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Analysis of Random Amplified Polymorphic DNA (RAPD) of *Artemisia capillaris* (Wormwood capillary) in East Coast of Peninsular Malaysia

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Abstract: The genetic variability among individuals of *Artemisia capillaris* from Terengganu and Kelantan, Malaysia was examined by using random amplified polymorphic DNA (RAPD). Samples were collected from different regions in Terengganu and Kelantan State. The genomic DNA was extracted from leaves using Sarkosyl method. The results showed clear RAPD banding pattern. Fifthty-seven oligonucleotide primers were screened and ten primers were selected (OPA 04, OPA 09, OPA 16, OPA 17, OPA 18, OPG 03, OPG 05, OPG 09, OPG 15 and 391) to amplify DNA from ten samples of *Artemisia capillaris*. A total of 335 (Terengganu) and 370 (Kelantan) RAPD fragments which were all polymorphic fragments (100%) with size ranging from 150-3000 bp were scored for Terengganu samples, while 124 polymorphic fragments (95%) with size ranging from 200-2500 bp were scored for Kelantan samples. Genetic distance for samples ranges from 0.0000 to 0.26000 (Terengganu) and 0.1300 to 1.5300 (Kelantan). Similarity index ranges from 0.0000 to 0.7838 (Terengganu) and 0.1167 to 0.8758 (Kelantan).

Key words: *Artemisia capillaris* . genomic DNA . sarkosyl . genetic variability . RAPD

INTRODUCTION

Artemisia capillaris is a species from class Magnoliopsida and family Asteraceae. It is know as wormwood or wormwood capillary in European [1] Yin Chen Hao in China [1, 2] and in common names are Pokok Ru Nyamuk, Pokok Daun Ru, Pokok Halau Nyamuk and Pokok Jata Hitam in Terengganu. *Artemisia capillaris* is a member of the parsley family, is a strong-smelling, fennel-like, annual plant reaching a height of about 4 feet or more. This plant was introduced to this country from Asia [3, 4] America and Europe [3]. It is cultivated in China, Japan, Taiwan [1] and some extent in this country. Small acreages of *Artemisia capillaris* have been grown successfully as a commercial crop.

Artemisia plants, particularly *A. iwayomogi*, *A. capillaris*, *A. princeps* and *A. argyi*, are important medicinal materials that are utilized in traditional Asian medicines [5]. *Artemsia* herbs are used for various purposes, such as medicine, spices, food and ornamentation [6]. *Artemisia capillaris* is considered to be a bitter and cooling herb, clearing "damp heat" from the liver and gall ducts and relieving fevers [7]. It is widely used in Asia to prevent and treat neonatal jaundice, also effective remedy for liver problems, works on stomach and spleen [7-9]. Modern research has confirmed that the plant has a tonic and strengthening effect upon the liver, gallbladder and digestive system [7]. Studies from [10] suggest that *Artemisia capillaris* can be a useful therapeutic agent for endotoxin-induced inflammation and injuries of the liver.

Owing to recent innovation in molecular biological techniques, such as Polymerase Chain Reaction (PCR) and DNA automated sequencing, nucleic acid data are becoming more and more important in biology [11]. One of the modern marker techniques for studying genetic variability is Random Amplified Polymorphic DNA, RAPD [12]. The technique requires no prior knowledge of the genome and it needs only a small amount of DNA [13]. Using this technique polymorphism can be detected in closely related organism.

RAPD preferred by many researchers as one an effective method to use for identification of genetic variation within and among populations in forest trees [14-16]. Beside that RAPD markers also show levels of polymorphism similar to isoenzyme markers [17]. RAPD analysis also can target amplifying a large number of loci [17, 18].

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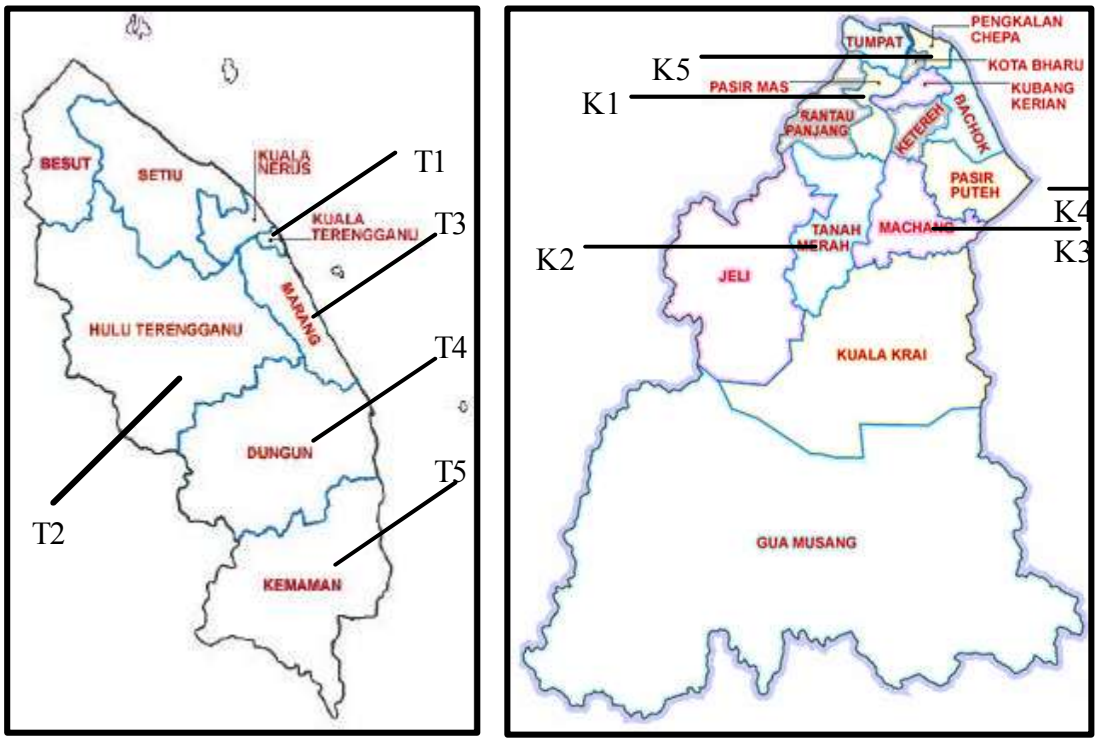

Fig. 1: Sampling sites

Genetic variation within and between populations is essential for the establishment of effective and efficient conservation for plants. Previous study of *Artemisia vulgaris* had reported that this *Artemisia vulgaris* had morphological and physiological variability in differences ecological regions [19] and this is same to *Artemisia capillaris*. As an old crop, the variety (wild) of *Artemisia capillaris* in Malaysia has also been presenting a wide range of morphological variation that could provide an important source of genetic material for selection and improvement of the crop. There are lacks of molecular data for this species in paper publish for molecular study and there is not yet study in molecular field for this *Artemisia capillaris* in Malaysia. Such ambiguous classification was not followed accordingly by the grouping based on the other characters. This may create a misunderstanding when identifying and selecting the materials for genetic improvement and conservation. The objectives of this study are to assess the degree of polymorphism of *Artemisia capillaris* and to establish the genetic database on the genetic variability of *Artemisia capillaris.*

MATERIALS AND METHODS

Sample collections: *Artemisa capillaris* samples were collected from different regions in state of Terengganu and Kelantan. All samples were collected randomly. Figure 1 shows the sampling sites.

DNA extraction: Sarkosyl Nitrogen Method was used for DNA extraction method [20]. Fresh and healthy leafs were used, then were placed in a mortar and transformed to powder using liquid nitrogen. 3ml of DNA extraction buffer were added in a fresh mortar and were homogenized. Then, 1 ml phenol was added and was homogenized again. The mixture was transferred to a test tube (with cap), 2 ml phenol were added and were centrifuged for 5 minute at 12 000rpm to separate phase. The upper aqueous phase was transferred into new tube. Then, two volumes of ice-cooled ethanol (95%) were added to the aqueous phase to precipitated DNA. The tubes were centrifuged for 5 minutes, 12 000rpm. Precipitated DNA was washed with cooled 70% ethanol. DNA was dissolved in 0.5 ml of TE and 2 µg RNAase were added. After incubation at 37°C for 30min, 0.25 ml phenol and 0.25 ml chloroform were added and tubes were well shaked. They were centrifuged and the upper aqueous phase was transferred into new tube. Two volumes of ice-cool 95% ethanol were added to the aqueous phase for ethanol precipitated DNA and were centrifuged for 5 minutes, 12 000rpm. Ethanol was poured from tube. Precipitated DNA was washed with ice-cool 70% ethanol. Pellet was dissolved in 0.2 to 0.5 ml of TE.

Table 1: Code, sequence and nucleotide length of primers used in the RAPD analysis

	Primer Code	P Primer sequence 5' to 3'	Nucleotide length
1	OPA 04	AATCGGGCTG	10-mers
2	OPA 09	GGGTAACGCC	10-mers
3	OPA 16	AGCCAGCGAA	10-mers
4	OPA 17	GACCGCTTGT	10-mers
5	OPA 18	AGGTGACCGT	10-mers
6	OPG03	GAGCCCTCCA	10-mers
7	OPG05	CTGAGACGGA	10-mers
8	OPG09	CTGACGTCAC	10-mers
9	OPG13	CTCTCCGCCA	10-mers
10	391	GCGAACCTCG	10-mers

Gel electrophoresis: Product was separated by agarose gel electrophoresis through 1.0 % agarose gel in 1.0 X TBE (10 mM Tris, 1mM EDTA pH 8.0, 1µl EtBr). Electrophoresis was conducted at 75 volts for 1 hour for genomic DNA and 55 volts for 1 to 2 hours for PCR amplifications. Gels were photographed with Image Master VDS.

Measurement of DNA purity and quality: The yield of DNA was measured using a UV-Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm [21]. The DNA concentration was determined by the formula:

$$\text{DNA concentration} = OD_{260} \times 50\ \mu g/ml \times \text{dilution factor [22].}$$

Screening of RAPD primers: Decamer oligonucleotides were used in this study. 57 RAPD primers (Table 1) were screened from a single individual. Primers that gave clear and polymorphic profiles were chosen for further study [23].

DNA amplification of selected primers: The selected primers were used to amplify the genomic DNA for ten samples of *Artemisia capillaris*. The total reaction volume of 25 µl was used with the final concentration containing 1.0× of reaction buffer included the concentration of genomic DNA 50ng, Fermentas Magnesium Chloride 4.0mM, Fermentas Taq DNA Polymerase 2units, Fermentas dNTP-mixture 0.4 mM and primer 10 pM.

The DNA was amplified by using a DNA Engine Thermal Cycler with Dual Alpha Unit (BIO-RAD). The amplification was programmed at 45 cycles for 30 seconds of denaturation at 94°C, 30 seconds of annealing temperature at 36°C, 1 minute of primers extension at 72°C and final extension of 2 minutes at 72°C.

PCR products were electrophoresed on 1.5% (w/v) agarose gel in 1.0 × TBE buffer at 55 V for 1 to 2 hour depending on the size of amplified fragment from each primer. The gel was stained in 1µg/mL Ethidium Bromide (EtBr) for 20 to 30 minutes. Then the gel was washed with distilled water for 5 to 10 minutes and photographed with Image Master VDS. A 100bp ladder plus maker (Fermentas) was used as a molecular weight standard . Each set of PCR products included with the negative control to ensure that the observed banding pattern was reproducible, repeatable and uncontaminated before scoring.

Data analysis: The RAPDistance Package Software Version 1.04 [24] and Numerical taxonomy and Multivariate Analysis System (NTSYS-pc) were used in this study. The molecular weights of bands were estimated based on the standard bands from Gene Ruler DNA Ladder Marker. The presence of band was scored from the photograph. Only clear and reproducible bands were given consideration.

These bands were considered as polymorphic when they were absent in some sample in frequency greater than 1% [25] and change in band intensity were not considered as polymorphism. Clear bands were scored as present (1) or absent (0) at particular position or distance migrated on the gel. The data matrix of 1's and 0's was prepared from the scorable bands and was entered into the data analysis package [24].

The indexes of similarity were calculated across all possible pair wise comparisons of individual within and among population following the method of Nei and Li [26]. The formula was:

$$SI = 2NXY/(NX+NY)$$

NXY is the number of RAPD bands shared in common between individuals X and Y, NX and NY are the total number of bands scored in X and Y respectively.

The similarity index was used to calculate the genetic distance values and to construct the dendrogarm. The dendrogarm provides a visual representation of the differences in the population of *Artemisia capillaris*. The dendrograms were constructed using the Unweighted Pair-Group Method of Arithmetic (UPGMA) employing Sequential, Agglomerative, Hierarchical and Nested Clustering (SAHN) from NTSYS-pc program [27].

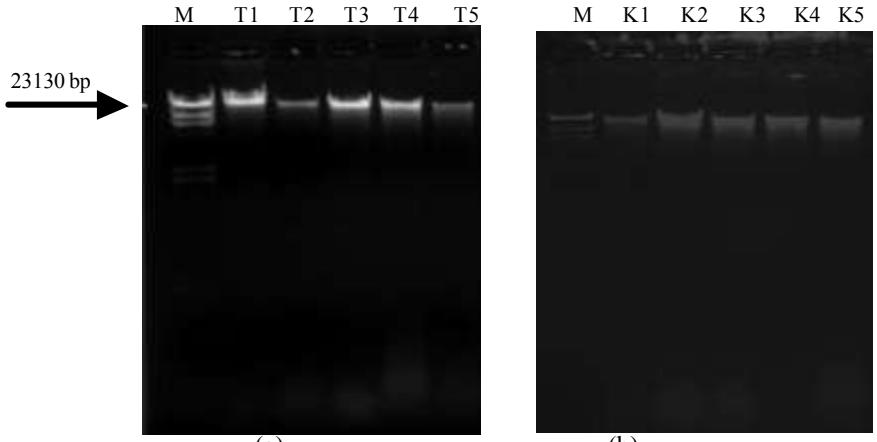

Fig. 2: Genomic DNA extracted on 1.0% agarose gel, λ DNA/Hind III marker (lane M) and representative samples of *Artemisia capillaris* a (lane T1 to T5) and b (lane K1 to K5)

Table 2: RAPD analysis for Terengganu population

Primer	Number of fragments	Size of fragments (bp)	Total number of fragments	Number of polymorphic fragments	Percentage of polymorphic (%)
OPA 04	0-7	300-1750	9	9	100.00
OPA 09	0-11	350-2500	14	14	100.00
OPA 16	0-11	650-3000	11	11	100.00
OPA 17	0-10	450-2000	12	12	100.00
OPA 18	0-10	750-2500	12	12	100.00
OPG 03	0-7	300-1200	10	10	100.00
OPG 05	0-14	250-2500	20	20	100.00
OPG 09	0-11	250-1350	13	13	100.00
OPA 13	0-14	250-1750	17	17	100.00
391	0-12	150-2500	12	12	100.00
Total	-	-	130	130	100.00

RESULTS AND DISCUSSION

Extraction of DNA: Genomic DNA was successfully extracted and observed to have sharp band. Using this method, the clear band and purity of DNA was obtained (Fig. 2). Extraction DNA from samples is the first step for all molecular marker type. DNA can be extract either from fresh, lyophilized, preserved or dried samples but to have good quality DNA fresh material is recommended [28]. There are difficulties to get plant DNA free from contaminating proteins and polysaccharides. In this method sarkosyl was used as for proteins remover. Most of the plant cells had very tough cell wall and make used vigorous method to breaking the cell [29]. The excessive force makes the degradation very high molecular weight molecules thought the shearing. The force make also can produce DNase and made low quality of DNA.

Screening of RAPD primers: Chosen suitable primers is very important process for PCR-RAPD to get clear and good band. Fifty-seven primers from the Operon and UBC (University of British Columbia, Canada) 10 mers were used during the screening of the RAPD primers. The banding patterns which were clear and reproducible bands selected. The selected primers were OPA 04, OPA 09, OPA 16, OPA 17, OPA 18, OPG 03, OPG 05, OPG 09, OPG 15 and 391. These primers were selected to generate RAPD pattern for all individuals of *Artemisia capillaris* samples.

Some polymorphisms were easy to score whereas other bands appeared to produce ambiguous fragments [12]. The best primers will produce more than three clear fragments. The number of fragments generated depends on the primer sequence rather than the nucleotide length.

RAPD profiles: RAPD technique has been used in genetic study for wheat [30] and for rice species [31]. Ten primers were applied to ten individuals of *Artemisia capillaris* for DNA amplification. The results showed different primers generated different fragment numbers and length of DNA amplification products as shown in Table 2 and 3. Different primers produced different fragment patterns.

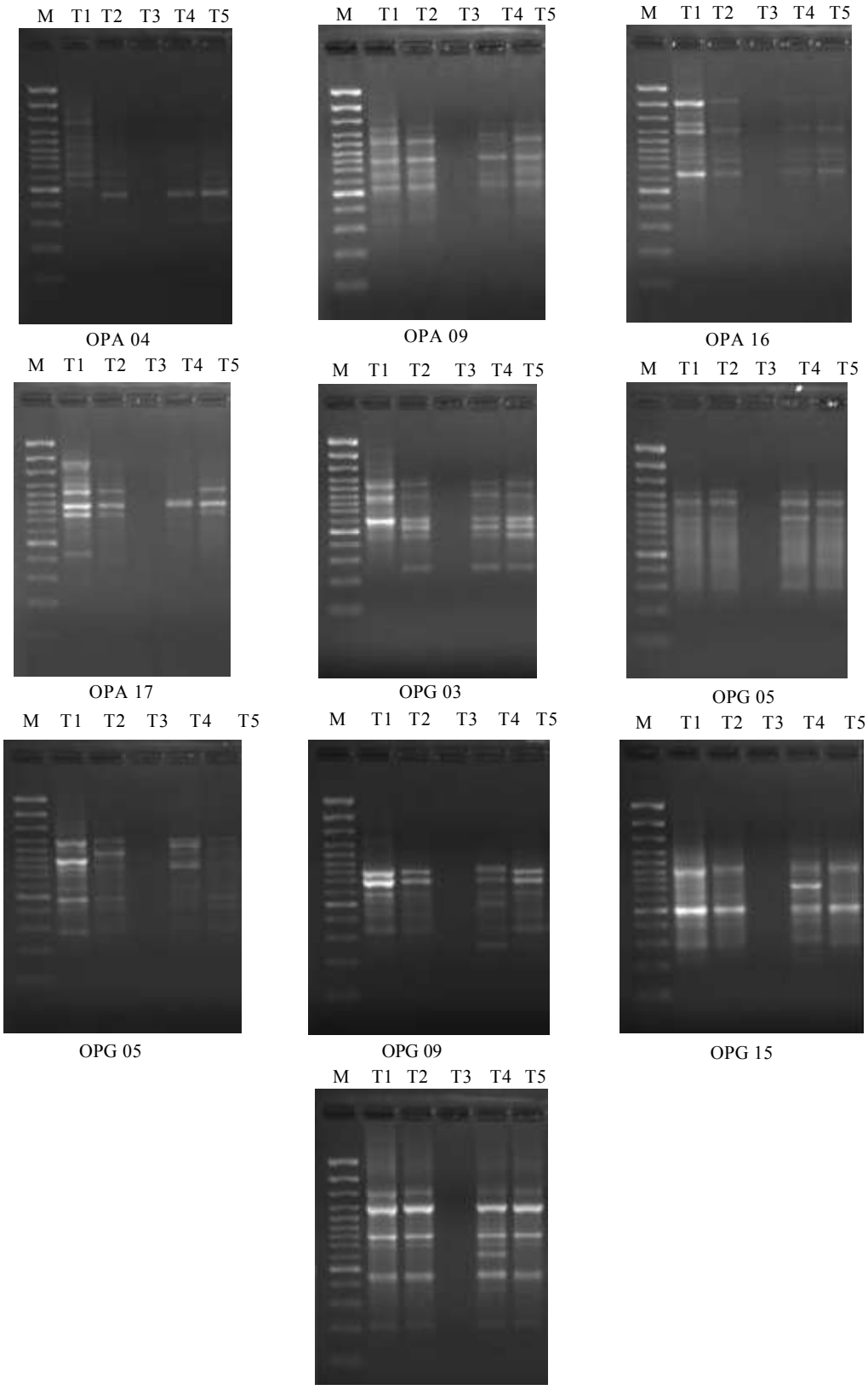

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Fig. 3: Banding patterns of RAPD fragments of *Artemisia capillaris* individuals from Terengganu. (Lane M is a marker 100 bp ladder plus. Individuals T1 to T5, left to right)

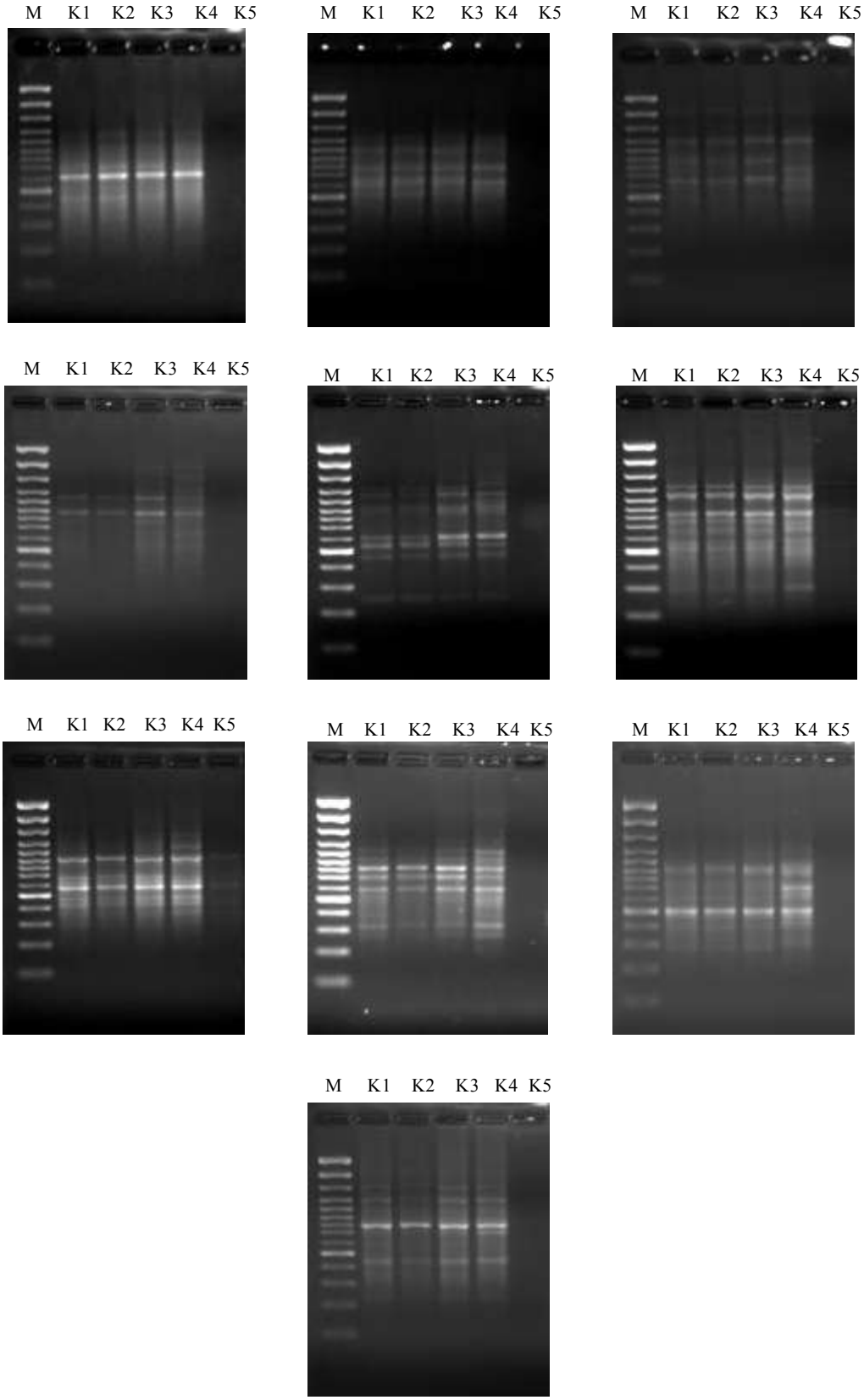

Fig. 4: Banding patterns of RAPD fragments of *Artemisia capillaris* individuals from Kelantan. (Lane M is a marker 100 bp ladder plus. Individuals K1 to K5, left to right)

Table 3: RAPD analysis for Kelantan population

Primer	Number of fragments	Size of fragments (bp)	Total number of fragments	Number of polymorphic fragments	Percentage of polymorphic (%)
OPA 04	0-9	400-1116	11	11	100.00
OPA 09	0-5	550-1200	6	6	100.00
OPA 16	0-9	450-2500	10	10	100.00
OPA 17	0-11	350-2500	13	13	100.00
OPA 18	0-12	250-1500	14	14	100.00
OPG 03	5-12	250-1350	16	14	87.50
OPG 05	6-13	300-1350	15	11	73.33
OPG 09	0-14	200-1500	16	16	100.00
OPA 13	0-15	250-1500	16	16	100.00
391	0-12	250-2000	13	13	100.00
Total	-	-	130	124	95.38

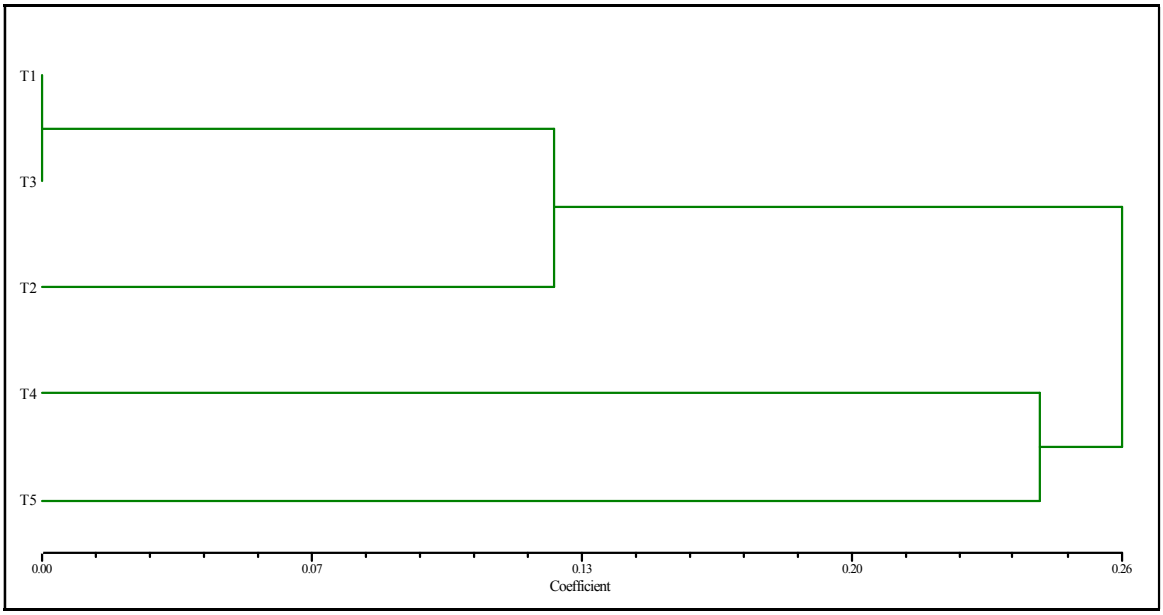

Fig. 5: Dendrogram of *Artemisia capillaris* from Terengganu State generated by primer OPA 04, OPA 09, OPA 16, OPA 17, OPA 18, OPG 03, OPG 05, OPG 09, OPA 13 and 391

335 fragments were generated by the ten primers for Terengganu samples. OPA 04 generated 16 fragments, OPA 09 generated 33 fragments, OPA 16 generated 29 fragments, OPA 17 generated 22 fragments, OPA 18 generated 35 fragments, OPG 03 generated 28 fragments, OPG 05 generated 43 fragments, OPG 09 generated 41 fragments, OPG 13 generated 45 fragments and OPG 391 generated 43 fragments (Fig. 3). The ten primers yielded 0 to 14 amplification products. The percentage of polymorphism generated for all primer was 100%. The size of bands generated by OPA 04, OPA 09, OPA 16, OPA 17, OPA 18, OPG 03, OPG 05, OPG 09, OPG 13 and 391 ranged from 150bp to 3000bp. The results show that all samples had different banding patterns.

Compare to samples from Terengganu, Kelantan samples produced a total of 370 fragment bands. The ten primers yielded 0 to 15 amplification products. OPA 04 generated 33 fragments, OPA 09 generated 19 fragments, OPA 16 generated 24 fragments, OPA 17 generated 28 fragments, OPA 18 generated 41 fragments, OPG 03 generated 46 fragments, OPG 05 generated 54 fragments, OPG 09 generated 42 fragments, OPG 13 generated 42 fragments and OPG 391 generated 41 fragments (Fig. 4). The percentage of polymorphism generated for all primer was 96%. The size of bands generated by OPA 04, OPA 09, OPA 16, OPA 17, OPA 18, OPG 03, OPG 05, OPG 09, OPG 13 and 391 ranged from 200bp to 2500bp.

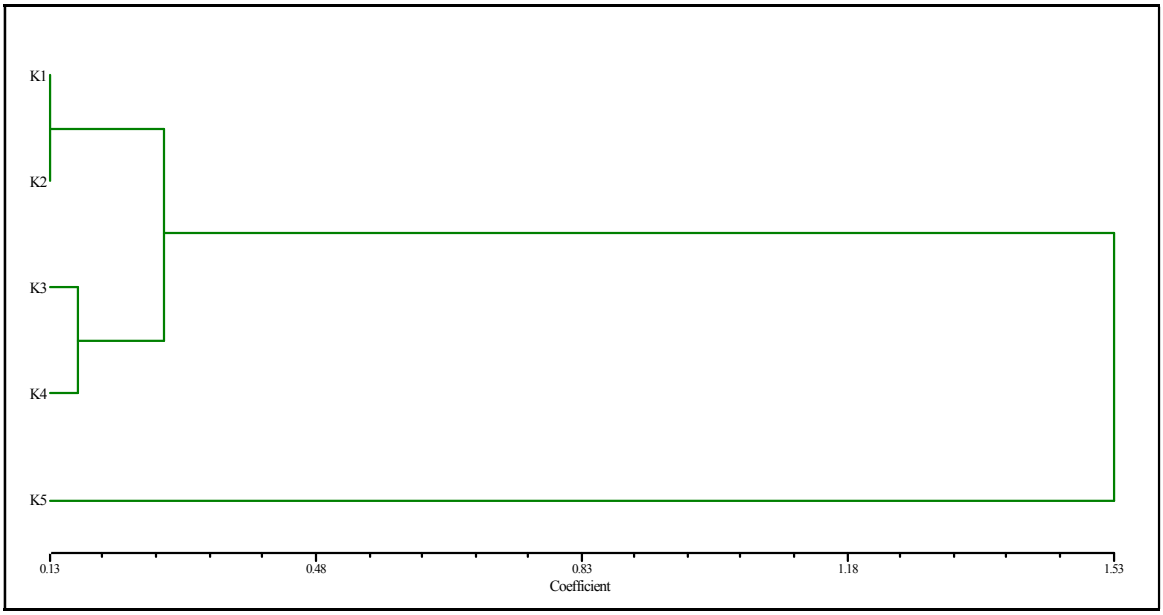

Fig. 6: Dendrogram of *Artemisia capillaris* from Kelantan State generated by primer OPA 04, OPA 09, OPA 16, OPA 17, OPA 18, OPG 03, OPG 05, OPG 09, OPA 13 and 391

The absence of amplification for sample T3 and K5 could be due to DNA quality. According to Nguyen [32] RNA and other contaminants would not have been easily detected when performing gel electrophoresis. Ratios above 2.0 correspond to RNA contamination, while ratios below 1.6 suggest protein contamination [33]. For some genomes, the purity of the DNA sample will not affect the amplification reaction [34]. Beside that genomic DNA concentration is an important factor in whether the reaction is productive or not. According to Chen [35] if there is too much DNA, there would be increased chances for contaminants. The PCR cycle conditions can be one of the factors, like at certain temperatures, the primers might not bind to the template DNA [36]. Other than DNA, this could result from imprecise pipetting or unintentional overlooking when doing master mix for PCR reaction. Another factor for unsuccessful PCR product might be due to the primers not finding complementary bases on the template DNA [37].

Dendrogram analysis: The dendrogram produced of Terengganu samples (Fig. 5) shows two main clusters. The first cluster consisted of samples T1, T2 and T3. The second cluster was consisted of samples T4 and T5. These two groups were joined together at about 0.260 genetic distance level. Samples T1 and T3 were linked together at a cluster 0.000 genetic distance level, the sample T2 were linked together at a cluster 0.125 genetic distance level, the sample T4 and T5 were linked together at a cluster 0.240 genetic distance level and were linked together with all sample at a cluster 0.260 genetic distance level. For the all cluster, they consisted of all individuals members T1, T2, T3, T4 and T5. Genetic distance levels of *Artemisia capillaris* ranged from 0.000 to 0.260.

Dendrogram cluster for Kelantan samples shows three main clusters (Fig. 6). The first cluster consisted of samples K1 and K2. The second cluster was consisted of samples K3 and K4. Last cluster only sample K5. These three groups were joined together at about 1.530 genetic distance level. Samples K1 and K2 were linked together at a cluster 0.130 genetic distance level. The sample K3 and K4 were linked together at a cluster 0.165 genetic distance level and then were link with first cluster (K1 and K2) at 0.277 genetic distance level. The all samples were linked together at a cluster 1.530 genetic distance level. For the all cluster, they consisted of all individuals members K1, K2, K3, K4 and K5. Genetic distance levels of Kelantan samples ranged from 0.130 to 1.530.

The similarity index for *Artemisia capillaris* within Terengganu ecotypes ranged between 0.0000 and 0.7838. This high similarity in samples indicated that low variability between individuals in that area. While the similarity index for *Artemisia capillaris* within Kelantan ecotypes ranged between 0.1167 and 0.8758. The similarity indices among individuals in each sample of *Artemisia capillaris* are represented in Table 4 and 5. Some samples got very low similarity indices and some got high similarity indices. The similarity indices show the relationship of the

Table 4: Similarity index of Terengganu population

Sample	T1	T2	T3	T4
T2	0.7742			
T3	0.0000	0.0000		
T4	0.6057	0.7355	0.0000	
T5	0.6145	0.7296	0.0000	0.7838

Range = 0.0000-0.7838, Average = 0.42433, SD = 0.369824

Table 5: Similarity Index of Kelantan population

Sample	K1	K2	K3	K4
K2	0.8758			
K3	0.8023	0.8118		
K4	0.6984	0.6813	0.8447	
K5	0.1319	0.1667	0.1296	0.1167

Range = 0.1167-0.8758, Average = 0.529064, SD = 0.340730

individual in each sample. Higher similarity indices suggest that the individuals in the population have closer genetic relation among them, while lower similarity indices suggest that the individuals in the population have farther genetics relation.

Similarly, quantity and purity of extracted genomic DNA also plays crucial role for analysis of molecular diversity and optimization of different parameters for PCR [38, 39]. The genetic similarity of the samples slightly correlated with their close geographic locations. The past study from Ash *et al*. [40] showed that there is distinct genetic variability within the *Cartahmus lanatus* population in Australia. Vellend and Waterway [41] also observed the genetic diversity within *Carex rariflora* population form different habitat and locations in Canada.

Diversity within terengganu and kelantan ecotypes: The study showed the presented of diversity in both Terengganu and Kelantan samples (Fig. 5, 6 and Table 4, 5). From the 57 primers screened, ten primers were polymorphic to both samples (Table 2 and 3). The size of ten primers ranged from 250 to 3000 bp. Out of these, the number of amplified fragments ranged from 335 to 370 with 73.33 to 100% of polymorphism fragments. The ten primers used for assessment of the both stated samples were found to be variable within the regions. The differences number of amplified fragments and percent polymorphic fragments between Terengganu and Kelantan samples is due to the difference samples there are from difference places.

The genetic similarity within Terengganu samples ranged from 00 to 78%. While the genetic similarity within Kelantan samples ranged from 12 to 88%. Similarity index >0.8 concidered high and crop is having a narrower genetic variability [42]. For Terengganu population, samples T1, T2, T3 and T4 showed similarity index >0.6, mean more variably. For Kelantan population, sample K1, K2, K3 and K4 showed similarity index >0.68, while sample K5 compared to other samples showed similarity index >0.1.

Study from Burtos *et al*. [43] suggested the level of intra and inter-population variation detected with RAPD markers to be different from each taxonomic unit, partially depending on the reproductive system of each for the genus *Hordeum* population. The genetic similarity of the Terengganu and Kelantan samples slightly correlated with their close geographic locations. This phenomenon was also observed in the morphological characteristics of barnyardgrass in Indonesia [44]. Some of the Terengganu samples were found to be closely correlated with the distance of geographical locations, for example samples T4 and T5. This may be due to seed dispersal through animal or air movement.

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