GENETIC ANALYSIS OF SOME SPECIES OF*Pinanga* (PALMAE) BY USING ISSR MARKERS

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ABSTRACT

A genetic analysis of 13 species *of Pinanga* (Palmae) was conducted by using Inter Simple Sequence Repeat (ISSR) markers. The markers were used in this study belonged to UBC primer set #9 (UBC 801-900) and each primer contains 15 to 22 mer nucleotides. Based on primer screening, nine UBC primers had clear and reproducible polymorphism bands. According to Dice's and Jaccard's similarity coefficients, cluster analysis by UPGMA among the 13 Pinanga species showed two clusters. Cluster A consisted of nine species: *P. javana, P. arinasae, P. patula, P. salicifolia, P. coronata, P. scortechini, P. disticha, P. grandis* and *P. densiflora;* and cluster B consisted of four species from five accessions: *P. caesia, P. copelandi, P. rumphiana-1, P. rumphiana-2* and *P. insignis.* The genetic similarity among the 13 species of Pinanga had a correlation with their geographical distribution. In cluster A, all of the accessions are from Sundaland and the adjacent region of Thailand, whereas all of the accessions in cluster B were distributed in the Philippines, Wallacea, and the New Guinea regions. Possibly this genetic similarity was caused by their geographical history and the natural barriers between them. This is the early conclusion was conducted using genetic markers on *Pinanga.* Further studies such as sequencing (plastid and nuclear ribosomal DNA) and applying more accessions of *Pinanga* species from broader geographic distributions may provide a better understanding of the relationships. The 1SSR markers application is a simple and quick way to analyze genetic relationships because no prior sequence data is needed, a large number of markers can be generated, and the supplies and equipment required are minimal.

Key words: Genetic analysis, ISSR markers, *Pinanga.*

INTRODUCTION

Systematic studies often use genetic markers to assay organismal relationships, biogeography, speciation, gene flow, genetic variability, and population structure (Soltis *et al.,* 1992). Genetic marker methods, such as *RAPD {Random Amplified Polymorphism DNA)* and *ISSR (Inter Simple Sequence Repeat)* are the most popular and widely applied in a wide variety of fields. Commonly, ISSR was conducted to investigate putative hybridization and gene flow (Sankar and Moore, 2001; Archibald *et al.,* 2004), genetic relationships within species, such as varieties, cultivars and clones identification (Wolff *et al.,* 1995; Charters *et a!.,* 1996; Fang and Roose, 1997; Tani *et al.,* 1998; Qian *et al.,* 2001; Camacho and Liston, 2001; Li and Ge, 2001; Nan *et al.,* 2003), and among species (Joshi era/., 2000; Martin and Sanchez-Yelamo, 2000; Awasthi *eta].,* 2004).

ISSR genetic markers were developed from the common simple repeat sequence (microsatellites) motifs present in eukaryotic organisms. The basic premise is that microsatellites loci are dispersed evenly throughout the genome (Tautz and Renz, 1984; Gupta *etai,* 1994; Zietkiewicz ef a/., 1994). ISSRs are DNA fragments located between adjacent, oppositely oriented microsatellites amplified by PCR using microsatellites core sequences and a few selective nucleotides as a single primer. ISSRs involve the use of designed primers to anchor a subset of tandem *di-, tri-, tetra-* or pewta-nucleotide repeats in general, resulting in amplification of the DNA sequence between two repeat units (Zietkiewicz *et al.,* 1994). The sequences that ISSRs target are abundant throughout the eukaryotic genome (Fang and Roose, 1997).

ISSR markers seem to produce more reliable and reproducible bands than RAPDs because of the longer length of their primer and their higher annealing temperature (Nagaoka and Ogihara, 1997; Wolfe and Liston, 1998; Qian *etal.,* 2001; Bornet and Branchard, 2001). Like RAPDs (Williams *et al,* 1994; Welsh and McClelland, 1990), ISSRs are a simple and quick method to check and estimate diversity level because no prior sequence data is needed, a large number of markers can be generated and the supplies and equipment required is minimal (Zietkiewicz *et al..* 1994).

Pinanga consists of 132 accepted species

(Govaerts and Dransfield, 2005) ranging from the Himalayas and south China to New Guinea, with the greatest diversity in the wet areas of the Sundaland. Almost all species of the genus are plants of the forest undergrowth and confined to humid tropical rain forest. In altitude the genus ranges from sea level to ca. 2800 m in the mountains (Uhl and Dransfield, 1987). *Pinanga* displays great variation in size and form and they are popular in landscape cultivation in countries with subtropical to tropical regions.

In the present study, the ISSR method was conducted to analyze genetic variation and to provide basic relationships information among thirteen species of *Pinanga* (Palmae).

MATERIALS AND METHODS

Plant Materials

Thirteen species, six species of solitary and seven species of clustered *Pinanga* were obtained in the wild status in tropics in Southeast Asia and the cultivated status in the Indonesian Botanic Gardens (Bali Botanic Garden, Bogor Botanic Garden, and Cibodas Botanic Garden) (see Table 1).

Methods

Genomic DNA Extraction

All of genomic DNA was extracted from silica gel dried-leaf tissues (Chase and Hills, 1991). The leaves were first ground into a fine powder in liquid nitrogen using a pestle and mortar, following the CTAB (cetyl trimethylammonium bromide) protocol described by Shaw (1988) and purified using Gene Clean II Kit (Bio 101, Inc.). DNA quantification was done using the uv/ visible spectrophotometer Ultrospec 3000 (Pharmacia Biotech). The template DNA samples were diluted with TE Buffer to make the working solution of 10 ng/il for PCR analysis.

PCR (Polymerase Chain Reaction)

PCR mixtures were performed in a volume 10 i 1 containing sterilized distilled water (sdw), 10 ng template DNA, 1 Ox Taq Buffer per reaction, 200 i M dNTP Mixtures, 100 i M UBC primer and 0.25 U Taq DNA Polymerase (Takara). PCR conditions were ordered as follows: (1) one cycle denaturation at 94° C for 5 min; (2) 35 cycles of 94° C for 60 s (denaturation), 50°C for 45 s

Table 1. *Pinanga* species used.

No	Species	Hab.	Abbrev.	Country of Origin and Distribution
1	*Pinanga arinasae* Witono	Solitary	*PA*	Indonesia: Bali
2	*Pinanga caesia* Blume	Solitary	*PCs*	Indonesia : Celebes
3	*Pinanga copelandi* Becc.	Solitary	*PCo*	Philippines : Luzon, Negros And Mindanao
4	*Pinanga coronata* (Mart. ex Blume) Blume	Clustered	*PC*	Indonesia : Sumatera, Java and Lesser Sunda Islands
5	*Pinanga densiflora* Becc.	Clustered	*PD*	Indonesia: Sumatera
6	*Pinanga disticha* Blume	Clustered	*PDi*	Indonesia: Sumatera; Malaysia; Thailand
7	*Pinanga grandis* Burret	Clustered	*PGr*	Indonesia: Sumatera
8	*Pinanga insignis* Becc.	Solitary	*PI*	Philippines : Luzon, Mindoro, Leyte and Mindanao
9	*Pinanga javana* Blume	Solitary	*PJ*	Indonesia: Java
10	*Pinanga patula* Blume	Clustered	*PP*	Indonesia: Sumatera, Kalimantan; Malaysia
11	*Pinanga rumphiana* (Mart.) J. Dransf& Govaerts	Solitary	*PR,*	Indonesia : Moluccas
12	*Pinanga rumphiana* (Mart.) J. Dransf. & Govaerts	Solitary	*PR:*	Indonesia : New Guinea
13	*Pinanga salicifolia* Blume	Clustered	*PS*	Indonesia: Sumatera, Borneo
14	*Pinanga scortechini* Becc.	Clustered	*PSc*	Thailand; Malay Peninsula

(annealing), 72°C for 120 s (extension); (3) final extension 72° C for 5 min and followed by a soaking at 4°C.

Electrophoresis

The amplified DNA fragments were separated by electrophoresis on 1.5% (w/v) agarose gel submerged in IX TAE Buffer, stained with an Ethidium Bromide and shaking gently at a mild shaker for 10 minutes. The amplified pattern was visualized on a uv transluminator. Each gel contains three lines of 10 kbp DNA Standard Marker (Novagen) on each side and one in the middle of the gels as a standard size marker.

Data Analysis

The data was obtained with ISSR primers and compiled into a matrix for clustering analysis using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) version 2.1 (Rohlf, 2000). Genetic similarity among species was calculated according to Dice's and Jaccard's similarity coefficients using SIMQUAL (Similarity for Qualitative Data). The similarity coefficients were then used to construct the dendrogram using UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) through the S AHN (Sequential, Hierarchical, Agglomerative, and Nested Clustering).

RESULTS

The average sample of genomic DNA was 24.5-161 i g/ml extracted from the 0.15-1.50 gr of silica gel dried-leaf tissues. ISSR primer obtained from the University of British Columbia (UBC) nucleic acid-protein service unit (UBC Primer Set # 9). It consists of 100 primer sequences (UBC 801 -900). Each primer contains 15 mer to 22 mer nucleotides. The primer that generated bright, distinguishable bands and polymorphism was tested by amplifying multiple individuals. Based on primer screening using 2 of the 14 samples *(Pinanga rumphiana-2* and *P. densiflora),* 22 UBC primers were revealed bands. These primers were UBC 807,808,809, 810,811,813,815,822,823,824,834,842,844,845,852, 865,866,881,886,888,890 and 891. Then by using all of the samples (13 species), nine of 22 UBC Primer had clear and reproducible polymorphism bands (Table 2).

The nine UBC primers used in the study revealed 458 scored bands, 444 of which were used for further analysis. The total number of bands generated by the nine primers in fourteen species varied from 25 in *Pinanga rumphiana-1 (PR),* followed 30 in *P. caesia (PCs), P. disticha (PDi), P. patula (PP),* and *P. scortechini (PSc),* 31 in *P. javana (PJ),* and/? *insignis (PI),* 32 in *P. densiflora (PD),* 34 in *P. rumphiana-2 (PRJ* and *P. salicifolia (PS),* 35 in *P. arinasae (PA),* 38 in *P. coronata (PC)* and *P. grandis (PGr),* and 40 in *P. copelandi (PCo).* Polymorphic bands of each primer varied from 10 to 19 and scored bands varied from 23 to 66 (table 2.). In the study, UBC 815 revealed the maximum number of polymorphic bands (19) and also total scored bands (66). In contrast, UBC 881 revealed the minimum number of polymorphic bands (10) and total scored bands (37). Perhaps, it is caused by the

Table 2. UBC primer Set # 9 with the total number of amplified and polymorphic bands

No	Primer	Sequence	DNA Repeats	No. of bands Amplified	Polymorphic Bands (%)	Scored Bands
1.	UBC 807	AGA GAG AGA GAG AGA GT	$(AG)_8T$	12	12 (100%)	44
2.	UBC 808	AGA GAG AGA GAG AGA GC	$(AG)_8C$	12	12 (100%)	50
3.	UBC 810	GAG AGA GAG AGA GAG AT	$(GA)_8T$	16	16(100%)	60
4.	UBC 813	CTC TCT CTC TCT CTC TT	$(CT)_8T$	11	11(100%)	**49**
5.	UBC 815	CTC TCT CTC TCT CTC TG	$(CT)_8G$	**19**	19(100%)	66
6.	UBC 834	AGA GAG AGA GAG AGA GYT	$(AG)_SYT$	14	14 (100%)	55
7.	UBC 844	CTC TCT CTC TCT CTC TRC	$(CT)_8RC$	16	16(100%)	**52**
8.	UBC 845	CTC TCT CTC TCT CTC TRG	$(CT)_gRG$	11	11 (100%)	45
9.	UBC 881	GGG TGG GGT GGG GTG	$(GGGTG)_3$	11	10 (90,9%)	**37**

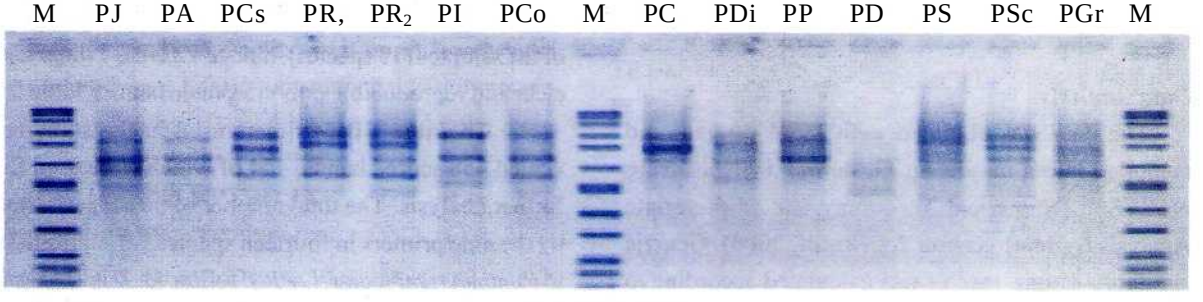
Note : Y = pyrimidine (C or T) and R = purine (A or G)

primer which has different DNA sequence repeats (penta-nucleotide repeats). Based on the study, the most successful primers were those consisting of 'A + G' and 'C + T' repeats. Two of nine primers that were used for further analysis were shown in Figure 1.

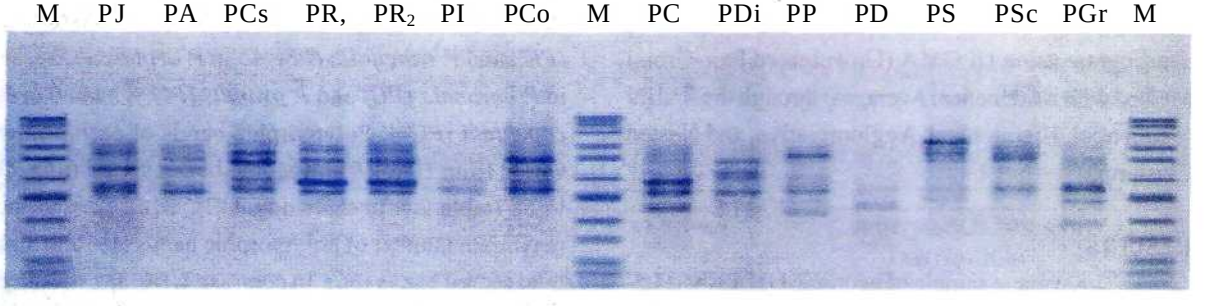
According to Dice's coefficients, the pairwise genetic similarity among 13 *Pinanga* species is shown in Table 3.

DISCUSSION

Using Dice's and Jaccard's similarity coefficients, cluster analysis by UPGMA on 13 *Pinanga* species showed two similar clusters (Figure 2). There was cluster A consisted of 9 species : *PJ, PA, PP. PS, PC, PSc, PDi, PGr* and *PD;* and cluster B consisted of 4 species: *PCs, PCo, PR, PR2* and *PI.* Based on Dice's similarity coefficients, genetic similarity among all



(a) **UBC** 813



(b) UBC 844

Figure 1. ISSR profiles of 13 *Pinanga* species using the primer (a) UBC 813, and (b) UBC 844. The DNA standard marker was put on each side and in the middle lanes

Table 3. Similarity matrix among 13 species of *Pinanga* according to Dice's coefficients.

	PJ.	*PA*	*PCs*	*PR,*	*PR:*	*PI*	*PCo*	*PC*	*PDi*	*PP*	*PD*	*PS*	*PSc*	*PGr*
PJ	1.000													
PA	0.844	1.000												
PCs	0.339	0.318	1.000											
PR,	0.222	0.207	0.491	1.000										
PR:	0.381	0.358	0.516	0.737	1.000									
PI	0.333	0.344	0.441	0.296	0.381	1.000								
PCo	0.290	0.329	0.559	0.444	0.556	0.551	1.000							
PC	0.328	0.338	0.455	0.295	0.371	0.299	0.474	1.000						
PDi	0.271	0.318.	0.414	0.377	0.419	0.271	0.441	0.394	1.000					
PP	0.441	0.508	0.276	0.151	0.258	0.271	0.353	0.364	0.483	1.000				
PD	0.164	0.308	0.200	0.182	0.219	0.984	0.286	0.294	0.300	0.267	1.000			
PS	0.413	0.478	0.323	0.175	0.333	0.318	0.389	0.314	0.387	0.613	0.375	1.000		
PSc	0.271	0.413	0.310	0.264	0.258	0.237	0.294	0.455	0.345	0.414	0.433	0.419	1.000	
PGr	0.239	0.338	0.333	0.197	0.257	0.239	0.342	0.324	0.424	0.333	0.382	0.457	0.424	1.000

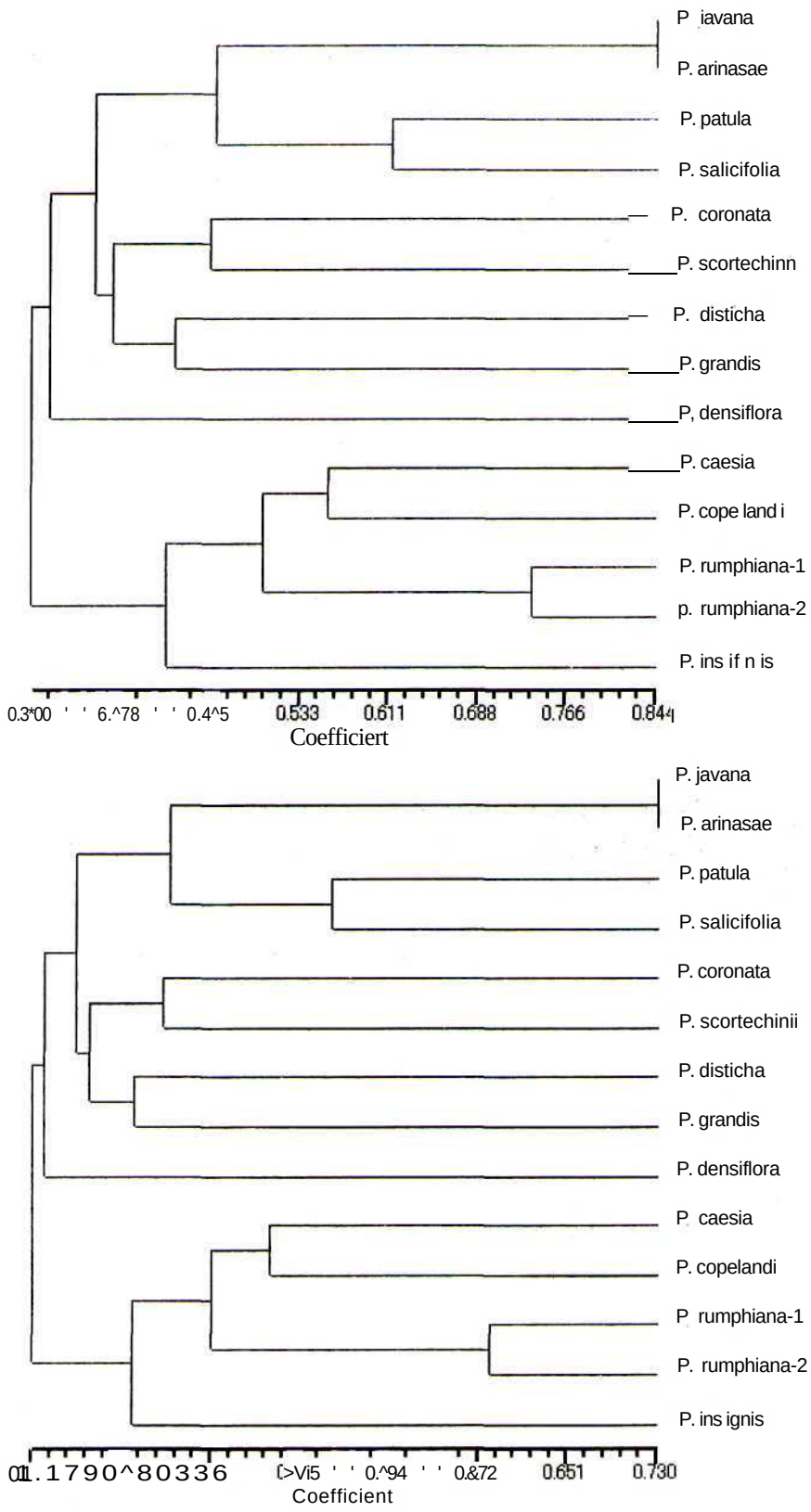


Figure 2. Dendrogram of genetic relationships among 13 *Pinanga* species generated by UPGM A cluster analysis according to (a) Dice's and (b) Jaccard's coefficient similarity.

Pinanga species varied between 0.30 to 0.84. In cluster A, the highest genetic similarity was revealed between PJ and/M (0.84); followed by *PP* and *PS* (0.61); *PJ, PA* and *PP, PS* (0.46); *PC* and *PSc* (0.45); *PDi* and *PGr* (0.42); *PC, PSc* and *PDi, PGr* (0.37); *PJ, PA, PP, PS* and *PC, PSc, PDi, PGr* (0.36); and finally PD and all *Pinanga* species in cluster A (0.32). In cluster B, the highest genetic similarity was obtained between PR_t and PR_2 (0.74); followed by *PCs* and *PCo* (0.56); $PR_,$, PR_2 and PCs, *PCo* (0.50); and finally *PI* and *allPinanga* species in cluster B (0.42). The genetic similarity between cluster A and cluster B is 0.30.

Cluster separation does not depend on the habitus (solitary or clustered), for example *PJ* and *PA* are solitary *Pinanga* but they belong to cluster A that consisted of all clustered *Pinanga.* Whereas, other solitary *Pinanga* such as *PCs, PCo,* PR_r PR_2 and *PI* separated in a different cluster (cluster B). A study has been conducted on morphological characters of four solitary *Pinanga,* there were *Pinanga javana, P. arinasae, P. insignis and P. rumphiana* and showed that they are closely related (Witono *et al,* 2002). This study showed a different result from the previous study which used morphological characters, because in this study *P. javana* and *P. arinasae* were not closely related to *P. insignis and P. rumphiana.*

It seems that the genetic similarity of the 13 *Pinanga* species is correlated with their geographical distribution (the country of origin and distribution are shown in Table 1). In cluster A, all of the *Pinanga* species are from Sundaland and the adjacent region of Thailand, whereas all of the accessions in cluster B are distributed in the Philippines, Wallacea, and the New Guinea regions. Possibly this genetic similarity is caused by their geographical history and the natural barriers between them.

Some workers suggested that Sundaland of the Malay Peninsula, Sumatra, Borneo, Java, and Bali, Wallacea of the Lesser Sunda Islands, Celebes and Moluccas, New Guinea and the Philippines regions should be treated as a single floristic element called "Malesia.". According to Dickerson's geomorphological analysis (1925) of the Philippines, the geologically unstable zone between Sundaland, Wallacea, and New Guinea, comprising almost all the Philippines, Celebes, the Lesser Sunda Islands and the Moluccas. Malesia was identified based not only on a shared tropical flora derived from Asia, but also on the presence of numerous species of the Australian flora. Sundaland is on Asia's relatively shallow continental shelf and was linked to Asia during the ice ages when sea levels were lower, and New Guinea is linked to Australia by a shallow continental shelf. The region between Sundaland and New Guinea is called *Wallacea.* Wallacea region was never linked to the neighboring continents, and has a flora and fauna that includes IndoMalaya and Australian elements. The *Wallace line* runs from South to North, passing the Lombok and Makasar Straits and ending in the southeastern part of the Philippines (Steenis, 1979; Whitmore (ed.), 1981; Holloway and Hall, 1998).

Indonesia contains one of the world's most remarkable geographical boundaries in its distribution of animals and plants. This early geographical separation explains why the tropical animal and plant species of Java, Sumatera and Borneo do not exist in the eastern part of Indonesia. Eventhough Celebes is close to Borneo, which is just across the Makasar Strait, most of animals and plant species are strikingly different between the western and eastern part of Indonesia. One possible reason for this is that Borneo and Celebes might have been separated by a deep strait at one point, while the great depth of the Banda Sea kept them apart during the glacial period. Apparently there is a correlation between the cluster separation of 13 *Pinanga* species by using ISSR markers and their natural distribution. This result is still early conclusion. However further studies utilizing more *Pinanga* species from a broader geographic distribution may provide a better understanding.

It can be concluded that ISSR was successful in generating a high degree of polymorphic markers in 13 *Pinanga* species. ISSR provided a high number of scorable and reproducible markers per primer. Also ISSR technique was simple to perform and cost-time efficient. However, this is the first study that was conducted with genetic markers on *Pinanga.* Further studies using plastid and nuclear ribosomal DNA sequencing are needed to understand genetic relationships among *Pinanga* species.

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