SAGO PALM 22: 6 − 12 (2014)

Comparison of species composition under different light conditions in sago forests

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Key words: chloroplast DNA (cpDNA), flora, sago palm

Introduction

In Indonesia, the sago palm, *Metroxylon sagu* Rottb. (Arecaceae), plays an important economic and cultural role, and it has various advantageous features of a crop species, as it is economically acceptable, relatively sustainable, and suitable for environmentally sound agro-forestry systems (Yamamoto, 2005). Although natural sago stands are found in several areas of Indonesia, spread over lowlands, coastal regions, and river deltas, many sago forests have been destroyed through commercial sago extraction, irrigation schemes, and conversion for wet rice production.

Little is known of transitions in the understory of a sago palm forest after its initial development. The sago palm has large pinnate compound leaves with an average size of approximately 10 m^2 per leaf (Nakamura et al., 2009). The shading of these leaves creates a dark environment in a sago palm forest. In fact, we could observe few species in a sago forest in Indonesia, and many plant species we could find were in the gap around fallen dead trees. From these observations, it appears that many plant species would increase by destroying the sago palm forests as that would allow light to reach the floor of the forests. Sago forests in Indonesia have been destroyed because of both extreme development projects and continuing rubber and oil palm expansions (Okamoto, 2000). The variation in solar environments in the sago forests is considered to affect the growth of many plant species. It is important to compare the plant species colonizing the different light conditions during sago forest development in order to monitor the invasion of alien plant species that pose a threat to sago conservation. However, most plant species are often seedlings, and they cannot be determined based only on morphological characteristics.

DNA barcoding is a technique for characterizing species by using a short DNA sequence from a standard and predetermined position in the genome (Hebert et al., 2003). Relative to the entire genome, DNA barcode sequences are very short for identifying an unknown sample in terms of a preexisting classification (Kress et al., 2005). Therefore, DNA barcodes are sometimes used in an effort to identify unknown species (Koch, 2010). For example, the cytochrome oxidase subunit I (COI) gene in mitochondrial DNA (mtDNA) is emerging as the standard barcode region for insects and other animals (Hebert et al., 2003); however, the use of the COI sequence is not appropriate for most species of flowering plants due to the much slower rate of COI

gene evolution in angiosperms (Kress et al., 2005). However, some chloroplast DNA (cpDNA) or nuclear DNA (nrDNA) sequences have a relatively fast mutation rate, which results in significant variation in these sequences between and/or within species; these have been proposed as potential barcodes (Fukuda et al., 2005; Kress et al., 2005; Kress and Erickson, 2008; Hayakawa et al., 2010; Hayakawa et al., 2011; Kumekawa et al., 2013). Thus, the aim of our study was to identify plant species that grow in different solar environments during transition in the understories of sago palm forests using molecular analyses based on two cpDNA genes, i.e., the *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) gene and the *trnL* (transfer RNA leucine) intron of ferns and angiosperms, respectively. These genes are important tools for molecular identification of land plants (CBOL Plant Working Group, 2009; Taberlet et al., 2007).

Materials and Methods

Plant materials

All plant materials used in this study were collected from one sago palm stand (1'95"S, 132'25"E) in the Malay Archipelago (Fig. 1). Within the study area, we

Fig. 1. Sampling area of this study.

selected four localities (A, B, C, and D), constructed 11 quadrats (four in A, three in B, one in C, and three in D) of 10 m x 10 m based on the brightness in each area (Fig. 2), and collected all plant species within them. Among these quadrats, A-1, A-2, A-3, A-4, B-3, and D-3 had high ratios of adult sago palms in a

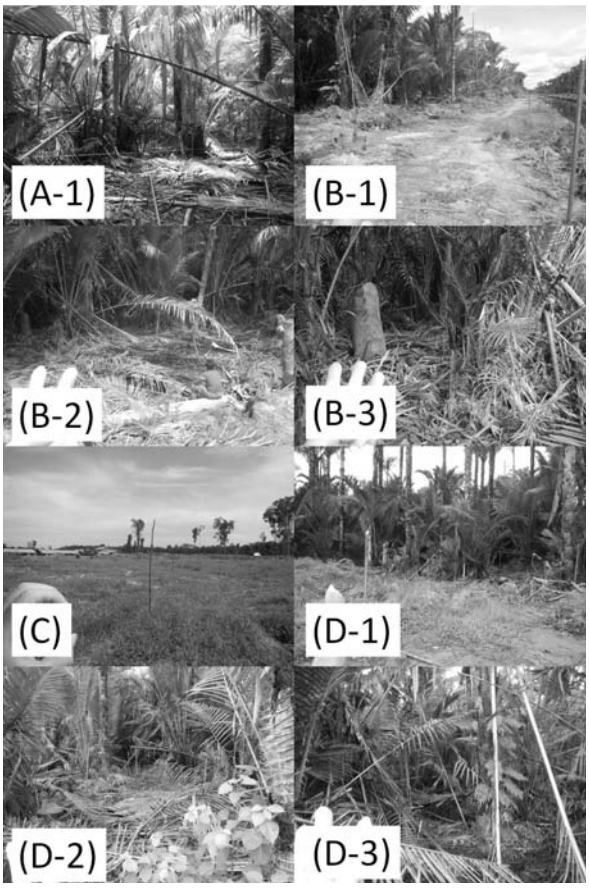

Fig. 2. Eight quadrates of A, B, C and D in this study. Photographed in A- 1 (dark), B-1 (bright), B-2 (bright), B-3 (dark), C (bright), B-1(bright), B-2 (bright), D-1 (bright), D-2 (bright) and D-3(dark).

forest and represented dark environments; B-1, C, and D-1 were in an open field and represented bright environments (Fig. 2). B-2 and D-2 had low ratios of adult sago palms with high ratios of the rosette stage (Fig. 2). The conditions in these intermediate quadrats were relatively bright, and so our study was included in the "bright environment" category.

DNA analyses

Total DNA was isolated from approximately 200-300 mg of an air-dried leaf using a Plant Genomic DNA Mini Kit (Viogene, Sunnyvale, USA) in accordance with the manufacturer's protocol. Isolated DNA was resuspended in Tris-EDTA (TE) buffer and stored at -20°C until use. DNA amplification by polymerase chain reaction (PCR) was carried out in a 50-μL reaction volume containing approximately 50 ng of total DNA, 10 mM Tris-HCl buffer (pH 8.3)

with 50 mM KCl and 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U *Taq* DNA polymerase (Toyobo, Osaka, Japan), and 0.5 μ M of each primer. We used the following thermal cycle profile for amplification: 40 cycles of 1.5 min at 94°C, 2 min at 48°C, and 3 min at 72°C, followed by a final extension step of 15 min at 72°C. We amplified *rbcL* and the *trnL* intron of cpDNA with primers designed by Hasebe et al. (1994) and Taberlet et al. (1991), respectively. After amplification, the reaction products were run on a 1% agarose gel stained with ethidium bromide and digitally photographed under UV light.

We also confirmed the sequences of these regions. After amplification, the reaction mixtures were subjected to electrophoresis on 1% low-melting-temperature agarose gels for the purification of the amplified products. We sequenced the purified reaction products using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, California, USA) and a Model 3730A automated sequencer (Applied Biosystems, California, USA) in accordance with the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification. Sequences for each region were pre-aligned with the CLUSTAL X program (Thompson et al., 1997), and ambiguously aligned regions were manually corrected to minimize the number of indels.

Our results were almost identical to previously published sequences, and the samples were assigned to the corresponding species published in the DNA Data Bank of Japan (DDBJ). In cases where there were no identical matches to previously published sequences, phylogenetic trees were constructed using our sequences and highly similar ones based on a homological search of the DDBJ. Our samples were assigned to the genus that included plant species of a sister group to our sequences in the phylogenetic tree (Fig. 3). For example, some of our sequences were similar to those of some species of *Ficus* (Moraceae); therefore, different sequences of this genus were named Ficus sp. 1, *Ficus* sp. 2, and so on (Fig. 4).

Results and Discussion

In total, we identified 70 species in 35 families (Table 1). Six species, i.e., *Nephrolepis* sp. (Lomariopsidaceae), *Stenochlaena* sp. (Blechnaceae), *Ficus* sp. 1 (Moraceae), *Fagraea volubilis* (Gentianaceae), *Freycinetia* sp. (Pandanaceae), and *Syzygium* sp. (Myrtaceae), were

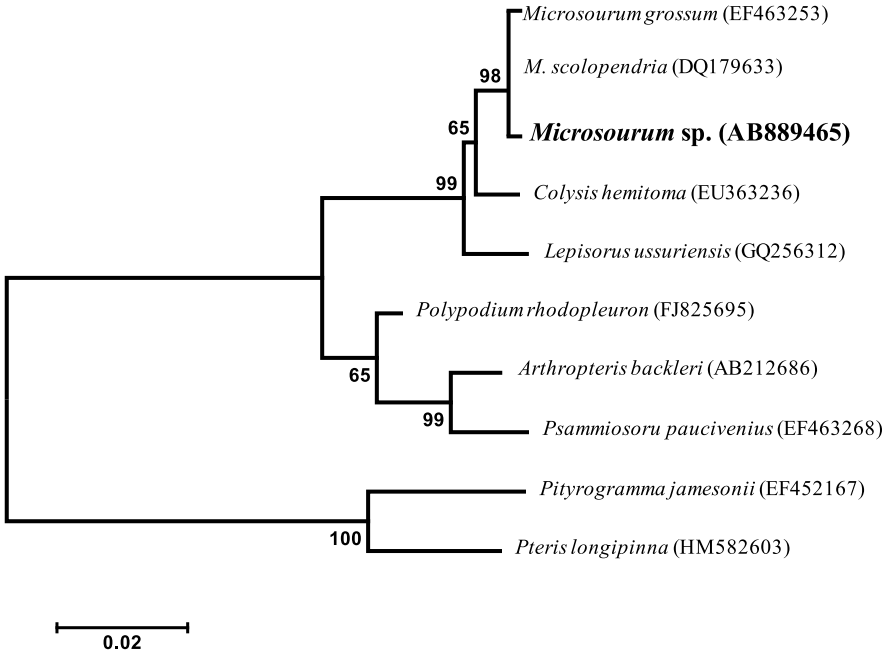

Fig. 3. Phylogenetic tree of Microsorum (Polypodiaceae) and its allied species. Numbers indicate bootstrap values. Bolded name indicates individual of our study. Parentheses indicate accession numbers.

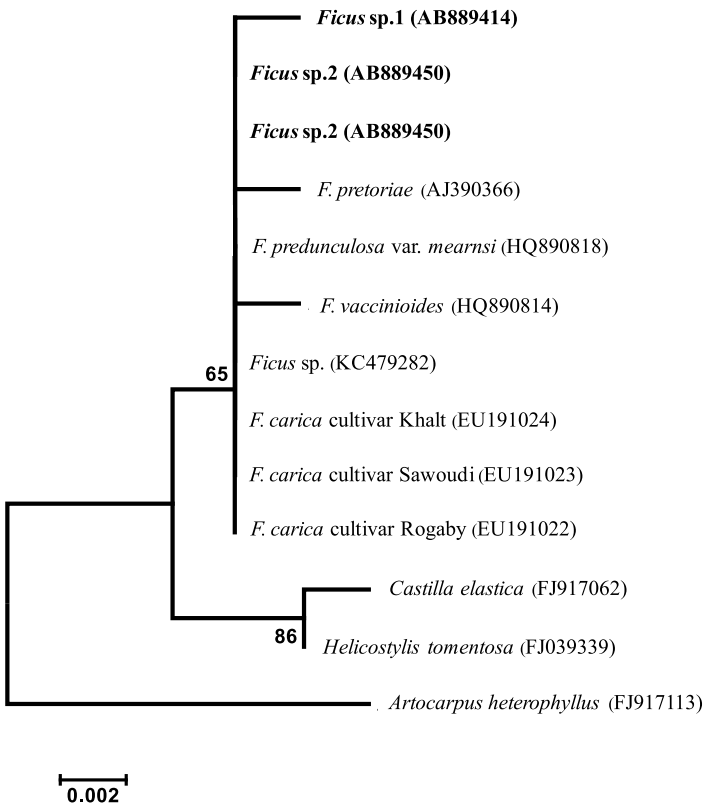

Fig. 4. Phylogenetic tree of Ficus (Moraceae) and its allied species. Numbers indicate bootstrap values. Bolded names indicate individuals of our study. Parentheses indicate accession numbers.

found in both bright and dark environments, suggesting that these plants are not dependent on a specific solar environment during the development of a sago palm forest. However, the remaining species inhabited either bright or dark environments; thus, they are considered effective species for distinguishing transitional stages of sago palm forest succession. We found 23 species (6 ferns and 17 angiosperms) in dark environments and 53 species (10 ferns and 43 angiosperms) in bright environments, indicating that the number of plant species in our quadrats decreased from bright to dark environments. This result supports a general tendency reported by Whittaker (1975), that it is a general tendency of succession that the number of species was large in bright environments and small in dark environments. Among our collected species, grasses of the Poaceae and Cyperaceae families grew in intermediate and bright quadrats, but none grew in dark environments. In fact, Table 1 shows 6 and 8 species, respectively, of the Poaceae and Cyperaceae families in bright environments. Therefore, our data supports the report of Whittaker (1975), and these plants could become standard indicator species of bright environmental conditions during the transition of sago palm forests. In general, grass, such as Poaceae and Cyperaceae, is a dominant species in the secondary succession of the forest (Robertson and Vitousek, 1981) and an early successional species (Tagawa, 1964), suggesting that locations C and D1 of our investigation are at the secondary succession stage (Table 1). Considering this general tendency of change reported by Whittaker (1975), our results suggest that locations A1 and A3 are late in the succession of the forest; in particular, A3 was at the climax stage. Therefore, our comparison of floras among these locations reconfirms the succession scheme of sago palm forests from the early to the late stages. Sago palm forests would also be formed in this pattern if deforestation has taken place in the region. Moreover, it is very interesting that the succession of sago palm forests from the early to the late stages of our study appears to correspond to a seed dispersal mode from wind to birds and animals, as many wind-dispersal species live in bright environments while birds and animals live in dark ones (Prach and Pyšek, 1999; Fuller and Moral, 2003). Praseeda and Jeyanth (2010) reported that invasive alien species include many grasses, climbers, colonizer shrubs, and trees with wind dispersal seeds; therefore, changes in the solar environment by sago palm deforestation would pose a serious risk to the habitat of native plant species in the region.

Table.1 The list of species, accession numbers, and locations. Taxonomic arrangement follows the Engler system. Black circles indicate the collected quadrates.

Taxon	Family	Species	Accession No.	location										
				Dark						Bright				
Pteridophyte				A-1	A-2	A-3	A-4	B-3	D-3	B-1	B-2	D-1	D-2	C
	Gleicheniaceae	*Dicranopteris linearis*	AB889467							●				
	Pteridaceae	*Acrostichum* sp.	AB889455									●		
		Ceratopteris thalictroides	AB889456								●			●
		Pityrogramma austroamericana	AB889401							●				
		Pityrogramma sp.	AB889468								●			
		Pteris sp.	AB889457							●				
		Taenitis blechnoides	AB889469					●						
	Aspleniaceae	*Asplenium* sp.	AB889470					●	●					
	Blechnaceae	*Artocarpus* sp.	AB889451						●					
		Blechnum orientale	AB889435									●		
		Stenochlaena sp.	AB889402	●	●		●	●	●				●	
	Dennstaedtiaceae	*Pteridium* sp.	AB889440									●		
	Lomariopsidaceae	*Nephrolepis* sp.	AB889466		●				●	●	●		●	
	Polypodiaceae	*Microsorum* sp.	AB889465		●									
Angiosperm														
Monocotyledoneae														
	Alismataceae	*Alisma* sp.	AB889427											●
	Dioscoreaceae	*Dioscorea alata*	AB889458					●						
	Pontederiaceae	*Pontederia* sp.	AB889426											●
	Poaceae	*Centotheca lappacea*	AB889442									●		
		Echinochloa frumentacea	AB889432											●
		Echinochloa sp.	AB889425											●
		Paspalum conjugatum	AB889406											●
		Paspalum sp.	AB889420							●	●	●		
		Pa. vaginatum	AB889405											●
	Araceae	*Epipremnum papuanum*	AB889417				●		●					
		Rhaphidophora sp.	AB889418				●		●					
	Pandanaceae	*Freycinetia* sp.	AB889410	●	●								●	
	Cyperaceae	*Cyperus* sp.	AB889399									●		
		Eleocharis palustris	AB889408									●		
		Erioscirpus sp.	AB889407											●
		Fimbristylis miliacea	AB889428											●
		Fuirena umbellata	AB889400									●		
		Schoenoplectus littoralis	AB889462							●				
		Unidentified1	AB889409							●				
		Unidentified2	AB889429											●
Dicotyledoneae														
Choripetalae														
	Moraceae	*Ficus* sp.1	AB889414			●				●	●		●	
		Ficus sp.2	AB889450						●					
		Ficus sp.3	AB889403								●			
	Urticaceae	*Cypholophus* sp.	AB889412		●									
	Menispermaceae	*Stephania* sp.	AB889461		●									
	Euphorbiaceae	*Endospermum moluccanum*	AB889423								●			
		Homalanthus populneus	AB889448										●	
		H. nutans	AB889449										●	
		Homalanthus sp.	AB889441									●		
	Phyllanthaceae	Unidentified	AB889443									●		
	Rutaceae	*Melicope elleryana*	AB889416				●							
	Meliaceae	*Chisocheton lasiocarpus*	AB889413			●		●						
	Celastraceae	*Lophopetalum* sp.	AB889453	●										
	Rhamnaceae	*Alphitonia* sp.1	AB889446										●	
		Alphitonia sp.2	AB889447										●	
	Malvaceae	Unidentified	AB889459									●		
	Melastomataceae	Unidentified	AB889460		●									
	Myrtaceae	*Syzygium* sp.	AB889415				●						●	

Taxon	Family	Species	Accession No.	location										
				Dark						Bright				
				A-1	A-2	A-3	A-4	B-3	D-3	B-1	B-2	D-1	D-2	C
Angiosperm														
Sympetalae														
	Apocynaceae	*Alstonia scholaris*	AB889439									●		
		Alstonia sp.	AB889421							●	●			
		Trachelospermum sp.	AB889454		●									
	Asclepiadoideae	Unidentified	AB889411		●									
	Gentianaceae	*Fagraea volubilis*	AB889437					●				●		
	Rubiaceae	Unidentified	AB889452	●										
		Breonia sp.1	AB889419							●				
		Breonia sp.2	AB889422								●			
		Cinchonoideae sp.	AB889436									●		
		Nauclea orientalis	AB889438									●		
	Convolvulaceae	*Ipomoea purpurea*	AB889433											●
	Linderniaceae	*Picria fel-terrae*	AB889463											●
		Torenia sp.	AB889434											●
	Phrymaceae	Unidentified	AB889444									●		
	Plantaginaceae	*Limnophila aromatica*	AB889404											●
		Limnophila sp.	AB889430											●
	Asteraceae	*Blumea* sp.	AB889445									●		
		Unidentified	AB889431											●

Our investigation using molecular identification was able to identify unknown samples without diagnostic morphological features in terms of a preexisting classification. For example, Ficus is easy to recognize using a characteristic feature of milky sap containing latex (Heywood, 1978); however, it was very difficult to recognize each fern without spores, which are important characters for identifying species in ferns (Iwatsuki, 1995). Moreover, although silica accumulation in the aerial parts is one of the most prominent characteristics of Poaceae (Jones and Handreck, 1967), it is difficult to identify infrafamilial taxa without flowers and fruits of this family. Our genetic information on different species growing in sago palm forests is an important research focus because this knowledge is needed to understand the succession of these important forests. Additional analyses should be conducted, including in other fields and locations, to clarify the succession of sago palm forests.

Acknowledgment

This research was supported by a research grant from The Mitsui & Co., Ltd., Environment Fund.

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