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Genetic Structure of the Tropical Tree *Eusideroxylon zwageri* in Indonesia Revealed by Chloroplast DNA Phylogeography

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Abstract: *Eusideroxylon zwageri* is a large tropical rainforest tree native to Indonesia, Malaysia, the Philippines, and Brunei. Because of its high economic value, illegal logging and overexploitation is threatening this species in several locations in Indonesia. In this study, in order to conserve genetic resources, we investigated the genetic structure of *E. zwageri* in Indonesia using chloroplast DNA sequencing. *Eusideroxylon zwageri* samples were collected from the Kalimantan (56 trees from seven populations) and Sumatra (16 trees from two populations) islands of Indonesia. Approximately 3137 bp of chloroplast DNA was sequenced for each tree. Twenty-one haplotypes were identified, of which six haplotypes were detected from two or three populations, whereas the other 15 haplotypes were detected from one population each. For each population, one to six haplotypes were detected, and phylogenetically closer haplotypes were detected within the same population. Although the haplotypes were roughly divided into two groups, geographically-close populations did not always have phylogenetically-close haplotypes. Our results suggest that in Indonesia, *E. zwageri* showed a high genetic diversity at the chloroplast DNA level, and populations within a population were derived from similar maternal lineages. Therefore, transplantation within a population may be a feasible option for *E. zwageri* conservation. However, transplantation among different populations should be conducted with careful consideration, because geographic distances are not always related to phylogenetic distances in *E. zwageri*.

Keywords: Borneo ironwood; chloroplast DNA sequencing; chloroplast haplotype; Kalimantan; Sumatra

1. Introduction

Eusideroxylon zwageri T.et B (Borneo ironwood) is a large tropical rainforest tree native to Indonesia, Malaysia, the Philippines, and Brunei. In Indonesia, this species is naturally distributed in the Kalimantan, Sumatra, and Bangka-Belitung islands. Owing to its strong and water-resistant wood, it has high economic value in the construction of bridges, ships, boats, and houses. However, this leads to illegal logging and overexploitation, which is threatening this species in several

locations in Indonesia [1]. Moreover, the lack of a traditional territorial forest management system by the indigenous people and the government also led to the destruction of stands [1]. Although transplantation of *E. zwageri* from other locations is a practical conservation strategy in such cases, an accurate understanding of the genetic structure of *E. zwageri* is necessary for consideration of its conservation, because wild populations often contain a spatial genetic structure linked to specific local ecosystems [2].

Evaluation of the intraspecific genetic variation in chloroplast DNA is a powerful tool for analyzing the genetic structure. In general, chloroplast DNA is maternally inherited in angiosperms; therefore, chloroplast DNA markers reveal gene flow from seed dispersal only. In addition, polymorphisms in haploid genomes are more affected by genetic drift than those in the nuclear genome [3]. Therefore, patterns in the spatial distribution of chloroplast DNA polymorphism—established by seed dispersal during range expansion—should be more slowly eroded by subsequent gene flow than the spatial patterns in nuclear genetic markers, especially in trees [4]. Previous studies on various plant species revealed expansion histories with chloroplast DNA analysis [5–7]. However, little is known about the genetic background of *E. zwageri*.

In this study, in order to gain a better understanding of the phylogeographic genetic structure of *E. zwageri*, we investigated chloroplast DNA polymorphism in *E. zwageri* populations in Indonesia using chloroplast DNA sequencing, which will be useful in designing a conservation strategy for this species.

2. Materials and Methods

2.1. Sample Collection

The plant material (*E. zwageri*) was collected from nine populations in Indonesia: two located in Sumatra and seven located in Kalimantan (Table 1 and Figure 1). These populations were considered natural or secondary forests, and those forests had different management policy by each managing organization (see Supplementary S1). Five to eleven *E. zwageri* trees were selected in each population, and a single leaf was collected per tree. The distance between two selected trees and diameter at breast height of the selected trees were more than 30 m and 10 cm, respectively. Each leaf was dried separately, with silica gel in a plastic bag, and stored at 25 °C (room temperature) until subsequent use.

Table 1. Regions and population from where *Eusideroxylon zwageri* samples were collected and sampling locations along with the number of detected haplotypes in each population in this study.

Region	Population	Longitude	Latitude	Number of Detected Haplotypes																				
				A	B *	C	D	E	F	G	H	I	J	K	L *	M *	N	O *	P	Q *	R *	S	T	U
Kalimantan	Kutai	117°35′29″ E	0°33′53″ N	1	6		1																	
Kalimantan	Berau	117°11′52″ E	0°35′03″ N								1				1			1	1	3	1			
Kalimantan	Bukit Soeharto	116°59′50″ E	0°35′41″ S									2	3	2	1					2		1		
Kalimantan	Kintap	115°09′05″ E	3°42′17″ S													7	1							
Kalimantan	Busang	113°55′43″ E	0°14′23″ S																		1		1	6
Kalimantan	Kapuas Hulu	111°59′23″ E	1°03′35″ N			6		2																
Kalimantan	Gunung Palung	109°59′11″ E	0°56′42″ N												1			1		3				
Sumatra	Musi Rawas	103°17′00′′ E	2°59′39″ S		2				1	5														
Sumatra	Jambi	102°53′17″ E	1°47′01″ S													8								

* haplotypes detected from two or three populations.

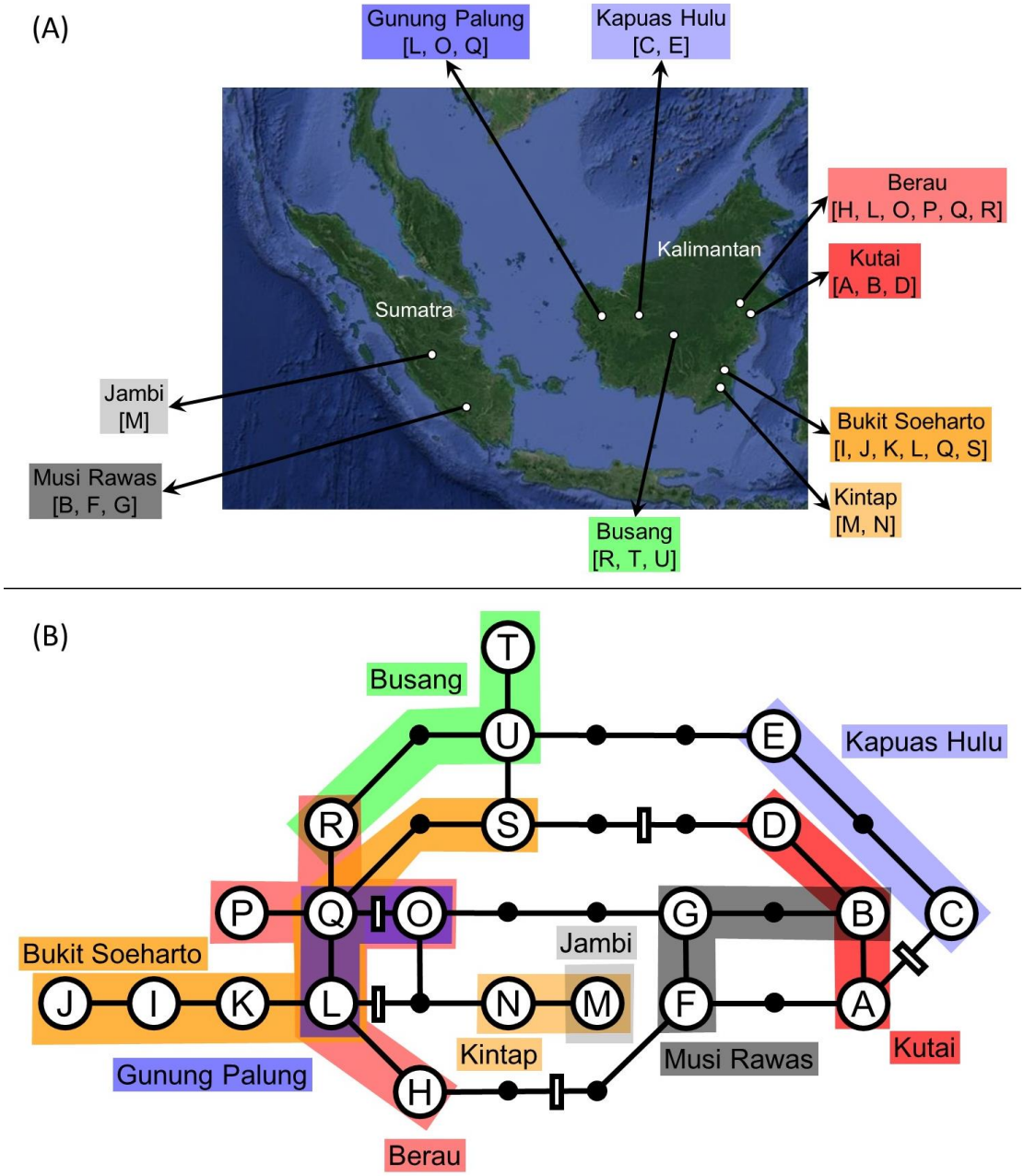

Figure 1. (**A**) Locations of population from where *Eusideroxylon zwageri* samples were collected on the Kalimantan and Sumatra islands. The letters in square brackets represent the detected chloroplast haplotypes at each location. (**B**) Statistical parsimony network of the 21 detected chloroplast DNA haplotypes. The solid circles denote missing or unsampled haplotypes. Each solid line without and with rectangle between the haplotypes and (or) solid circles represents a nucleotide substitution and sequence inversion, respectively. Haplotypes highlighted by the same colors are derived from the same locations.

2.2. DNA Extraction and Chloroplast DNA Sequencing

DNA was extracted from each leaf sample using the modified cetyltrimethylammonium bromide method [8]. The extracted DNA was stored at 4 °C until further analysis. Six chloroplast DNA regions (Ez_Cp_01, Ez_Cp_05, Ez_Cp_07, Ez_Cp_09, Ez_Cp_11, and Ez_Cp_12; [9], see Supplementary S2) were amplified by PCR, as described in [10]. Finally, chloroplast DNA sequencing reactions were

performed as described in [10], using an ABI 3100 Genetic Analyzer (Thermo Fisher Scientific K.K., Kanagawa, Japan).

2.3. Data Analysis

The sequence data were assembled using the program BioEdit Sequence Alignment Editor [11]. Sequences were aligned using the software package MEGA 5 [12]. The chloroplast DNA haplotypes were determined based on nucleotide substitutions and sequence inversions in the aligned sequences. A haplotype network was reconstructed with the statistical parsimony method [13] using TCS ver. 1.21 [14], with a 0.95 probability connection limit. Each indel was treated as one nucleotide gap.

3. Results

Seventy-two *E. zwageri* trees from nine populations in Indonesia were used to determine the 3137 bp sequence of six chloroplast DNA regions for each tree sample (see Supplementary S2). We found 12 chloroplast DNA mutations, including 11 nucleotide substitutions and one sequence inversion (Table 2), and identified 21 haplotypes (haplotypes A to U) (Table 2). The haplotype network was constructed, and showed roughly two haplotype groups: one group including haplotypes A to G, and the other including haplotypes H to U (Figure 1). Between these two groups, there were two or more missing or unsampled haplotypes detected (Figure 1).

Table 2. Chloroplast DNA characteristics of all haplotypes detected in *Eusideroxylon zwageri*.

	a	**Ez_Cp_01**			**Ez_Cp_05**			**Ez_Cp_07**	**Ez_Cp_09**	**Ez_Cp_11**		**Ez_Cp_12**	
	b	**530**			**648**			**490**	**467**	**505**		**497**	
	c	**63**	**264**	**56**	**164**	**166**	**560**	**468**	**171–175**	**45**	**137**	**28**	**431**
Haplotype	***d***	***SNP***	***SNP***	***SNP***	***SNP***	***SNP***	***SNP***	***SNP***	***SI***	***SNP***	***SNP***	***SNP***	***SNP***
A		C	A	A	A	A	G	C	CTACT	T	A	G	A
B		C	A	A	A	A	G	A	CTACT	T	A	G	A
C		C	A	A	A	A	G	C	AGTAG	T	A	G	A
D		C	A	A	A	A	G	A	CTACT	T	G	G	A
E		C	A	A	A	A	G	C	AGTAG	G	G	G	A
F		T	C	A	A	A	G	C	CTACT	T	A	G	A
G		T	C	A	A	A	G	A	CTACT	T	A	G	A
H		T	C	A	G	A	G	C	AGTAG	T	A	T	A
I		T	C	A	G	C	A	C	AGTAG	T	G	T	A
J		T	C	A	G	C	A	A	AGTAG	T	G	T	A
K		T	C	A	G	C	G	C	AGTAG	T	G	T	A
L		T	C	A	G	A	G	C	AGTAG	T	G	T	A
M		T	C	G	G	A	G	C	CTACT	T	G	T	C
N		T	C	A	G	A	G	C	CTACT	T	G	T	C
O		T	C	A	G	A	G	A	CTACT	T	G	T	A
P		T	C	G	G	A	G	A	AGTAG	T	G	T	A
Q		T	C	A	G	A	G	A	AGTAG	T	G	T	A
R		T	C	A	G	A	G	A	AGTAG	G	G	T	A
S		C	A	A	G	A	G	A	AGTAG	T	G	T	A
T		C	A	A	G	A	G	A	AGTAG	G	G	T	C
U		C	A	A	G	A	G	A	AGTAG	G	G	T	A

a: ID of primer pair, ***b***: sequence length (bp), ***c***: location in each sequence, ***d***: type of mutations, SNP: single nucleotide polymorphism, SI: sequence inversion. Haplotype names are the same as in Figure 1.

Six haplotypes (haplotypes B, L, M, O, Q, and R) were detected from two or three populations, whereas the other 15 haplotypes were detected from one population each (Table 1 and Figure 1). For each population, one to six haplotypes were detected (Table 1 and Figure 1). More haplotypes were detected from the natural populations (Kutai, Berau, Bukit Soeharto, Busang, Gunung Palung, and Musi Rawas) than from the populations including secondary populations (Kintap, Kapuas Hulu, and Jambi) (Table 1, Figure 1, and see Supplementary S1). Phylogenetically closer haplotypes were detected within the same population. Haplotypes A to G were found in Kutai, Kapuas Hulu, and Musi Rawas (Table 1). In contrast, Haplotypes H to U were found in Berau, Bukit Soeharto, Kintap, Busang,

Gunung Palung, and Jambi (Table 1). Although the populations in Kutai and Berau are geographically close (43.8 km), the haplotypes detected in these populations were not phylogenetically close (Table 1 and Figure 1).

4. Discussion

In this study, we detected 21 chloroplast haplotypes from nine populations (Table 1 and Figure 1) using our previously developed chloroplast DNA markers [9]. Previously, several studies have examined the genetic distribution of tropical woody species through the analysis of chloroplast haplotypes. Tnah et al. [15] reported that 21 chloroplast haplotypes were detected from 32 populations in *Neobalanocarpus heimii*. Kamiya et al. [16] detected 15 chloroplast haplotypes in *Shorea curtisii* in eight populations. Ohtani et al. [2] reported 28 chloroplast haplotypes in 27 *S. leprosula* populations. Similarly, many chloroplast haplotypes in *E. zwageri* were detected in our study. In addition, the populations in the natural forests (see Supplementary S1) had more chloroplast haplotypes (Table 1 and Figure 1). Assuming that more maternal lineages had more genetic variations, our results indicated that the secondary populations might lead to the loss of genetic variation in *E. zwageri* within population.

Multiple haplotypes were detected in each population, except Jambi (Table 1). Considering the extremely low mutation rate of chloroplast DNA, the existence of several haplotypes per population (Table 1) might be explained by the hypothesis that *E. zwageri* has been established in these populations for a long time. Because *E. zwageri* has heavy, gravity-dispersed seeds, the expansion of its distribution in nature may be slow. In this study, the haplotypes detected within each population were phylogenetically close (Table 1), supporting the slow expansion of *E. zwageri* at the population level, owing to its biological characteristics. Moreover, this result was consistent with the results of previous studies in *S. curtisii* [16] and *S. leprosula* [2]. In these species, there was a clear differentiation in genetic structure of chloroplast haplotypes between the Sumatra and Kalimantan populations. However, in our study, it was difficult to detect a clear relationship between geographic and phylogenetic distances for *E. zwageri* based on the results of phylogenetic analysis and haplotype distribution (Figure 1 and Table 1). For example, haplotypes B and M were found on both Kalimantan and Sumatra islands (Table 1). Moreover, haplotype B from groups A to G—which were phylogenetically close—were detected on both islands (Figure 1). According to many previous studies on phylogeographic analysis of chloroplast DNA in indigenous tree species, the distribution ranges of vastly different haplotypes are often spatially separate [7,17], and genetically close haplotypes are distributed continuously [6,7,17]. In contrast, the results obtained in *Brachypodium sylvaticum* [6], *Prunus serotina* [7], and *Ailanthus altissima* [8] suggest that introduced non-indigenous plant species with limited maternal lines can expand their distributions widely in each country and area of introduction. In addition, phylogenetically-divergent haplotypes in the same and nearby populations have been reported in several invasive plants [8,18–21]. Based on the results of previous studies and our present study, we propose a hypothesis that the nationwide distribution of *E. zwageri* has been affected by human activity in Indonesia. The long-term effect of human activity on the genetic structure in useful tree species is described in [22]. In Indonesia, *E. zwageri* is also one of the most important species as construction wood for making furniture, windows, door frames, heavy pilings, and fence posts [23]. Therefore, the long history of human usage of *E. zwageri* in Indonesia may support our results.

5. Conclusions

In this study, it was revealed that *E. zwageri* had genetic structure at a chloroplast DNA level in Indonesia. Since populations within a population were derived from similar maternal lineages, transplantation within a population may be a feasible option for *E. zwageri* conservation. In that case, when a secondary population is treated, seedlings should be prepared from the plural similar lineages. However, transplantation among different populations should be conducted with careful consideration, because geographic distances are not always related to phylogenetic distances in

E. zwageri, and additional aspects are required to accurately evaluate the production area of *E. zwageri* with the knowledge of chloroplast haplotypes.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/8/7/229/s1. Supplementary S1: Forest and management conditions of each location. Supplementary S2: All base arrangements of "Haplotype B" for six chloroplast DNA region that we used in this study. Yellow-highlighted characters represent where genetic variations detected and are corresponded with Table 2.

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Conflicts of Interest: The authors declare no conflict of interest.

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