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Assessment of genetic relationships within *Bouea* (Anacardiaceae) accessions in Peninsular Malaysia using inter simple sequence repeats (ISSR) markers

Mohd Norfaizal Ghazalli[1]*, Muhamad Fahmi Yunus[1] and Abdul Latiff Mohammad[2]

[1]Strategic Resources Research Centre, MARDI Headquarters, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia.
[2]School of Environmental and Natural Resource Science, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.

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Malaysia is a genetically diverse hotspot for wild relatives of *Mangifera* and other Anacardiaceae members. The genus *Bouea* is interesting in that it has small sweet-sour edible fruits. This genus consists of two species, namely *Kundang (Bouea macrophylla)* and *Remia (B. oppositifolia)* which are of economic value. In this study, we highlighted the molecular evidences using inter simple sequence repeats (ISSR) markers to understand its genetic relatedness among accessions. A total of 52 ISSR have been screened on selected accession of *Kundang* and *Remia* from Peninsular Malaysia. Ten of the 52 primers for *Kundang* generated 89 scorable bands, where 45 bands (50.2%) were polymorphic. The simple matching coefficient of similarity provided similarity values ranging from 0.659 to 0.955 while relationship among the different accessions was distributed among three main divergent clusters. For *Remia*, 10 of the 52 primers tested gave 89 scorable bands, where 54 bands (59.5%) were polymorphic. The simple matching coefficient of similarity values ranged from 0.591 to 0.977 while the cluster analysis separated 23 accessions into two well-defined clusters. These studies indicate that there was considerable ISSR variation among the accessions reflecting variation among the accessions, which were morphologically indistinguishable. DNA polymorphism detected by ISSR analysis offers a useful molecular marker for the identification of different accessions of *Kundang* and *Remia.*

Key words: Biodiversity, *Bouea*, genetic diversity, molecular marker, ISSR.

INTRODUCTION

Forest genetic resources in Malaysia and Asia in particular are now on a rapid decline and in some regions their future looks jeopardized. According to the convention on biological diversity (CBD), individual countries are responsible for the conservation and sustainable use of their biological diversity. Mango (*Mangifera indica*) is probably the most well-known and best-loved fruit of the South Asian tropics (Mukherjee 1948). Other than mangoes, *Bouea* is also a well-known genus in Anacardiaceae which bears edible fruits (Kochummen 1989; 1996). This genus usually release turpentine-smelling sap which becomes black when exposed to the air (Kosterman et al., 1993). *Bouea* is characterized by their tree forms, glabrous branches, with simple leaves, opposite-decussate, coriaceous, glabrous, entire and petiolate, inflorescence axillary or terminal panicles, small-

sized flowers, male and bisexual, calyx 3-5 lobed, petals 3-5, imbricate, glabrous, keeled, stamens 3-5, filaments subulate, glabrous, inserted on the disc, anthers basifixed, apiculate; disc small, rounded, glabrous, ovary ovoid or sub-globose, 1-locular, 1-ovuled, style almost central, short, stout, stigma capitate or 3-fid, drupe, 1-locular. There are about three species in South-east Asia and Malesia (Chayamarit, 2010). In this study, we tried to understand the genetic relatedness between *Bouea* species using molecular markers. Molecular techniques have greatly aided in evaluating the plant variation where it can lead to an early identification of useful variants. One of the recent applications of new techniques of molecular biology is the rapid development in molecular markers and gene mapping as a tool for early detection of any variation.

DNA molecular markers based on polymerase chain reaction (PCR) have been widely used nowadays. These markers, showed along the DNA sequences that specify the location of desirable genetic traits and indicate specific genetic differences or polymorphism. Polymorphism involves the existence of different alleles of the same gene in plants or a population of plants. Thus, the use of molecular markers is to track the inheritance of genes influencing a trait of interest in a genetic improvement programme, management and manipulation of plant genomes, and to study plant cell responses to mutation can be used for enhancement of crop yields and quality (Ahloowalia and Maluszynski, 2001; Ye et al., 2005; Mba et al., 2007; Yunus et al., 2013).

Various techniques have been used to mark individual characters in segregating populations and construct genetic maps such as Inter Simple Sequence Repeats (ISSR), simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP).

The molecular approach for identification of plant genotypes is more effective com-pared to the traditional morphological markers, as it allows direct access to the plant genome. ISSR markers are PCR based technique which is easy, quick, simple and economical; developed by Zietkiewicz et al. (1994). This type of marker are reproducible due to their better stringency (high annealing temperature), require no gene sequence information neither prior genetic studies (Archak et al., 2003; Thimmappaiah et al., 2009).

ISSR markers have been successfully used for the assessment of genetic diversity and gene pool origin in common bean (*Phaseolus vulgaris* L.) (Galvan et al., 2003), wild populations of Chinese liquorice (*Glycyrrhiza uralensis)* (Yao et al., 2008), African edible seeded *Citrullus lanatus* (Dje et al., 2010), for wild strawberry (*Fragaria vesca*) (Cekic, et al., 2001) and also Jatropha (*Jatropha curcas*) (Noor Alam et al., 2013). For Anacardiaceae family, ISSR marker has been successfully used to study variation among species such as Mango (*Mangifera indica*) (Gonzalez et al., 2002; Rocha et al., 2012; Damodaran et al., 2012) and also Indian cashew (*Anacardium occidentale*) (Archak et al., 2003).

Although studies on genetic diversity in Anacardiaceae are well documented, limited study and research are available for underutilized species of Anacardiaceae such as those *Bouea*. Presently, only one report is available on the use of SSR molecular markers in germplasm characterization of *Remia* (*B. oppositifolia)* (Damodaran et al., 2013).

Currently, no specific ISSR marker is available for this species, and the development of a new marker for this species would be time-consuming and costly. Therefore a more practical approach is to use ISSR marker from mango study on *Bouea.* To our knowledge, this is the first report on application of ISSR marker to detect variations among *Bouea* species.

MATERIALS AND METHODS

Plant materials and DNA extraction

A total of 56 accessions of *Kundang* (*B. macrophylla*) and 23 accessions of *Remia* (*B. oppositifolia*) were collected from Peninsular Malaysia and used for DNA using protocol based on Murray and Thompson (1980) with some minor modifications where the mixture between dry tissue powder and extraction buffer was incubated for 2 h at 65°C with occasional gentle mixing.

Polymerase chain reaction (PCR) and primer screening

PCR reactions were carried out in 25 µl volume containing 3 mM $MgCl_2$, 0.2 mM dNTP, 2.5 µL Buffer (10X), 0.2 µL Taq DNA polymerase, 0.2 µM of primer, and 50 ng of DNA template. The thermal profile of the reaction was programmed as follows: one cycle of initial denaturation at 92°C for 1 min, followed by 30 cycles with the following cycle profile: DNA denaturation at 92°C for a minute, annealing at 40 - 60°C for 2 min, extension at 72°C for 2 min. The final extension was at 72°C for 5 min followed by cooling

*Corresponding author. E-mail: mnfaizal@mardi.gov.my.

Abbreviations: NTSYS, Numerical Taxonomy System version; **UBC,** University of British Columbia; **UPGMA,** unweighed pair-group method using arithmetic averages.

Table 1. Primers name, total amplified bands, polymorphic bands and polymorphism percentage for *Kundang* (*B. macrophylla*) in Peninsular Malaysia.

Primer name	Sequences (5' - 3')	Total amplified bands	Polymorphic bands	Polymorphism percentage
UBC-808	$(AG)_8$ GC	9	5	55.6
UBC-826	$(AC)_7$ A	10	5	50.0
UBC-827	(AC)8 G	9	2	22.2
UBC-834	$(AG)_8$ YT	9	5	55.6
UBC-840	$(GA)_8$ YT	9	6	66.7
UBC-841	$(GA)_8$ YC	10	5	50.0
UBC-846	$(CA)_8$ RT	8	5	62.5
UBC-855	$(AC)_8$ YT	10	5	50.0
UBC-873	$(GACA)_4$	5	2	40.
UBC-886	VDV $(CT)_7$	10	5	50.0
	Total	89	45	
	Mean	8.9	4.5	50.3

to 4°C. PCR products were separated by electrophoresis through 1.5% agarose gels in 1X TAE buffer at 70V for 120 min. The gels were stained using SYBR® Safe DNA Gel Stain and were documented using GeneSnap gel doc system.

Primer selection

Fifty two (52) primers of inter ISSR have been screened on selected accession of *Kundang* and *Remia* from Peninsular Malaysia. These primers belong to the Set 9# of the University of British Columbia (UBC) (Gonzalez et al., 2002). Out of 52 ISSR primers screened, only 10 ISSR primers consisting of UBC-808, 826, 827, 834, 840, 841, 846, 855, 873 and 886 were able to produce polymorphic banding patterns. Each primer had different annealing temperatures that ranged from 45.0 to 59.3°C.

Analysis of ISSR data

DNA amplified fragments showing polymorphisms were scored using a binary system of 0 (absent) and 1 (present). Genetic relatedness among the *Kundang* and *Remia* accessions was estimated using simple matching coefficient of similarity. The unweighed pair-group method using arithmetic averages (UPGMA) cluster analysis was performed using the Numerical Taxonomy System version 2.01 (NTSYS-pc) (Rohlf, 2000).

RESULTS

ISSR analysis of *Kundang*

Markers were selected based on their ability to generate a visible polymorphism among the samples. For the ISSR analysis performed in this study, only 10 of the 52 primers tested gave 89 scorable bands ranging from 500-3000 bp in size. Of these, 45 bands (50.2%) were polymorphic. The number of bands for each primer varied from 5 (UBC-873) to 10 (UBC-826, UBC-841, and UBC-886) with an average of 8.9 bands per primer. The percentage of polymorphism per primer ranged from 22.2 to 66.7% with an average of 50.3% (Table 1). This indicates that there was considerable ISSR variation among the accessions. Figure 1 shows the amplification profiles, generated by primers UBC-840 and UBC-855, across the different accessions.

The simple matching coefficient of similarity provided similarity values ranging from 0.659 to 0.955, indicating the genetic variation level among accessions (Data not shown). These coefficients reflected the genetic relationship between the accessions. The highest level of similarity between two accessions was found between *Kundang* 60 and *Kundang* 61, with a similarity coefficient of 0.955 (the shortest genetic distance) while minimum similarity coefficient (0.659) was observed between *Kundang* 34 and *Kundang* 66 (the highest genetic distance). Accessions with 100% (1.0) of similarity were not found, which indicates the absence of duplicates. The dendrogram obtained by the UPGMA method from the 89 bands scored for the 56 accessions is shown in Figure 2.

In addition, relationship among the different accessions was distributed among three main divergent clusters (Figure 2). The first cluster consisted of three accessions *Kundang* 1, *Kundang* 7 and *Kundang* 34; The second cluster composed of three sub-clusters (2A, 2B, and 2C): The sub-cluster 2A consisted of 16 accessions, while the sub-cluster 2B consisted of 13 accessions and the sub-cluster 2C consisted of 22 accessions. Meanwhile, the third cluster composed only of *Kundang* 65 and *Kundang* 66 (Table 2). Based on these results, ISSR successfully detected variation between the accessions, which were morphologically indistinguishable. The DNA polymorphism detected by ISSR analysis offers a useful molecular marker for the identification of different accessions of *Kundang* (*B. macrophylla*).

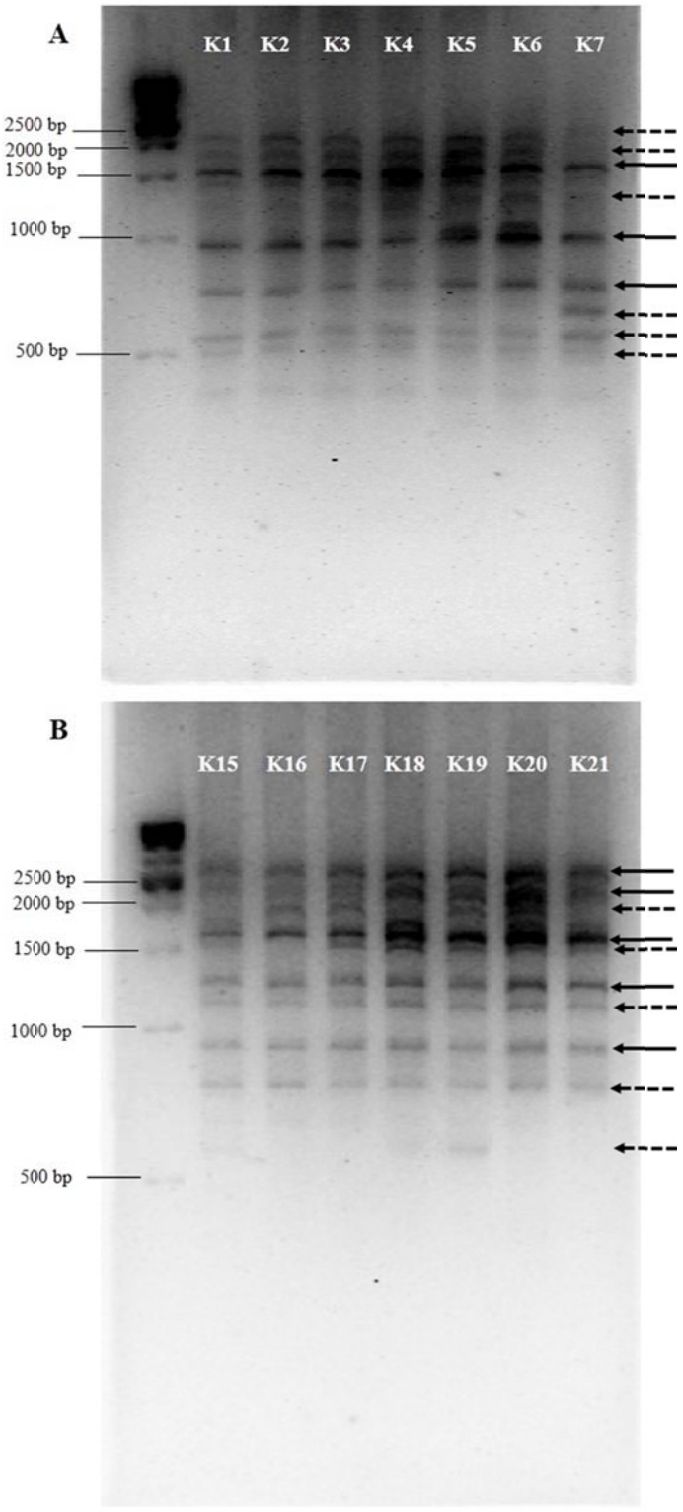

Figure 1. Amplification of genomic DNA from different accessions of Kundang (*B. macrophylla*) using various ISSR primers, A. primer UBC-840 and B. primer UBC-855. In each of the two panels, left lane corresponds to l0 kb DNA ladder; lanes 2 through 8 correspond to amplified DNA from 7 different accessions. Solid arrows point to the monomorphic bands, while the dashed arrows point to the polymorphic bands.

ISSR analysis of *Remia*

Markers were selected based on their ability to generate a visible polymorphism between the samples. For the ISSR analysis performed in this study, 10 of the 52 primers tested gave 89 scorable bands, ranging from 500-4000 bp in size. Of these, 54 bands (59.5%) were polymorphic. The number of bands for each primer varied from 5 (UBC-873) to 11 (UBC-855) with an average of 8.9 bands per primer. The percentage of polymorphism per primer ranged from 40 to 80% with an average of 59.5%. This indicates that there was considerable ISSR variation among the accessions (Table 3). Figure 3 shows the amplification profiles, generated by primers UBC-826 and UBC-827, across the different accessions.

Analysis based on the scoring data was used to determine the genetic relationship and genetic diversity of *Remia* accessions using simple matching similarity coefficient. The former values ranging from 0.591 to 0.977, indicating the genetic variation level among the accessions (data not shown). These coefficients reflected the genetic relationship among the samples. The highest level of similarity between two accessions was found between *Remia* 20 and *Remia* 21, with a similarity coefficient of 0.977 while minimum similarity coefficient (0.591) was observed between *Remia* 20 and *Remia 31.* Accessions with 100% similarity were not found, which indicates the absence of duplicates.

The dendrogram obtained by the UPGMA method from the 89 bands scored for the 23 accessions is shown in Figure 4. The cluster analysis separated 23 accessions into two well-defined clusters; Cluster 1 with 20 accessions and cluster 2 with only three accession; Remia 8, Remia 28, Remia 29. Cluster 1, could be separated into three sub-clusters; (A) consisted of 6 accessions, (B) consisted of 7 accessions, and sub-cluster (C) consisted of 7 accessions (Table 4). Based on these results, ISSR successfully detected variation among accessions, which were morphologically quite indistinguishable.

DISCUSSION

Traditionally, the classification of plants is mainly based on morphological and anatomical aspects. Moreover, there is little information about the relationships among the species in *Bouea* genus. The present study aimed to assess the genetic divergence among a wide range of *Bouea* germplasm collected from different geographical locations in Peninsular Malaysia using the ISSR markers. Currently, we obtained significant ISSR markers that proved their effectiveness for distinguishing the different genotypes in *Bouea* germplasm. Based on the results, there are considerable genetic variations among 56 accessions of *Kundang* and 23 *Remia* studied. Ten of the

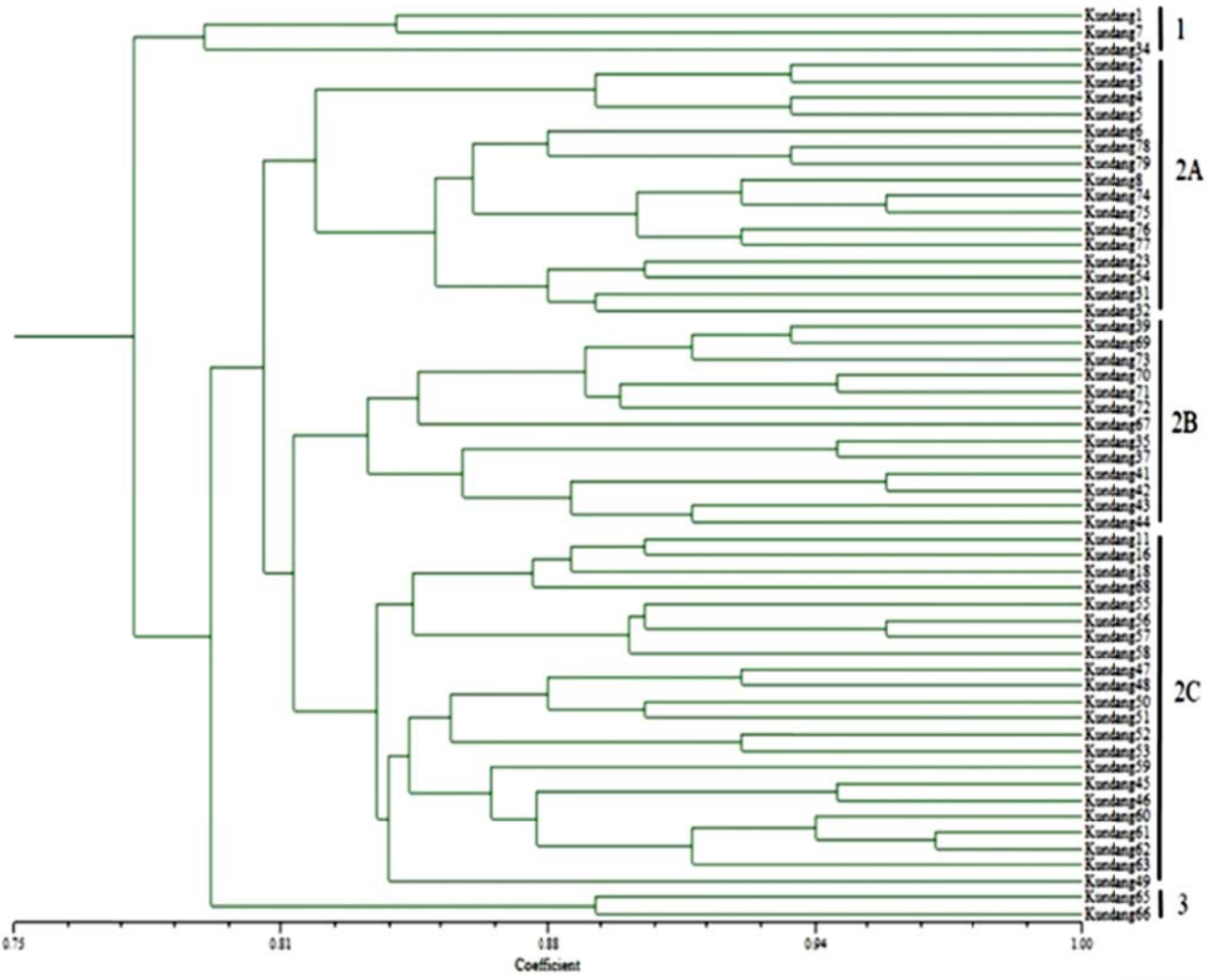

Figure 2. Dendogram constructed from simple matching coefficients from ISSR data, showing the clustering of the 56 accessions of *Kundang* (*B. macrophylla*) in Peninsular Malaysia.

52 primers for *Kundang* generated 89 scorable bands, where 45 bands (50.2%) were polymorphic. For *Remia*, 10 of the 52 primers tested gave 89 scorable bands, where 54 bands (59.5%) were polymorphic. For comparison, application of molecular markers as complementary approach for genetic characterisation of *Remia* has been reported using SSR Marker where 61 SSR products of which 29 were polymorphic (47.5%) (Damodaran et al., 2013). Thus ISSR marker was the more efficient marker system than SSR for detecting polymorphism and to determine diversity for *Remia*.

ISSR markers have been used for identification of Anacardiaceae by different researchers. The results are in confirmative of moderate diversity of 64% reported by Damodaran et al. (2012) earlier with Indian mango germplasm. However, He et al. (2007) reported that all Mango cultivars in China showed the higher percentage of polymorphism of 77.2%. Similar observation was also made by Rocha et al. (2012) where higher diversity was detected among the 102 accessions of 'Uba' mango cultivars. In the other study, another Anacardiaceae family that showed significant results by using ISSR to study the genetic variation is cashew (*Anacardium occidentale*) (Archak et al., 2003) where the degree of polymorphism was 78.9%. In addition, previous study by Thimmappaiah et al. (2009) showed that the degree of polymorphism for Indian cashew was 86.6%.

A moderate polymorphism between 50 - 60% observed with ISSR markers indicated moderate level genetic variation existing among *Kundang* and *Remia* analysed. The moderate level of genetic diversity observed in the present study could be explained in several ways. One

Table 2. Cluster comparison based on the ISSR marker and provenance of different accessions of *Kundang* (*B. macrophylla*) in Peninsular Malaysia.

Cluster	Accession's number	Provenance
Cluster 1	*Kundang 1*	Selangor
	Kundang 7	Selangor
	Kundang 34	Kelantan
Cluster 2A	*Kundang 2*	Selangor
	Kundang 3	Selangor
	Kundang 4	Selangor
	Kundang 5	Selangor
	Kundang 6	Selangor
	Kundang 8	Selangor
	Kundang 23	Perak
	Kundang 31	Kelantan
	Kundang 32	Kelantan
	Kundang 54	Perak
	Kundang 74	Perak
	Kundang 75	Perak
	Kundang 76	Perak
	Kundang 77	Perak
	Kundang 78	Perak
	Kundang 79	Pahang
Cluster 2B	*Kundang 35*	Kelantan
	Kundang 37	Kedah
	Kundang 39	Johor
	Kundang 41	Negeri Sembilan
	Kundang 42	Negeri Sembilan
	Kundang 43	Negeri Sembilan
	Kundang 44	Negeri Sembilan
	Kundang 67	Johor
	Kundang 69	Kelantan
	Kundang 70	Perak
	Kundang 71	Perak
	Kundang 72	Kedah
	Kundang 73	Kedah
Cluster 2C	*Kundang 11*	Negeri Sembilan
	Kundang 16	Terengganu
	Kundang 18	Pahang
	Kundang 45	Negeri Sembilan
	Kundang 46	Negeri Sembilan
	Kundang 47	Negeri Sembilan
	Kundang 48	Negeri Sembilan
	Kundang 49	P.Pinang
	Kundang 50	Kedah
	Kundang 51	Perak
	Kundang 52	Perak
	Kundang 53	Perak
	Kundang 55	Negeri Sembilan
	Kundang 56	Negeri Sembilan
	Kundang 57	Negeri Sembilan
	Kundang 58	Negeri Sembilan
	Kundang 59	Negeri Sembilan
	Kundang 60	Negeri Sembilan
	Kundang 61	Negeri Sembilan
	Kundang 62	Negeri Sembilan
	Kundang 63	Negeri Sembilan
	Kundang 64	Negeri Sembilan
	Kundang 68	Johor
Cluster 3	*Kundang 65*	Johor
	Kundang 66	Johor

Table 3. Primers name, number of amplified bands, polymorphic bands and polymorphism percentage for *Remia* (*B. oppositifolia*) in Peninsular Malaysia.

Primer name	Sequences (5' - 3')	Total amplified bands	Polymorphic bands	Polymorphism percentage
UBC-808	$(AG)_8$ GC	9	5	55.6
UBC-826	$(AC)_7$ A	9	4	44.4
UBC-827	(AC)8 G	9	6	66.7
UBC-834	$(AG)_8$ YT	9	7	77.8
UBC-840	$(GA)_8$ YT	9	5	55.6
UBC-841	$(GA)_8$ YC	10	8	80.0
UBC-846	$(CA)_8$ RT	8	4	50.0
UBC-855	$(AC)_8$ YT	11	6	54.5
UBC-873	$(GACA)_4$	5	2	40.0
UBC-886	VDV $(CT)_7$	10	7	70.0
	Total	89	54	
	Mean	8.9	4.5	59.5

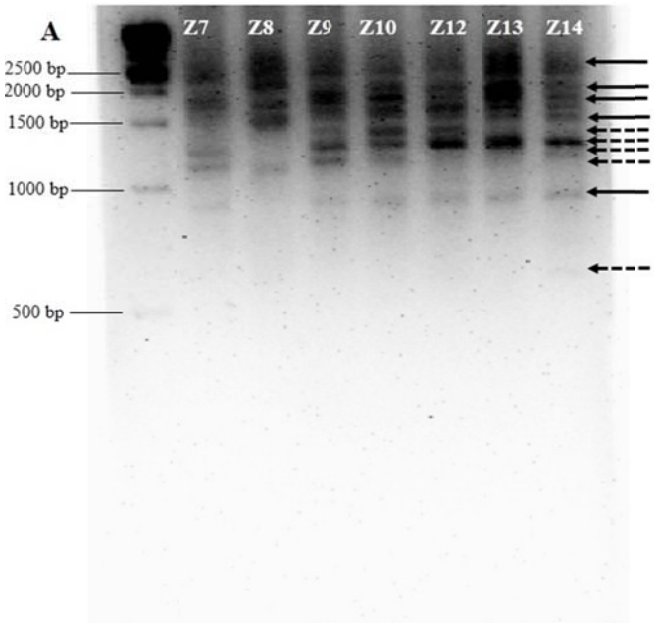

Figure 3. Amplification of genomic DNA from different accessions of Remia (*B. oppositifolia*) using two ISSR primers, A) primer UBC-826 and B) primer UBC-827. In each of the two panels, left lane corresponds to l0 kb DNA ladder; lanes 2 through 8 correspond to DNA from 7 different accessions. Note: solid arrows point to the monomorphic bands, while the dashed arrows point to the polymorphic bands.

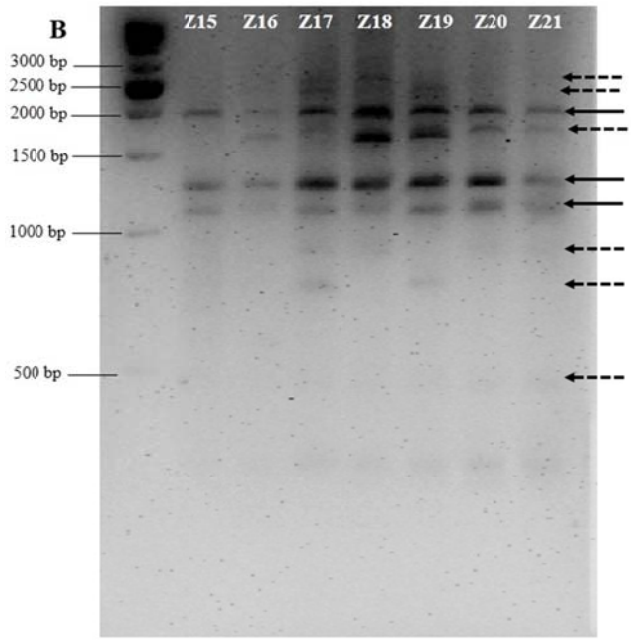

Figure 3. Contd.

hypothesis which could explain the moderate level of diversity would be the origin of the cultivation. Dje et al. (2010) suggested that it could be the resultant of one single entry point and then gradually by migration, the seeds were widespread in the entire country. Our results indicate the need for identification of more parental lines in enhancing the effectiveness of conservation and furthering the breeding success.

Based on the results for *Kundang* and *Remia*, accessions from different provenance were found to cluster together indicating no correlation between molecular groupings and their geographical origin. A similar result has also been revealed by Samal et al. (2012) where no clear-cut geographical separation was revealed among Indian mango cultivars. Our results from cluster analysis did not show distinct clusters by provenance might be

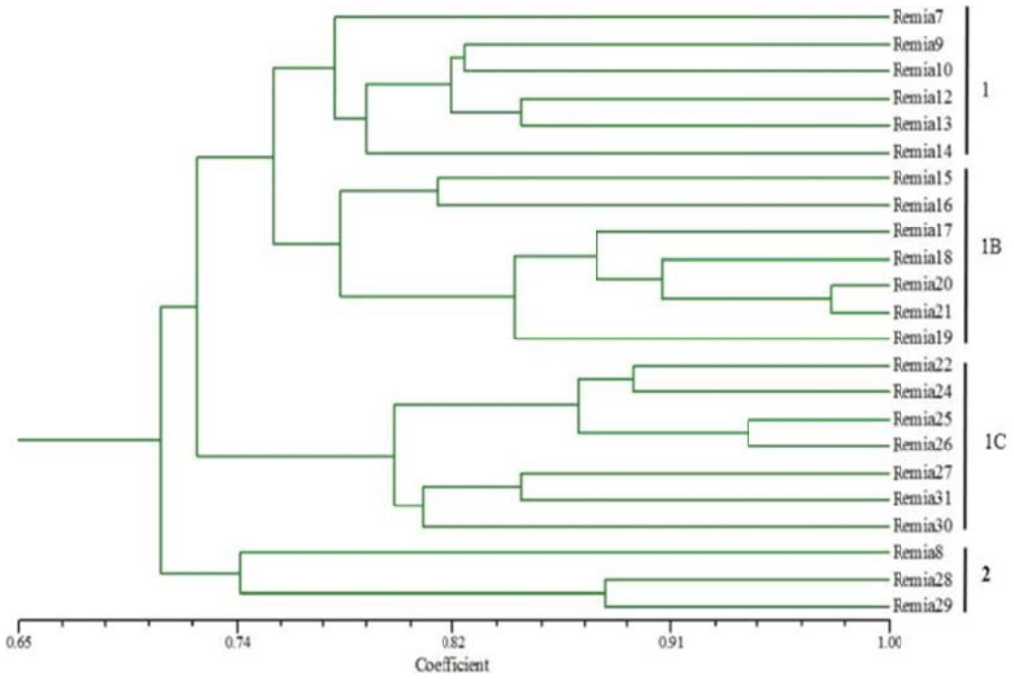

Figure 4. Phylogenetic relationship among 23 *Remia* (*B. oppositifolia*) accessions in Peninsular Malaysia constructed from simple matching similarity coefficients.

Table 4. Cluster comparison based on the ISSR marker and geographic origin of different accessions of *Remia* (*B. oppositifolia*) in Peninsular Malaysia.

Cluster	Accession's number	Provenance
Cluster 1A	*Remia* 7	Terengganu
	Remia 9	Kelantan
	Remia 10	Kelantan
	Remia 12	Pahang
	Remia 13	Kedah
	Remia 14	Kedah
Cluster 1B	*Remia* 15	Kedah
	Remia 16	Kedah
	Remia 17	Perlis
	Remia 18	Kedah
	Remia 19	Kedah
	Remia 20	Kedah
	Remia 21	Kedah
Cluster 1C	*Remia* 22	Kedah
	Remia 24	Pahang
	Remia 25	Pahang
	Remia 26	Kelantan
	Remia 27	Kelantan
	Remia 30	Perak
	Remia 31	Perak

Table 4. Contd.

	Remia 8	Kelantan
Cluster 2	*Remia* 28	Kelantan
	Remia 29	Kelantan

due to the movement of planting materials throughout the country.

Plus, this might have been due to free exchange of germplasm that has taken place between different provenances.

Molecular markers have several advantages over the traditional phenotypic markers since they are unaffected by environment and detectable in all stages of development. In this present study, a set of 10 ISSR markers were shown to be adequate to assess the genetic diversity among 56 accessions of *Kundang* and 23 accessions of *Remia* in Peninsular Malaysia. The results show the efficiency of these for the study of genetic diversity and the data can be used to assess genetic similarity levels among accessions. The findings can be helpful for the management of germplasm and furthering the breeding success for identification of diverse parental combinations to create segregating generation with maximum genetic variability for further selection.

Further efforts in *Kundang* and *Remia* breeding probably will result in new cultivars with better characteristics that will contribute to adding the *Bouea* sp. to the list of important underutilized fruits already available in the markets.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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