Cross-genera Transferability of Microsatellite Loci for Asian Palmyra Palm (*Borassus flabellifer* L.)

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Abstract. Asian Palmyra palm, found throughout south and southeast Asia, is important for local economies, especially for sugar palm production. Unlike its related species, such as oil palm and coconut, only a few genetic markers are available for Asian Palmyra palm. In this study, we tested the transferability of molecular markers derived from oil palm, and a set of selected markers were used for evaluating the diversity of Asian Palmyra palm growing in Thailand. From 545 primer pairs of expressed sequence tag-simple sequence repeat (EST-SSR) and genomic simple sequence repeat (gSSR) markers, 317 (58.17%) primer pairs were able to amplify the Asian Palmyra palm DNA, and 19 (5.99%) pairs were polymorphic. After extensively genotyping 164 samples from 12 populations, we obtained 25 loci with the polymorphism information content (PIC) average of 0.37 and allele numbers ranging from one to five. The observed and expected heterozygosity ranged from 0 to 1 and 0 to 0.76, respectively. A dendrogram showed separation of the palm populations into two clades, between north-eastern and southern-central regions. This study provides a set of microsatellite markers for use in further genetic studies of Asian Palmyra palm.

Asian Palmyra palm ($2n = 36$), found widespread in the Indian subcontinent and Southeast Asia, is a monocotyledonous dioecious woody perennial tree in the *Arecaceae* family. This palm tree is important for local agriculture and economies as its inflorescence sap is used for palm sugar production and its fruits are widely consumed (Lim, 2012; Morton, 1988). The Asian Palmyra palm has a very slow growth rate and requires 12–20 years to produce its first inflorescence flowers, only then the sex is revealed (Davis and Johnson, 1987). It is widely believed that Asian Palmyra palm

originated in Africa and was introduced to India and then into the southeast Asia more than a thousand years ago (Kovoor, 1983).

With such a long juvenile stage and without any direct sex determination, most growers are reluctant to cultivate new crops, and, with the expansion of farmlands and urbanization, Asian Palmyra palm population is in rapid decline. Conservation plans and improvement for use are operating in South and Southeast Asia to conserve this plant species (Barfod et al., 2015; Davis and Johnson, 1987; Sirajuddin et al., 2016). However, genetic data of Asian Palmyra palm are currently limited, and only a few molecular markers including RAPDs and ISSRs have been reported (George et al., 2016; Vinayagam et al., 2009). Markers with higher information such as microsatellites are needed to evaluate the genetic groups and diversity of the Asian Palmyra palm before establishing effective conservation plans.

New microsatellite markers require a high developing cost. Nonetheless, many microsatellite markers have been shown to be transferable across plant species and genera, providing an economical way for marker

development (Karaca et al., 2013; Whankaew et al., 2011; Zehdi et al., 2012). Oil palm (*Elaeis guineensis*), a closely related species to Asian Palmyra palm, has available genome sequences and a wide range of developed microsatellite markers including genomic SSR (gSSR) and EST-SSR (Billotte et al., 2005; Singh et al., 2013; Ukoskit et al., 2014). Thus, it is feasible to apply these markers on the Asian Palmyra palm. In this study, we evaluated the cross-genera transferability of the gSSRs and EST-SSRs derived from oil palm and determined the diversity of some Asian Palmyra palm populations in Thailand.

Materials and Methods

Plant material, DNA isolation, and marker analysis. Leaf samples were collected from 164 accessions located in 12 provinces in three geographical regions in Thailand (Table 1). Oil palm was used as an out-group. Because the original CTAB (Cetyltrimethylammonium bromide) method (Gawel and Jarret, 1991) gave low yields when isolating DNA from the leaf samples of Asian Palmyra palm. A medication was made by adding 10% polyvinylpyrrolidone in the extraction buffer to help improving the DNA yield. DNA samples from one oil palm and 10 randomly selected Asian Palmyra palm accessions were used for an initial screening using markers previously developed from the oil palm: 289 EST-SSR (Ukoskit et al., 2014) and 256 gSSRs (Billotte et al., 2005). The polymerase chain reaction (PCR) reactions were performed in a 20 μ L volume containing 20 ng of total DNA, 20 μ M of each dNTP, 0.25 μ M of each primer, 10X PCR buffer (with 1.5 mM $MgCl_2$), and 0.5 U *Taq* DNA polymerase (Vivantis Technologies, Selangor Darul Ehsan, Malaysia). Thermocycling parameters included an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1.30 min annealing temperature following Temp in Table 2, 30 s extension step at 72 °C, and 8 min at 72 °C for the final extension step. The PCR products were separated and resolved by 6% polyacrylamide gel electrophoresis with silver staining. Primer pairs, which generated polymorphic markers, were then used for analyzing the 164 accessions. Fragments were scored based on the Low Molecular Weight DNA Ladder (New England Biolabs, Ipswich, MA) and converted into binary data.

Data analysis. Linkage disequilibrium (LD) was calculated using PowerMarker version 3.2.5 (Liu and Muse, 2005), and sequential Bonferroni correction (Holm, 1979) was performed according to the multiple comparisons of the LD tests. Some loci were excluded after observing significant LD. The Hardy–Weinberg equilibrium (HWE) was tested using POPGENE version 1.31 (Yeh et al., 1999). Polymorphic EST-SSR and gSSR loci were used for calculating the number of alleles per locus (N_A), observed heterozygosity (H_o), expected heterozygosity (H_e), and polymorphism information

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Table 1. Location and the number of Asian Palmyra palm samples used in this study.

Part of Thailand	Province	Code	Sample numbers	Location
North-Eastern	Buriram	BU	11	14°36'53.9"N 102°47'17.1"E
	Sisaket	SS	9	14°50'23.5"N 104°11'37.2"E
	Nakhon-Ratchasima	NR	10	15°09'04.3"N 102°10'54.6"E
	Kalasin	KB	6	16°20'09.9"N 103°38'09.3"E
Central	Phetchaburi	PH	18	13°03'33.8"N 99°54'07.8"E
	Nakhon Pathom	NP	11	13°52'13.4"N 100°10'18.9"E
	Kanchanaburi	KA	22	14°03'04.9"N 99°32'46.6"E
	Phachinburi	PC	18	14°07'30.7"N 101°31'20.3"E
	Chainat	CN	14	15°06'22.6"N 100°14'37.7"E
	Nakhon Sawan	NS	14	15°52'11.7"N 100°15'35.2"E
Southern	Songkhla	SK	18	7°27'40.8"N 100°26'29.6"E
	Surat Thani	ST	13	9°24'56.8"N 99°12'11.3"E

Table 2. Characteristics of 19 polymorphic microsatellite markers tested in the Asian Palmyra palm.

Primer name	Repeat motif	Temp (°C)	Allele size (bp)	N _a ^z	H _o ^y	H _e ^x	PIC ^w
ESSR75	(AAG) ₅	60	270–285	2.17	0.4	0.5	0.38
			175–190	2.33	0.1	0.38	0.48
			350–365	3.33	0.97	0.62	0.54
ESSR76	(AGC) ₅	60	190–205	2.75	0.23	0.51	0.55
			200–215	2	0.54	0.35	0.39
			230–242	2.67	0.77	0.51	0.42
ESSR82	(GCT) ₅	60	220–239	2.08	0.87	0.51	0.38
ESSR332	(AT) ₆	60	240–254	2.83	0.4	0.44	0.48
ESSR553	(A) ₁₉	60	125–139	2.92	0.35	0.52	0.6
ESSR566	(AG) ₇	60	150–164	2.58	0.55	0.41	0.38
ESSR609	(GA) ₇	59	200–228	1.92	0.24	0.33	0.34
ESSR650	(AG) ₁₄	59	150–168	2	0.26	0.34	0.32
ESSR652	(GAG) ₆	60	150–168	1.83	0.23	0.19	0.18
ESSR673	(GGC) ₈	59	75–113	2	0.3	0.37	0.34
ESSR681	(AAAT) ₅	59	350–370	2.42	0.46	0.43	0.47
mEgCIR2332	(GA) ₁₄	55	250–278	3.25	0.49	0.43	0.38
mEgCIR3295	(GT) ₇ (GA) ₂₃	55	300–360	4.17	0.33	0.61	0.65
			250–310	2.17	0.14	0.17	0.17
mEgCIR3311	(GA) ₁₅	55	180–210	2.25	0.51	0.36	0.34
mEgCIR3413	(GA) ₁₈	55	250–286	2.5	0.8	0.52	0.43
mEgCIR3477	(GA) ₂₂	55	170–214	2.17	0.11	0.15	0.16
mEgCIR3592	(GA) ₂₀	55	150–190	2.58	0.37	0.34	0.34
mEgCIR3755	(GA) ₁₅	55	380–410	1.17	0	0.02	0.02
			140–170	1.67	0.12	0.1	0.12
mEgCIR3788	(GA) ₁₈	55	180–216	2.58	0.73	0.49	0.41
				2.41	0.41	0.38	0.37

^zNumber of alleles.^yObserved heterozygosity.^xExpected heterozygosity.^wPolymorphic information content.

content (PIC) value (Botstein et al., 1980) using POPGENE version 1.31 (Yeh et al., 1999). A dendrogram was constructed using the EST-SSR and gSSR loci based on Nei's standard genetic distance (Nei, 1972) and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method in Populations software version 1.2.32 (Langella, 1999).

Results and Discussion

An initial marker screening using 289 EST-SSR and 256 gSSR primers derived from oil palm provided 154 (53.3%) and 163 (63.7%) amplifiable markers, respectively. Polymorphic bands were observed from 11 EST-SSR and eight gSSR primers, and these were subsequently used for evaluating the polymorphism of the palm populations. Analysis of 164 accessions using these 19 primers provided 36 polymorphic loci (21 EST-SSR and 15 gSSR loci), but only 15 EST-SSR and 10 gSSR loci were not significant

in the LD and HWE tests after the sequential Bonferroni correction ($P > 0.05$) (Table 2). The average PIC value of combined EST-SSRs and gSSRs was 0.37. An average 2.41 alleles per locus was observed. The means of observed and expected heterozygosity were 0.41 and 0.38, respectively.

A dendrogram of 12 populations showed that the populations were clustered into two main clades, clearly separated from the oil palm outgroup (Fig. 1). The north-eastern clade was separated from those of the southern and central clade. The two clades were geographically separated on the map of Thailand and this likely resulted from geographical barriers (Pyšek et al., 2008). Phayayen hill and the Phetchabun mountain ranges located between the two areas had interrupted human movement in the past and, thereby, limited the anthropogenic spread of the Asian Palmyra palm. By contrast, there is no geographical barrier between the southern and central regions resulting in a considerable widespread of the species.

Transferability of SSRs between species or genera has been reported in many plant species including cereals (Castillo et al., 2010; Ince et al., 2010; Sim et al., 2009; Tang et al., 2006) and woody species (Gasic et al., 2009; Park et al., 2010; Yu et al., 2011). The cross-genera transferability of EST-SSRs and gSSRs shown in this study ranged between 53.3% and 63.7%, which are within the 30% to 65% reported for the transferability in other plant species including legumes and grasses (Choudhary et al., 2009; Karaca et al., 2013; Whankaew et al., 2011; Yu et al., 2013; Zeid et al., 2010; Zhou et al., 2013). The transferred EST-SSR and gSSR markers will be a valuable tool for a variety of genetic analyses for the Asian Palmyra palm. In addition, with the availability of genome sequence of date palm (*Phoenix dactylifera*) (Al-Dous et al., 2011; Al-Mssallem et al., 2013), a closely related species to Asian Palmyra palm, the numbers of transferable markers for the Asian Palmyra palm could be further expanded.

Fig. 1. (A) UPGMA dendrogram representing the relationship among Asian Palmyra palm populations in Thailand. Clade1 consists of southern (SK and ST) and central (PH, NP, CN, NS, KA, and PC) populations while clade2 is only north-eastern (NR, BU, SS, and KB) populations. (B) A map of Thailand indicating the distribution of two distinct genetic groups based on the UPGMA dendrogram.

The genetic study of the Asian Palmyra palm was, so far, limited to a few markers, for example, RAPDs developed for sex determination (George and Karun, 2011; George et al., 2007), and ISSRs used for diversity assessment in India (Vinayagam et al., 2009). These, however, were unable to provide a genetic resolution for the population structure of the Asian Palmyra palm because of their limitation as dominant markers. This study provides a set of codominant microsatellite EST-SSR and gSSR markers, which would be used to further study the genetics of Asian Palmyra palm. Furthermore, we found that the population diversity of Asian Palmyra palm in Thailand is very low as indicated by the genetic variation indices (PIC, N_a , H_o , and H_e). This observation is somewhat similar to previous studies in India using ISSRs that showed 0.84 average similarity coefficient and PIC ranging between 0 and 0.56 (Vinayagam et al., 2009) and RAPDs that had 0.76 average similarity coefficient (George et al., 2016; Ponnuswami, 2010; Ponnuswami et al., 2008; Raju and Reji, 2015). The small number of alleles per locus coincides with the notion that Asian Palmyra palm is an introduced species (Kovoor, 1983), and, with limited numbers of originally introduced plants, this eventually resulted in low genetic diversity.

In conclusion, the evaluation of marker transferability in this study provided a set of microsatellite markers for studying the genetic diversity and population structure of the Asian Palmyra palm in Thailand. This data could be useful for the management of the Asian Palmyra palm plantation and advance its breeding efforts.

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