity coefficients indicated the narrow genetic diversity. Moreover, the results indicated that the 'Nam Hom' coconut samples both 'Kon Chip' and 'Kon Klom' strains collected in different locations were for the main part genetically similar with cophenetic correlation (r) = 0.9347.

Keywords: 'Nam Hom', coconut, AFLP, genetic diversity, Cocos nucifera L.

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1. Introduction

The coconut palm (*Cocos nucifera* L.) is an outbreeding perennial crop in the tropical zone. Owing to its importance for rural communities, it has been termed the 'tree of life'; from the roots to the fronds, all coconut palm constituents are utilized for either nutritional or non-food purposes [1]. Coconut belongs to the monotypic genus with a single species *Cocos nucifera*. It is presumes the generic name *Cocos* as well as the popular name coconut. This tree belongs to the family Arecaceae (Palmae) [2]. Coconut products provide food, shelter and energy to farm households and can be made into various commercial and industrial products. Coconuts varieties can be classified as tall and dwarfs coconut [3]. Tall coconut are fast growing and predominantly allogamous (cross-fertilising) whereas, Dwarf coconut have a reduced growth habit and are mostly autogamous (self-fertilising). The latter varieties are thus considered to be fixed lines, while the former constitute polymorphic populations and cultivars are either population maintained under natural pollination [4]. Many researchers have studied its morphological characteristics for coconut variety classification and for genetic diversity evaluation. However, morphological characteristics are more or less affected by environmental conditions, a fact which has impeded exact classification and evaluation. In Thailand, Thai coconut verities are classified into two main categories, namely: (i) tall palms which are late-bearing and produce a bole at the base of the stem, and (ii) dwarf palms which are early-bearing and without a bole. A third category, 'miscellaneous', may be added to include several other peculiar, a number of varieties of coconut whose affinities are not clearly understood, and which occur sporadically as uncommon variants among other coconuts in certain localities [5]. Aromatic coconut called 'Nam Hom' coconut in Thailand is a special type of green dwarf coconut, the liquid endosperm (coconut water) of which is characterized by a pleasant "pandan-like" aroma due to the presence of 2-acetyl-1-pyrroline (2AP). 'Nam Hom' coconut arose as a mutant of 'Mu Si Khieo' in Nakhon Chaisri district of Nakhon Pathom province, central Thailand. The main production areas of 'Nam Hom' coconut are Damnoen Saduak district in Ratchaburi province, Ban Phaeo district in Samut Sakorn province, Amphawa district in Samut Songkram province and Sampran district in Nakhon Pathom province. Those production areas are in the western

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Table 1 Accession of coconut used in the variation study

No.	Name	Collection site*	No.	Name	Collection site*
1	'Kon Chip' 1	Sam Phran 1	42	Tall coconut 2	Sam Phran
2	'Kon Chip' 2	Sam Phran 1	43	Tall coconut 3	Ban Phaeo
3	'Kon Chip' 3	Sam Phran 2	44	Tall coconut 4	Ban Phaeo
4	'Kon Chip' 4	Sam Phran 2	45	'Kon Chip' 23	Damnoen Saduak 5
5	'Kon Chip' 5	Sam Phran 3	46	'Kon Chip' 24	Damnoen Saduak 5
6	'Kon Chip' 6	Sam Phran 3	47	'Kon Chip' 25	Damnoen Saduak 6
7	'Kon Chip' 7	Ban Phaeo 1	48	'Kon Chip' 26	Damnoen Saduak 6
8	'Kon Chip' 8	Ban Phaeo 1	49	'Kon Chip' 27	Damnoen Saduak 7
9	'Kon Chip' 9	Ban Phaeo 2	50	Tall coconut 5	Damnoen Saduak
10	'Kon Chip' 10	Ban Phaeo 2	51	Tall coconut 6	Damnoen Saduak
11	'Kon Chip' 11	Damnoen Saduak 1	52	'Kon Chip' 28	Damnoen Saduak 8
12	'Kon Chip' 12	Damnoen Saduak 1	53	'Kon Chip' 29	Damnoen Saduak 8
13	'Kon Chip' 13	Damnoen Saduak 2	54	'Kon Klom' 1	Damnoen Saduak
14	'Kon Chip' 14	Damnoen Saduak 2	55	'Kon Klom' 2	Damnoen Saduak
15	'Kon Chip' 15	Damnoen Saduak 3	70	Mutant coconut 2	Sam Phran
16	'Kon Chip' 16	Damnoen Saduak 3	71	Mutant coconut 3	Damnoen Saduak 1
17	'Kon Chip' 17	Damnoen Saduak 4	72	Mutant coconut 4	Damnoen Saduak 2
18	'Kon Chip' 18	Damnoen Saduak 4	73	'Kon Chip' 30	Sam Phran 4
19	'Kon Chip' 19	Ban Phaeo 3	74	'Kon Chip' 31	Sam Phran 4
20	'Kon Chip' 20	Ban Phaeo 3	75	'Kon Chip' 32	Sam Phran 5
21	'Kon Chip' 21	Ban Phaeo 4	76	'Kon Chip' 33	Sam Phran 5
22	'Kon Chip' 22	Ban Phaeo 4	77	'Kon Chip' 34	Sam Phran 6
26	'Nam Wan' 1	Ban Phaeo 1	78	'Kon Chip' 35	Sam Phran 6
27	Mutant coconut 1	Ban Phaeo	79	'Kon Chip' 36	Ban Phaeo 5
28	'Nam Wan' 2	Damnoen Saduak 1	80	'Kon Chip' 37	Ban Phaeo 5
29	'Nam Wan' 3	Damnoen Saduak 2	81	'Kon Chip' 38	Ban Phaeo 6
30	'Nam Wan' 4	Damnoen Saduak 3	82	'Kon Chip' 39	Ban Phaeo 6
41	Tall coconut 1	Sam Phran			

*Name of district and number of collection site

region of Thailand. However, there are the minor morphological differences in 'Nam Hom' coconuts grown in these areas which the main cultivar is coconut fruit with three lobes at the end of fruit called 'Kon Chip'. The other is the fruit with round shape at the end of the fruit called 'Luk Klom'or 'Kon Klom' [5]. Moreover, some production areas have 'Nam Wan' cultivar, one of dwarf palms with sweet liquid endosperm but they do not have a pleasant "pandan-like" aroma. These different characters of 'Nam Hom' coconut could make confusion to the consumer. However, 'Nam Hom' coconut from the different area has the different flavor and aroma. It has been still unclear that these differences come from the genetic variation or environmental difference. If we could prove that all the 'Nam Hom' coconut trees from the different production areas were the same genetic, the different flavor and aroma should then be the consequence of the different environment or orchard management.

Thus, information on the genetic diversity and relationships among 'Nam Hom' coconuts in the different areas would be useful to eliminate the confusion and help future germplasm collections. Increasingly, molecular marker technologies are playing an important role in assessing genetic diversity, identifying genetic relationships, and aiding germplasm fingerprinting in plant collections. Over the last few decades a variety of different genetic analytical techniques have emerged in the field of molecular genetics along with several PCR-based genetic markers that have now been established and are used to provide information on genetic variations in plant species. Initially, RAPD was employed for genetic analyses but problems regarding reproducibility had been reported [6], so the amplified fragment length polymorphism (AFLP) technique was then introduced because it has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques giving a reliable and reproducible marker system [7].

AFLP, a relatively new DNA fingerprinting technique [7] uses selective amplification of restriction fragments. It has a high multiplex ratio, does not require DNA

probes or prior sequence information, and is now preferred over other DNA-based marker systems in instances where little is known about the genomic structure [8 - 11]. In addition, a larger number of loci are detected *per* reaction in comparison with RAPD and it is seen to give a higher precision than RAPD. For example, at the species level the technique is proficient at revealing diversity and effective in covering a wide area of the genome in a single assay [12]. The procedure is simple, largely, requires only small amounts of DNA and can be performed without the use of radioactivity [13]. This PCR-based method generates complex banding patterns of DNA types amplifying up to at least 100 fragments in each reaction. However, despite a few drawbacks to the procedure, it is more intensive and expensive than other procedures, such as RAPD, and it has the potential to be very useful in genetic analysis [14 - 16]. Within perennial fruit cultivars [17] have detected substantial genetic variations and also demonstrated that cultivars can be discriminated on the basis of their genetic characteristics. Recent reports have focused on using DNA based markers, particularly AFLP markers, to measure the genetic diversity and relationships in fruit species; such as, cherry (*Prunus avium*) [18], lemon (*Citrus lemon* L.) [19], mango (*Mangifera indica* L.) [17], peach (*Prunus persica* L.), pear (*Pyrus* sp.) [20], litchi [21], citrus [16, 22] and pummelo [23]. For coconut, there have been some researchers reported the genetic diversity and variation in coconut using AFLP technique [24][4]. However, the information on the genetic diversity and relationships between 'Nam Hom' coconuts in the different production area at the western region of Thailand is not studied.

Therefore, the objective of this study is to estimate the genetic diversity and relationships among 'Nam Hom' coconut grown in the different production area at the western region of Thailand using AFLP analyses.

2. Materials and Methods

2.1 Plant material

Fresh mature coconut leaf samples were taken at various locations of the western region of Thailand (Sam Phran district, Nakhon Prathom province, Damnoen Saduak district, Rachaburi province and Ban Phaeo district, Samut Sakhon province). Leaves were collected from 2 plants *per* collection site; a total of samples see Table 1. The coconut cultivars consisted of 'Nam Hom' coconut ('Kon Chip' and 'Kon Klom' strain), 'Nam Wan' coconut, Tall coconut and mutant coconut. Fifty five accessions in total were studied for genetic variation in this research.

2.2 DNA extraction

DNA was extracted using a modified CTAB protocol [25]. From the leaves collected 20 mg were ground and placed in a 1.5 ml microfuge tube over liquid nitrogen. Next, 700 µl of preheated extraction buffer containing 2% CTAB, 100 mM Tris-Cl, 1.4 M NaCl, 20 mM EDTA, 0.625% 2-Mercaptoethanol and 3% PVP was added and the mixture incubated for 30 min at 65°C and then placed on ice for 10 min. Following this, a further mixture was made by adding 300 µl of 5 M potassium acetate and placed back on the ice for a further one hour. The resulting cooled mixture was then placed in a centrifuge and spun for 10 min at 14,000 rpm. The supernatant was poured into a new microfuge tube and 700 µl of chloroform: isoamylalcohol at 24:1 was added. This mixture was then centrifuged for 20 min at 14,000 rpm and the supernatant poured into a new microfuge tube and mixed with an equal amount of ice-cold 95% ethyl alcohol (EtOH) for 5 min. Using the centrifuge again the mixture was centrifuged for 5 min at 14,000 rpm and the supernatant poured into a new microfuge tube and mixed with 500 µl of 70% EtOH and centrifuged for 5 min at 14,000 rpm after which the supernatant was drained out. The subsequent produced DNA pellet was dried at 65°C and then 200 µl of 1X TE buffer was added to the pellet and incubated at 65°C for 1 hr. Finally, the DNA was kept at -20°C in freezer waiting for AFLP analysis.

2.3 Amplified fragment length polymorphism analysis

AFLP analysis was conducted as described by [7] with some modifications. Initially/first Genomic DNA (100 ng) was digested for 3 hrs at 37°C to a final volume of 25 µl with 10 units of *Eco*RI and 10 units of *Mse*I in 1X R/L restriction/ligation buffer (33 mM Tris-HCl, pH 7.5, 10 mM potassium chloride, 0.5 mM DTT). To this mixture was added 10 µl of ligation mix containing 7.5 pmol adapter for *Eco*RI and 75 pmol adapter for *Mse*I, 1.2 units T4-DNA ligase, 1.2 mM ATP and 1x ligation buffer. Next the ligation reaction was performed at 37°C for 3 hrs after which a DNA template was prepared by diluting DNA with 10 X dH_2O and 3 µl of the resulting digestion-ligation mixture (DNA template) was used for PCR pre-amplification by adding 0.25 mM of primer, 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, and 0.3 units of *Taq* DNA polymerase, in a final volume of 10 µl. The thermal conditions for PCR were: 24 cycles of 30 s at 94°C, 1 min at 56°C and 1 min at 72°C. A GeneAmpR PCR System 9700(Applied Biosystem) was used. A template for selective amplification was made from 2 µl of pre-amplification product and a mixture of 0.25 µM of primer *Mse*I, 0.25 µM primer *Eco*RI, 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 mM dNTP, and 0.3 units *Taq* DNA polymerase (Euroclone) to a final volume of 10 µl. The following PCR conditions were observed and the annealing temperature was reduced

Table 2 List of AFLP primers, their sequence, number of bands, polymorphism (%) of AFLP analysis

No. of primer	Sequence	Total number of band	AFLP fragment score		Polymorphism %
			Monomorphic	Polymorphic	
1	AAC-CAG	47	29	18	38.30
2	AAC-CAT	42	30	12	28.57
3	AAG-CAC	61	37	24	39.34
4	AAG-CAT	45	19	26	57.78
5	ACC-AAC	54	36	18	33.33
6	ACC-AAG	33	24	9	27.27
7	ACC-AAT	44	25	19	43.18
8	ACC-CAA	29	22	7	24.14
9	ACC-CAG	31	16	15	48.39
10	ACC-CAT	30	9	21	70.00
Total	10	416	247	169	
Average		41.6	24.7	16.9	41.03

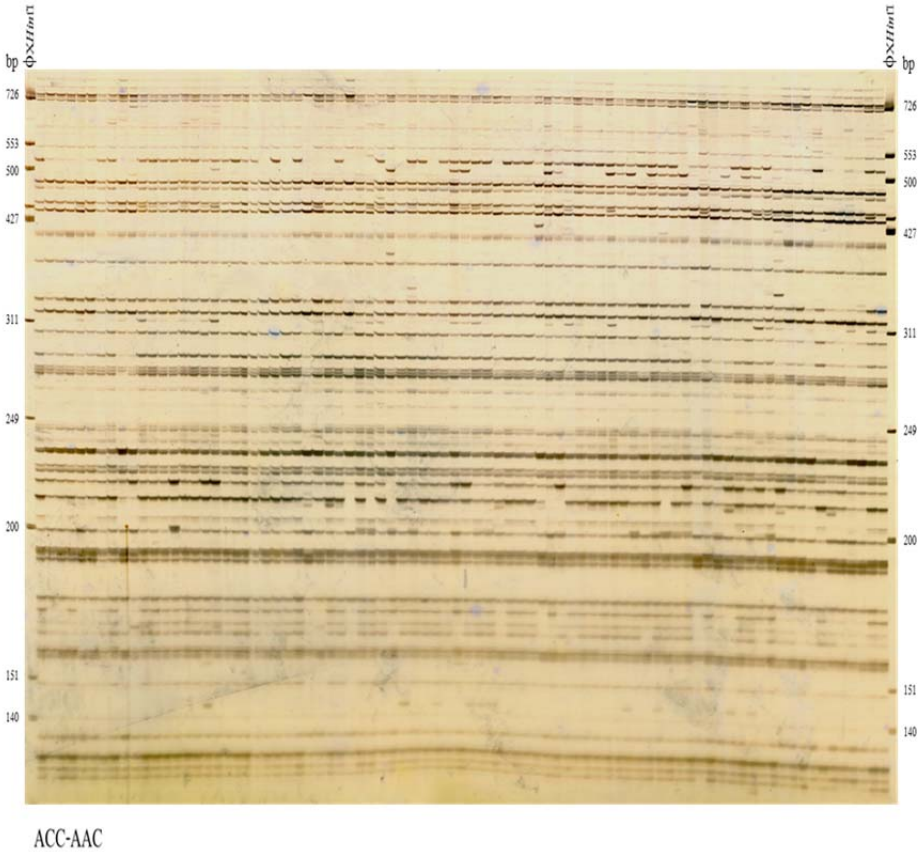

Figure 1 An AFLP profile of 'Nam Hom' coconut genomic DNA using primer combination E-ACC/M-AAC

every cycle by 1°C: nine cycles of 30 s starting at 94°C down to 65°C and a further 1 min at 72°C. The next stage involved a further 30 cycles for 30 s at 94°C, 30 s at 56°C, 1 min at 72°C and hold at 4°C until the reaction was complete. It was stopped with the addition of 5 µl of loading buffer (10 mM EDTA pH 8.0, 98% formamide, Bromophenol Blue & Xylene cyanol). Selective PCR was performed in A GeneAmp® PCR System 9700 (Applied Biosystem). Amplified fragments were separated by 4.5% (w/v) polyacrylamide gel electrophoresis: silver staining. The DNA bands were

visualized by autoradiography using silver staining and manually scored for their presence or absence.

2.4 Data analysis

The NTSYS program was used for cluster analysis and based on a similarity matrix. The matrix was analyzed by the unweighted pair-group method with arithmetic mean (UPGMA) [26] and relationships between the cultivars were illustrated as a dendrogram. AFLP polymorphic bands were scored as either present (1) or absent (0) to process a binary matrix. The Jaccard similarity index was computed for each pair of cultivars, [27, 28].

3. Results and Discussion

3.1 Polymorphism as detected by AFLPs

The ten pairs of primers generated a total of 416 bands of which 169 bands (41.03%) were polymorphic. The mean number of band per assay was 41.60. Part of a typical gel is shown in Figure 1. Ten primer pairs were selected from 64 pairs of *Eco*RI/*Mse*I primers based on their sharp electropherogram and specific polymorphism. Genomic DNA of the sample was tested using AFLP analysis with the primer pairs. For coconut, [24] have stated that the eight pairs of primers generates a total of 332 scoreable products among the 42 genotypes studied, of which 198 bands (61.4%) were polymorphic. Whereas, the study on African coconut found that AFLP analysis with 12 primer combinations gave a total of 1106 bands, of which 303 were polymorphic (27%) [4]. The size of AFLP fragments generated by the different primer combinations in this study ranged from 140 to 726 bp (Figure 1) and the number of bands produced by the different primer combinations ranged from 30 to 61. Table 2 shows the obvious differences in the total bands amplified by various primers. The maximum number of polymorphic bands was amplified with the E- ACC/M-CAT primer pair identifying 70.00% polymorphism and the minimum number of polymorphic bands was amplified with the E-ACC/M-CAA primer pairs identifying 24.14% polymorphism. A total of 416 AFLP bands were identified with 10 primer pair combinations. Similar to the results seen by [29], who also applied the AFLP technique, with grapevine accessions and obtained a 49% polymorphism level, this study has found a total of 169 (41.03%) polymorphic bands with a range from 24.14% to 70.00% and an average number of polymorphic bands of 16.9 *per* AFLP primer combination (see Table 2). In citrus fruit, [30] have reported from their experiment that six primer combinations generated, 571 of 599 fragments were polymorphic with the range of polymorphic bands per primer combination being 63 to 119 (mean of 95.2) with the average polymorphic rate of AFLP markers was 95.3%. Previous reports have shown a high level of polymorphism but it was studied on different species of a single plant. The lower level of polymorphism in our study is possibly due to the samples being obtained from the same species but of different production area and some morphological characteristic so it is likely there will not be a great genetic difference. Similar finding to this study were reported by [31] in a study of apple cultivars that reported 208 (57.5%) polymorphic bands from 362 bands were observed. [32] studied AFLP fingerprinting of Egyptian date palm cultivars and found that the number of polymorphic amplicons was 233 representing a level of polymorphism of 53.81%. Our results are different from the previous study in coconut by [24] who have stated that the eight pairs of primers generates a total of 332 scoreable products among the 42 genotypes studied, of which 198 bands (61.4%) were polymorphic. It could be that our results studied in the same coconut species but the previous one studied in different genotypes. Thus, the polymorphic bands amplified by any AFLP primer in our study were sufficient to discriminate all ‘Nam Hom’ coconut accessions. An example of the pattern of amplified products obtained with one AFLP primer pair is shown in Figure 1.

3.2 Cluster analysis of ‘Nam Hom’ coconut

From the AFLP cluster analysis, performed with a similarity coefficient as illustrated in the dendrogram of the phylogenetic tree (Figure 2), the similarity coefficients ranged from 0.85-1.00. The dendrogram, constructed from 10 AFLP markers, indicated that 55 coconut accessions in this study can be clearly divided into four groups at 0.88 of similarity coefficients. This grouping observation is consistent with existing morphological classification of coconut tree. The first, second and third group in this study contains tall coconut group and mutant coconut from dwarf coconut group. While, the forth group contains all coconut which belongs to the dwarf coconut group including ‘Nam Hom’ and ‘Nam Wan’ coconut from all production area with 0.895-1.00 of similarity coefficients indicated the narrow genetic diversity. Our result is according to [3] who reported that coconuts varieties can be classified as tall and dwarfs coconut. However, [24] reported that coconut from Sri Lanka can be classified as tall, intermediate and dwarf accessions using AFLP technique. While, [5] reported that coconut in Thailand can be classified as tall, dwarf and miscellaneous coconut (a number of varieties of coconut whose affinities are not clearly understood, and which occur sporadically as uncommon variants among other coconuts in certain localities).

The similarity coefficient values within dwarf coconut were very high (Figure 2). This indicates that the genetic variation within dwarf coconut, grown in different locations, is very narrow including ‘Nam Hom’

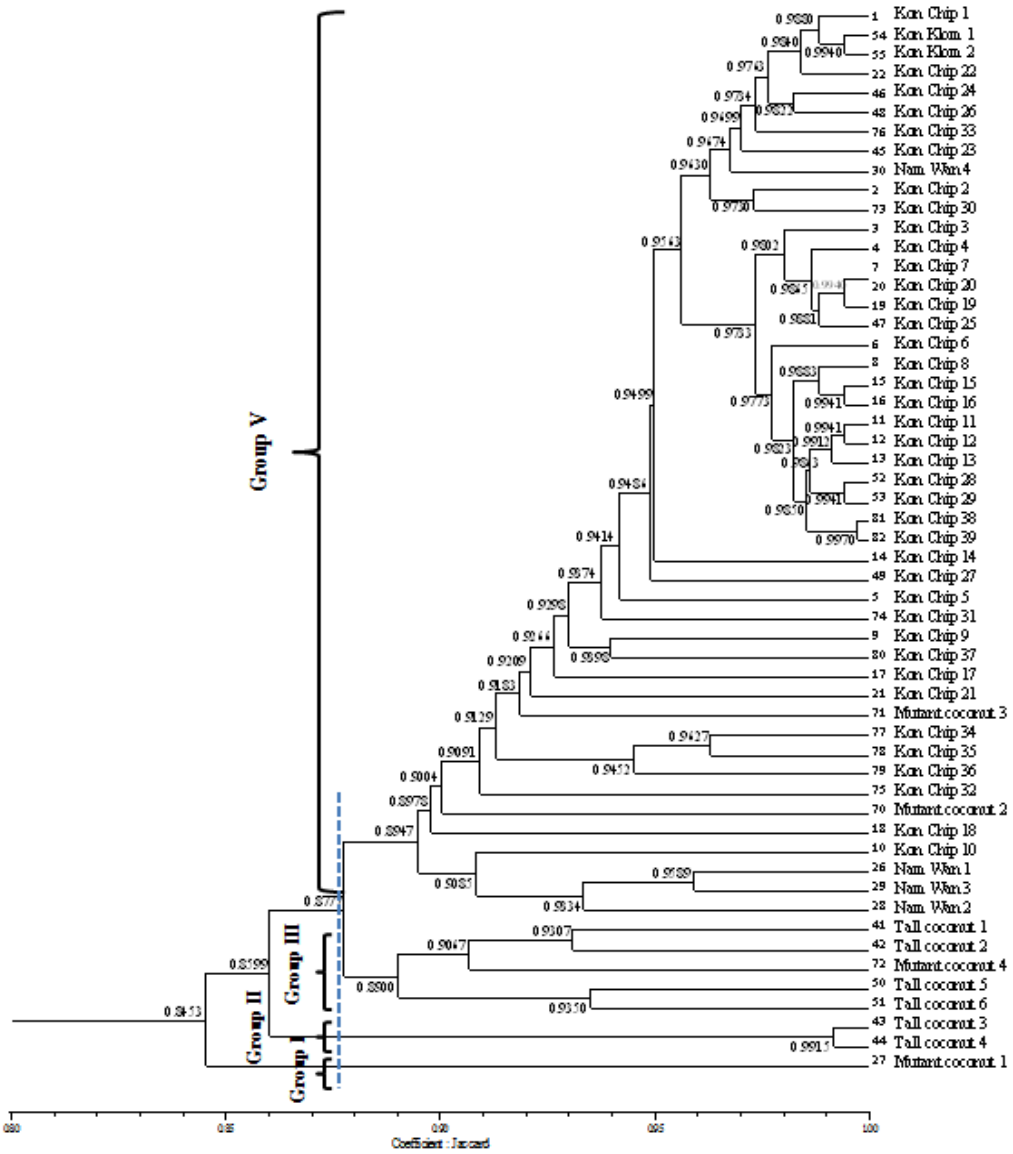

Figure 2 Dendrogram of phylogenetic tree with the similarity coefficient value showing genetic relationship among 'Nam Hom' coconut accessions.

coconut fruit with three lobes at the end of fruit ('Kon Chip'), 'Nam Hom' coconut fruit with round shape at the end of the fruit ('Kon Klom') and 'Nam Wan' coconut from all production area. Therefore, it is likely that they are from the same progenitor material. Similar phenomena have been reported by [33, 34] of the high degree of genetic similarity between coffee and macadamia cultivars. As consideration within dwarf groups, there were very high similarity coefficient at 0.89-0.99 which included 'Kon Chip', 'Kon Klom' and 'Nam Wan' coconut. This indicated that those cultivars might be from the same progenitor and very close in genetic; therefore the primers in our study could not detect any difference in those coconut trees and more studies employing genetic markers will be required to distinguish between the two groups of coconut trees. Similar result found in pummelo cultivar studied by [23] who found that the DNA primers in their study were not capable to detect any of the genetic differences between *Khaonamphueng* and *Khaoyai* cultivars. Consequently it is still unclear whether or not *Khaonamphueng* and *Khaoyai* are the same cultivar.

Moreover, as consideration in the genetic variation especially in 'Nam Hom' cultivar ('Kon Chip'), we found that the accessions collected from different production area were very close in the genetic

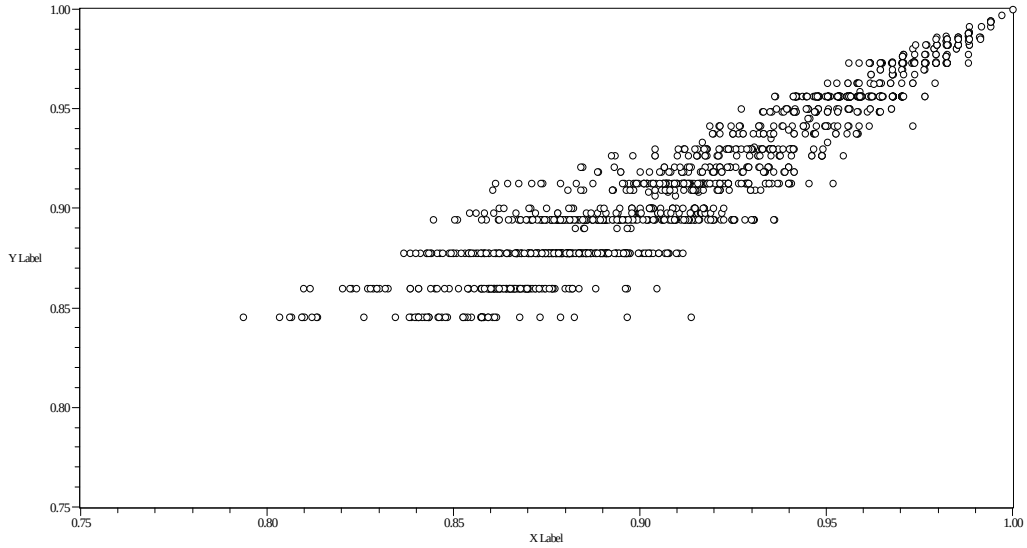

Figure 3 Data distribution of 'Nam Hom' coconut grouping from 55 samples, cophenetic correlation (r) = 0.934

variation (Figure 2). Our results found that the leaf samples collected from the same cultivar, but in different locations (Table 1) were genetically similar. Those mean the same cultivars grown in the different production areas. This is according to [5] who indicated that the dwarf coconut is self-pollinating and thus genetically more stable, producing fairly uniform populations and little genetic diversity [35]. For example, 'Kon Chip' strain (No. 1-22 in Table 1) collected from Sam Phran, Ban Phaeo and Damnoen Saduak district showed a 0.9209 similarity coefficient and 'Nam Wan' coconut (No. 26, 28-30 in Table1) collected from Ban Phaeo and Damnoen Saduak district showed a 0.9334 similarity coefficient. This is probably due to self – fertilization in the coconut. Consequently, the difference in flavor and aroma in coconut fruits from different area may be resulted from the different environment and orchard management as the cophenetic correlation was very high (r=0.9347) (Figure 3). Effect of orchard management on flavor and aroma may be that coconut grower often grow the tall coconut nearby or around the 'Nam Hom' coconut orchard, this could make the opportunity for cross pollinating between tall coconut and 'Nam Hom' coconut. This may create the variation of 'Nam Hom' coconut genetic, resulting in the variation of flavor and aroma. However, it is obvious that 'Nam Hom' and 'Nam Wan' coconut was very close in the genetic variation and morphological characteristic resulting in the difficult isolation. Normally, the different characteristic among these coconut used for the isolation was flavor and aroma of liquid endosperm (coconut water). The coconut water from 'Nam Wan' coconut has the lower pleasant aroma but sweeter liquid endosperm than those from 'Nam Hom' coconut.

The results from this study clearly demonstrated the efficiency of the AFLP marker system for coconut cultivar fingerprinting identification and typing using only a small number of primer combinations. These results are inconsistent with the findings of different plant species. In conclusion, the phylogenetic tree dendrogram showed that 55 coconut accessions grown in western region of Thailand could be classified into four groups. The first group to the third group consisted of tall coconuts and the mutant coconut. The forth group consisted of dwarf coconut including 'Nam Hom' coconut ('Kon Chip' strain), 'Nam Hom' coconut ('Kon Klom' strain) and 'Nam Wan'coconut. Moreover, the results indicated that the samples collected from the 'Nam Hom' coconut ('Kon Chip' strain) in different locations were for the main part genetically similar.

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