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Study of genetic variability of Wormwood capillary (*Artemisia capillaris*) using inter simple sequence repeat (ISSR) in Pahang region, Malaysia.

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Abstract

The genetic variability of five individuals of *Artemisia capillaris* from state of Pahang, Malaysia was examined using Inter Simple Sequence Repeat (ISSR) technique. Samples were collected from different regions in Pahang State. Results showed distinctive ISSR banding pattern in this species. Twenty five primers were applied and only ten were selected (807, 809, 825, 834, 841, 862, 866, 876, nIssr 1 and nIssr 3) as reliable amplifying ISSR markers. A total of 90 ISSR bands including 62 polymorphic (68.89%) with amplicon size ranging from 150–2500 bp were scored. Genetic distance for samples ranged from 0.0500 to 0.4200. For similarity index samples were ranged from 0.6400 to 0.9545.

Keywords*: Artemisia capillaries*; phylogenic; variability; polymorphism; wormwood.

Abbreviations: ISSR_Inter Simple Sequence Repeats.

Introduction

Artemisia capillaris is known as wormwood or wormwood capillary in English. *Artemisia* is a big genus and about 500 plants belong to this genus. *Artemisia capillaris* was introduced to Malaysia from Asian countries (Duane and Martha, 2006). It has usually been cultivated in China, Japan, Taiwan and some extent in this country. *Artemisia capillaris* is a perennial plant (Torrell et el., 2003), from a member of the parsley family with some distinct properties such as strong fennel-like smell and height about 4 feet or more.

Artemsia herbs are used for various purposes, such as medicine, food, spices and ornamentation (Lee et al., 2006). The Korean Herbal Pharmacopoeia, (2002) stated that *Artemisia capillaris* is important medicinal materials that are utilized in traditional Asian medicines. *Artemisia capillaris* considered to be a bitter and cooling herb, clearing "damp heat" from the liver and gall ducts and relieving fevers (Chevallier, 1996). *Artemisia capillaris* widely used in Asia to prevent and treat neonatal jaundice, also effective remedy for liver problems, works on stomach and spleen (Chevallier, 1996; Huang et al., 2003; Abestmall, 2006). The studied from Hong et al., 2004 suggests that *Artemisia capillaris* can be a useful therapeutic agent for endotoxin-induced inflammation and injuries of the liver. *Artemisia capillaris* also used as anti platelet aggregation activity and against HIV replication in H9 lymphocytic cells (Wu et al., 2001). This herbal drug has been widely used as a choleretic and anti-inflammatory agent for hepatitis and fatty liver (Nan et al., 2002). Owing to the recent innovation in molecular biological techniques, such as polymerase chain reaction (PCR) and DNA automated sequencing, nucleic acid data are becoming more and

Primer	Code	Primer sequence 5' to 3'	Length
1	801	ATATATATATATATATT	17-mers
2*	807	AGAGAGAGAGAGAGAGT	17-mers
3*	809	AGAGAGAGAGAGAGAGG	17-mers
4	811	GAGAGAGAGAGAGAGAC	17-mers
5	818	CACACACACACACACAG	17-mers
6	820	GTGTGTGTGTGTGTGTC	17-mers
7*	825	ACACACACACACACACT	17-mers
8	830	TGTGTGTGTGTGTGTGG	17-mers
9*	834	AGAGAGAGAGAGAGAGYT	18-mers
10	836	AGAGAGAGAGAGAGAGYA	18-mers
11*	841	GAGAGAGAGAGAGAGAYC	18-mers
12	848	CACACACACACACACARG	18-mers
13	857	ACACACACACACACACYG	18-mers
14*	862	AGCAGCAGCAGCAGCAGC	18-mers
15*	866	CTCCTCCTCCTCCTCCTC	18-mers
16	870	TGCTGCTGCTGCTGCTGC	18-mers
17*	876	GATAGATAGACAGACA	16-mers
18	878	GGATGGATGGATGGAT	16-mers
19	881	GGGTGGGG TGGGG TG	15-mers
20	888	BDBCACACACACACACA	17-mers
21	890	VHVGTGTGTGTGTGTGT	17-mers
22*	nIssr1	GATAGATAGATAGATA	16-mers
23	nIssr2	GACAGACAGACAGACA	16-mers
24*	nIssr3	CAGCAGCAGCAGCAG	15-mers
25	nIssr4	CAACAACAACAACAA	15-mers

Table 1. Sequence and length of primers used in the ISSR analysis. *=primers that were selected.

more important in biology (Hillis et al., 1996). ISSR-PCR technique has been chosen to investigate genetic variation among five different populations in this study. This method is widely used for population studies because they are highly variable, require less investment in time, money, labor than other methods (Wolfe and Liston, 1998; Harris, 1999). According to Esselman et al., (1999), ISSRs can generate higher percentages of polymorphic loci than other PCR methods. Ajibade et al., (2000) and Galvan et al., (2003) concluded that ISSR would be a better tool for phylogenetic studies. ISSR technique is also very simple, fast, cost-effective, highly discriminative and reliable (Pradeep et al., 2002).

ISSRs had reported that used in studies of cultivated species to produce genetic linkage maps (Kojima et al., 1998; Cekic et al., 2001) and to determine the relatedness of lines of agriculturally important species (Jain et al., 1999; Chowdhury et al., 2002; Mondal, 2002). The ISSRs have also been instrumental in determining variability and correcting misidentifications in large germplasm collections (Fang et al., 1997; Gilbert et al., 1999; Lanham and Brennan, 1999; Charters and Wilkinson, 2000).
Understanding of the genetic variation within and between populations is essential for the establishment of effective and efficient conservation for plants. *Artemisia capillaris* has shown morphological and physiological variation in different ecological regions (Barney and Di Tommaso, 2003). Barney and Di Tommaso, (2003) suggested that *Artemisia vulgaris* has high morphological and genetic variability and could possibly be classified as different species. As an old cultivated plant, *Artemisia capillaries* (wild type) in Malaysia has shown a wide range of morphological variation that could provide an important source of genetic material for selection and improvement of this crop. However, there is not sufficient molecular study and data for *Artemisia capillaris* in Malaysia. In this study we evaluated the genetics variation of some important populations of *Artemisia capillaries* in Malaysia for further classification purposes.

Materials and methods

Sample collections

The wild type samples of *Artemisa capillaris* were collected from some areas of Pahang state randomly, including Kuantan, Pekan, Maran, Temerloh and Rompin. These sampling sites were far enough from each other.

DNA extraction

The Sarkosyl method was used for DNA extraction from plant material (Hasan et al., 2008).

ISSR

Twenty five ISSR primers from UBC (University British Columbia) were examined (Table 1). Primers that showed sharpness, clarity existence of polymorphism were chosen for further studies (D'Amato and Corach, 1997). The total reaction volume of 25 µl was used with the final concentration containing 1X reaction buffer, 50 ng genomic DNA, 3.0 mM of $MgCl_2$, *Taq* polymerase (2.5 units), 0.4 mM dNTPs and 10 µM primer. ISSRs were amplified using a DNA Engine Thermal Cycler With Dual Alpha Unit (Bio-Rad). The amplification was programmed for 1 cycle for 2 min at 93^0C, 2 min at 35^0C and 2 min at 72^0C. Then, 93^0C at 1 min for denaturation, 2 min of annealing temperature at 36^oC, 2 min of primers extension at 72^oC at 38 cycles and final extension of 10 min at 72^oC. PCR products were run on 1.5% (w/v) agarose gel in 1x TBE buffer at 55 V for 1 to 2 h depending on the size of amplified fragment produced from each primer. The gel was stained in 1 µg/mL ethidium bromide for 20 to 30 min and photographed with Image Master VDS.

Data Analysis

The Numerical taxonomy and Multivariate Analysis System (NTSYS-pc) were used in this study. The molecular weights of band were estimated based on the standard bands from Gene Ruler and DNA Ladder Marker. The presence of band was scored from the photograph. Only clear and reproducible bands were considered.

These bands were considered as polymorphic when they were absent in some sample in frequency greater than 1 % (Jorde, 1995). 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 been prepared from the scorable bands and was entered into the data analysis package (Armstrong et al., 1994). The indces of similarity were calculated across all possible pair wise comparisons of individual within and among populations following the method of Nei and Li (1979). The formula was: SI=2NXY/(NX + NY) NXY is the number of ISSR 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 index similarity was used to calculate the genetic distance values and to construct the dendrogarm. The dendrogarm provides a visual representation of the relationship of different populations 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 (Rohlf, 1994).

Results and discussion

Optimum PCR condition

Genomic DNA was successfully extracted and observed to be confirmed to have bright distinctive bands (Fig 1). DNA can be extracted either from fresh, lyophilized, preserved or dried samples but for obtaining high quality DNA fresh material is recommended (Semagn et al., 2006). There were several difficulties to get plant DNA free from contaminating proteins and polysaccharides. In here Sarkosyl methods was used to remove proteins. The DNA purity of *Artemisia capillaris* ranged from 1.1616 to 1.4590 measured by UV-Biophotometer which is the optimum purity for amplification (Sambrook et al., 1989).

ISSR primer screening

Twenty five primers from the UBC (University of British Columbia, Canada) were used during the screening of the ISSR primers. The clear and reproducible bands were selected as suitable polymorphic primer. Selected primers are 807, 809, 825, 834, 841, 862, 866, 876, nIssr 1 and nIssr 3. These primers were selected to generate ISSR pattern of genomic DNA for all individuals of *Artemisia capillaris* samples.

The best primers produced more than three fragments. The number of amplified fragments is associated to the primer sequence rather than to the nucleotide length. Some polymorphisms were easy to score, according to Godwin et al, (1997). In some cases a single ISSR primer can generate more than 100 bands in vascular plants.

Table 2. Characterization of amplified DNA fragments.

Primer	Number of fragments	Size of fragments (bp)	Total number of fragments	Number of polymorphic fragments	Percentage of polymorphic (%)
807	4 – 8	350 – 2000	12	11	91.67
809	4 – 6	300 – 1031	6	2	33.33
825	4 – 6	250 – 966	6	2	33.33
834	5 – 7	200 – 2000	10	8	80.00
841	6 – 8	150 – 900	10	6	60.00
862	3 – 3	350 – 800	4	2	50.00
866	6 – 7	350 – 2000	10	6	60.00
876	3 – 8	250 – 2000	9	7	77.78
nIssr1	1 – 13	250 – 2500	15	15	100.00
nIssr3	5 - 8	300 - 900	8	3	37.50
Total	-	-	90	62	68.89

ISSR profiles

ISSR technique had already been used in genetic study for *Alliaria petiolata* (Meekins et al., 2001) and also for identification and differentiation of *Ficus carica* L. (Guasmi et al., 2006). Ten primers were applied on five individuals of *Artemisia capillaris* for DNA amplification. The results showed that different primers generated various numbers of fragments with different lengths of amplified products as shown in Table 2. The repeated sequence of the primer is also important. Study of ISSR profiles in mulberry that were generated from CA/TG repeat anchored primers showed that these repeats are abundant in the *Morus* genome (Awasthi et al., 2004). Vijayan and Chatterjee, (2003) also observed amplification of AC rich repeat based on ISSR primers. However, Fang et al., (1997) observed no amplified products using $(AT)_8$ repeats on trifoliate orange (*Poncirus trifoliata* Raf.), but $(CA)_n$ based primers have given excellent fingerprint patterns.

In our investigation 298 fragments were generated by the ten different primers. The primers 807, 809, 825, 834, 841, 862, 866, 876, nIssr1 and nIssr3 generated 31, 26, 25, 33, 34, 15, 32, 33, 41 and 28 fragments, respectively (Fig 2). The total percentage of polymorphism generated for all primers was 68.89%. The fragments were ranged from 150 bp to 2500 bp. The results show that samples had different banding patterns but some samples had same patterns which mean genotype similarity. Hassel and Gunnarsson, (2003) stated that primers had several characteristics that can affect the number and quality of DNA fragments amplified during PCR.

Phylogenic analysis

The dendrogram was produced for *Artemisia capillaries* samples that shows two main clusters (Fig 3). The first cluster consisted of individual numbers of P2, P3, P4 and P5. The second cluster was consisted of individual number of P1. These two groups were joined together on 0.420 genetic distance level. Sample P2 and P3 were linked together at a cluster with 0.050 genetic distance, the sample P4 linked at a cluster with 0.130 genetic distance, the sample P5 were linked together at a cluster 0.380 genetic distance level and were linked together with all sample at a cluster 0.420 genetic distance. For all clusters, they consisted of all individuals members P1, P2, P3, P4 and P5.

Genetic distance levels of *Artemisia capillaris* ranged from 0.050 to 0.420. The UPGMA cluster analysis, based on the genetic distance, was generated from Nei and Li's (1979). The function of dendrogram is to indicate the genetic relationship among samples means samples could potentially be originated from the same ancestor. The environmental effect might also be one of the reasons of different phylogenic information (Shibata, 2002). Low level of genetic distance indicates that these populations have very close phylogenic relationship among the region. However they were distinctly separated within clustering dendrogram. Genetic variations observed in some of the landraces are very narrow because they have long cultivation history in the geographical region and adaptation or local agroclimatic conditions have affected on species (Seehalak et al., 2006).

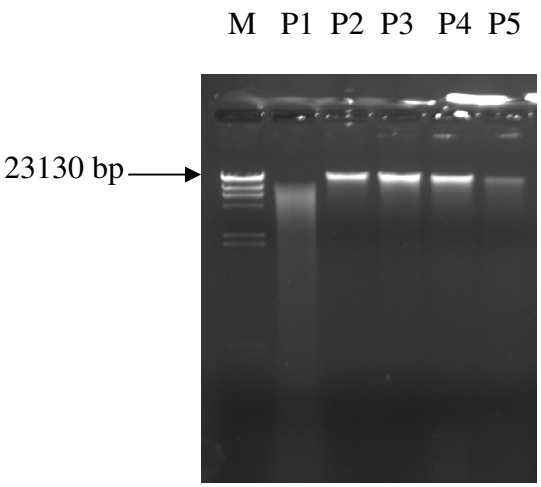

Fig 1. Genomic DNA extracted on 1.0% agarose gel and stained with 0.5 µg/mL ethidium bromide (EtBr), λ DNA/Hind III marker (lane M) and representative samples of *Artemisia capillaries* (lane P1- Kuantan, P2- Pekan, P3- Rompin, P4- Maran and P5- Temerloh).

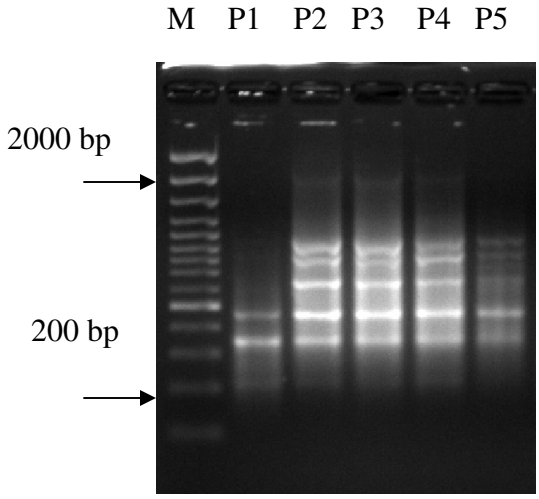

Fig 2. Banding patterns of ISSR fragments of *Artemisia capillaris* individuals using primer 834. (Lane M is a marker 100 bp ladder plus. Individuals P1 to P5, left to right).

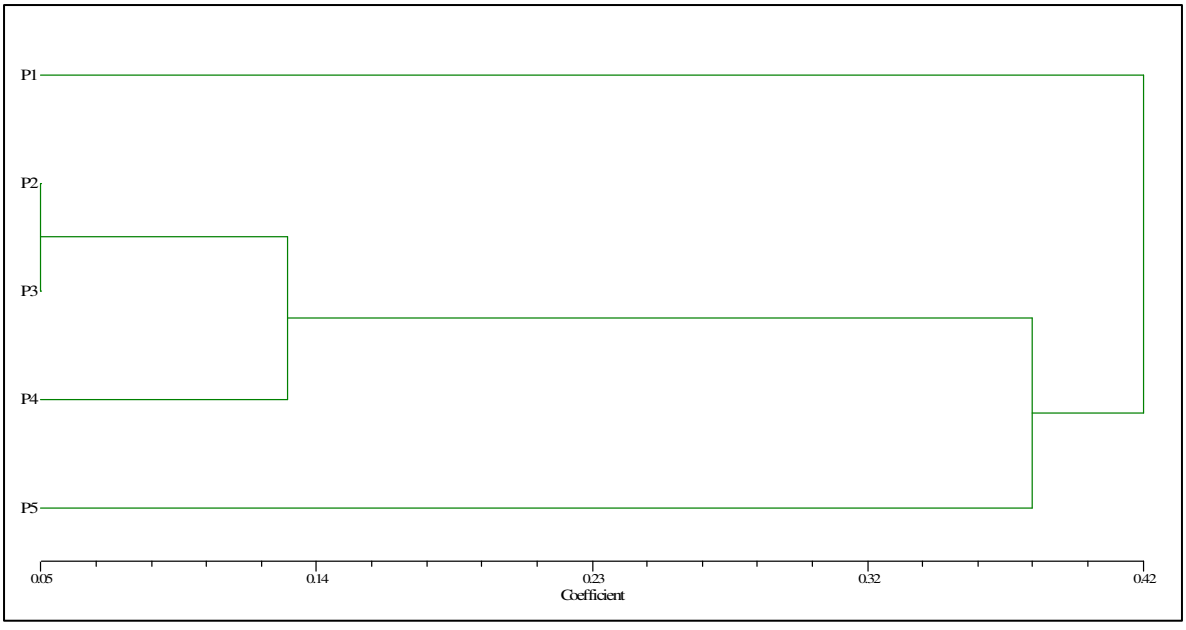

Fig 3. Dendrogram based on the genetic distance generated from Nei and Li's indices of *Artemisia capillaris* from Pahang State.

Table 3. Similarity index of *Artemisia capillaries*

Sample	P1	P2	P3	P4
P1				
P2	0.6605505			
P3	0.6422018	0.9545455		
P4	0.6422018	0.8636364	0.8939394	
P5	0.6400000	0.6504065	0.6666667	0.7317073

Range=0.6400000 – 0.9545455
Average = 0.7345856
SD=0.1218536

The average similarity index for *Artemisia capillaris* was calculated about 0.6400 to 0.9545. The similarity indices among individuals in each sample of *Artemisia capillaris* are represented in Table 3. This high similarity in samples indicated that there is low variability between individuals in studied area. The similarity indices show the relationship of the individual in each sample. Higher similarity indices suggest that the individuals in the population have closer genetic relation and vice versa. The high similarity indices slightly correlated with their close geographic location for Barnyardgrass (Tasrif et al., 2004). Fernández et al., (2002) reported high similarity index in 16 barley cultivars form different countries. This might be due to highly polymorphic, abundant nature of the microsattelites due to slippage in DNA replication.

Similarly, quantity and purity of extracted genomic DNA also plays crucial role for analysis of molecular diversity and optimization of different parameters for PCR (Staub et al., 1996). Some studies show ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants (Carvalho et al., 2004; Martins et al., 2004; Ramage et al., 2004; Modgil et al., 2005). ISSR also has been used to detect varieties and diversity in rice (Blair et al., 1999) and the endemic and endangered plant *Cycas guizhouensis* (Cycadacceae)(Long et al., 2004) that confirms capability of this method to evaluate genetic variation in plant species.

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