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DNA Genotyping of Borneo Ironwood using M13 Universal Primer and SCAR Marker Development in *Eusideroxylon zwageri*

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Abstract Borneo Ironwood, consists of two species, i.e. *Eusideroxylon zwageri* and *Potoxylon melagangai*, is the most well known durable tropical hardwood timber tree of Borneo. Both species are morphologically similar, except the wood characteristics. This has resulted in taxonomic difference between them. We used PCR with M13 universal primer to determine the genetic relatedness and SCAR marker to distinguish both species. Cluster analysis grouped both species into two major clusters, suggesting that there are two genetically distantly related species. One diagnostic band (locus M13-16) was successfully converted to SCAR marker. A single fragment (~720 bp) was amplified in all *E. zwageri* individuals but completely absent in *P. melagangai*. Differentiating the species by SCAR marker is very useful for screening large numbers of Borneo Ironwood seedlings collected from natural populations. The identification of individuals at species level constitutes one of the first basics in any effective conservation programme. In fact, the Borneo Ironwood has been counted as one of the endangered species in Sarawak.

Keywords Borneo Ironwood • genotyping • M13 • universal primer • diagnostic band • SCAR

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Introductions

Belian or its popular name, Borneo Ironwood is the most famous and well-known durable tropical hardwood timber tree of Borneo, which comes from Lauraceae family. It is officially announced as the State Tree of Sarawak on 20th March 1984. There are two species of Borneo Ironwood, namely *Eusideroxylon zwageri* and *Eusideroxylon melagangai* (De Wit, 1949). Both species are not easy to be distinguished in the field because of similar morphological characteristics, except the wood structure (Browne, 1955; Soerjanegara and Lemmens,

1994). *E. melagangai* has lighter colour of wood, lower durability and natural strength, lower height rays, reddish colour, rather fine texture and longer bands of soft tissue linking up the pores compared to *E. zwageri*. Besides, it has lighter density (0.70 – 0.80 g/cm³) compared to *E. zwageri* (0.88 – 1.19 g/cm³) that enabled it to float on the surface of water. The variation in wood characteristics has resulted in taxonomic difference between both species. In 1979, Kostermans finally referred *E. melagangai* to a new monotypic genus as *Potoxylon* and named as *Potoxylon melagangai* Kosterm., which is synonym to *E. melagangai* Sym. (Soerjanegara and Lemmens, 1994). However, the results could be inconclusive due to developmental and environmental influences on the morphology, problems with low juvenile-mature correlation and the close phenotype resemblance among clones from selection response for similar characteristics (Chong et al. 1995). Thus, it would be best to use PCR-based genetic markers coupled with morphological characteristics to differentiate Borneo Ironwood.

PCR-based genetic markers are developmentally stable, governed by complex genetic interactions and not affected by the environment. M13 universal primer is one of the marker systems that have been widely recognized in DNA fingerprinting of plants, animals and microorganisms (Chong et al. 1995; Ho et al. 2006). It is a dominant marker derived from protein III gene of M13 bacteriophage that later proved useful in generating multiple banding patterns in all plant species and potentially an individual-specific banding pattern. Sequence characterized amplified region (SCAR) markers have been developed recently to produce more specific and reproducible results that could be used for species or cultivar identification (Scheef et al. 2003).

Reliable SCAR markers have been successfully developed for several crops and woody species, including common bean (Adam-Blondon et al. 1994), rice (Nagvi and Chattoo, 1996) and apple (Evans and James, 2003). To date, there is no published report on the development of species-specific markers in Borneo Ironwood. Hence, the objectives of this study were (1) to genotype Borneo Ironwood by using M13 universal primer and (2) to convert M13 amplified diagnostic band to species-specific sequence characterized amplified region (SCAR) marker in *E. zwageri* for Borneo Ironwood typing.

Materials and Methods

A total of 16 individuals of *E. zwageri* and *P. melagangai* maintained in the green house were selected for the present study. *E. zwageri* were collected from Samunsam Wildlife Sanctuary, Lundu meanwhile *P. melagangai* were collected from Mulu National Park, Miri, Sarawak on August 2000. The total genomic DNAs of *E. zwageri* and *P. melagangai* were isolated using a modified CTAB method from Doyle and Doyle (1987). DNA concentration was determined spectrophotometrically and its integrity and concentration were further determined by 0.8% agarose gel electrophoresis.

PCR amplification reaction was performed in volumes of 25 µl containing 1X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 3.0 mM $MgCI_2$, 0.2 mM each of dNTP, 0.4 µM M13 universal primer, 1 unit of *Taq* DNA polymerase (Promega) and 10 ng of genomic DNA. The amplifications were done in a GeneAmp PCR System Model 2400 (Perkin-Elmer) thermocycler, programmed for 35 cycles of 1 minute of denaturing at 94°C, 1 minute of annealing at 48°C and 2 minutes of extension at 72 °C after an initial 2 minutes denaturing at 94°C. The program was then followed by a final extension step at 72 °C for 10 minutes. The PCR products were separated on a 1.2% (w/v) agarose gel for 3 hours at 80 V in 1X TBE buffer and stained with Gelstar® nucleic acid gel stain (FMC Bioproducts).

The PCR bands were named after the primer and a hyphenated number corresponding to the order of their migrations, e.g., the PCR amplified bands were designated as M13-01, M13-02, and etc. The presence of a band was scored as 1 and absence was scored as 0, based on the following criteria: (1) each locus was assumed as independent or non-allelic, (2) there was no bias in scoring monomorphic fragments versus polymorphic fragments and (3) only fragments in the range of 300 bp to 2.1 kb were considered in order to increase the data reliability. Pairwise genetic similarity was calculated based on the similarity index, $J_{ij} = C_{ij} / (n_i + n_j - C_{ij})$ (Jaccard, 1908). The data was then subjected to cluster analysis by the Unweighted Pair-Group Method with Arithmetical Averages (UPGMA) method as described by Sneath and Sokal (1973) using NTSYS-pc software (Rohlf, 1998).

Table 1
Jaccard similarity matrix generated by M13 universal primer (M: *Potoxylon melagangai*; Z: *Eusideroxylon zwageri*)

	M7	M8	M9	M10	M11	M12	M14	M15	Z6	Z9	Z10	Z11	Z12	Z17	Z18	Z20
M7	1.00															
M8	1.00	1.00														
M9	0.56	0.56	1.00													
M10	0.76	0.76	0.80	1.00												
M11	0.76	0.76	0.80	0.84	1.00											
M12	0.68	0.68	0.88	0.84	0.92	1.00										
M14	0.68	0.68	0.88	0.76	0.84	0.84	1.00									
M15	0.68	0.68	0.80	0.84	0.84	0.84	0.84	1.00								
Z6	0.44	0.44	0.48	0.52	0.60	0.52	0.52	0.44	1.00							
Z9	0.48	0.48	0.36	0.40	0.48	0.40	0.40	0.32	0.88	1.00						
Z10	0.52	0.52	0.40	0.44	0.52	0.44	0.44	0.36	0.92	0.96	1.00					
Z11	0.40	0.40	0.44	0.48	0.56	0.48	0.48	0.40	0.96	0.92	0.88	1.00				
Z12	0.52	0.52	0.40	0.44	0.52	0.44	0.44	0.36	0.92	0.96	1.00	0.88	1.00			
Z17	0.40	0.40	0.44	0.48	0.56	0.48	0.48	0.40	0.96	0.84	0.88	0.92	0.88	1.00		
Z18	0.40	0.40	0.44	0.48	0.56	0.48	0.48	0.40	0.96	0.84	0.88	0.92	0.88	0.92	1.00	
Z20	0.48	0.48	0.36	0.40	0.48	0.40	0.40	0.32	0.88	1.00	0.96	0.92	0.96	0.84	0.84	1.00

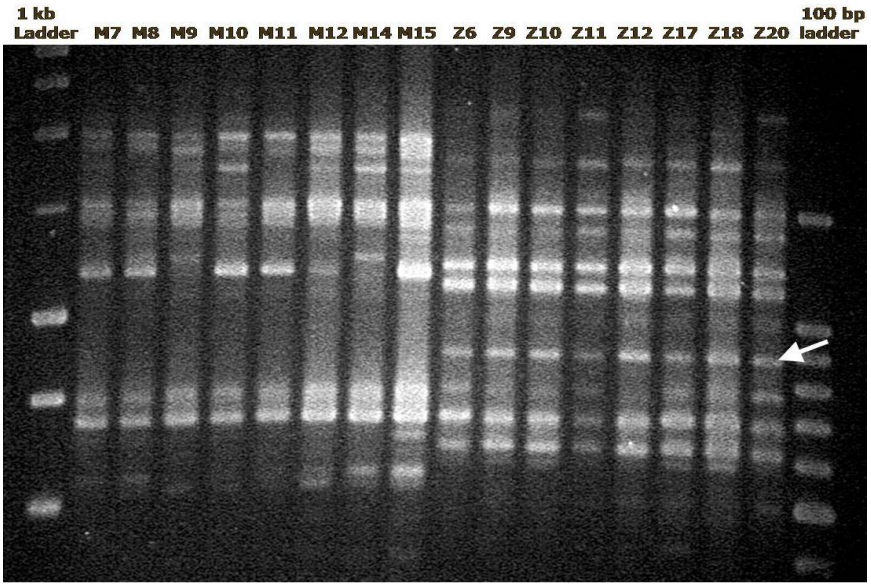

Figure 1: DNA profiles of *P. melagangai* (lane 2 – 9) and *E. zwageri* (lane 10 – 17) generated by using M13 universal primer. Arrow (locus M13-16) indicates species-specific, strong and reproducible band that was amplified in *E. zwageri* and converted into species-specific SCAR marker

The diagnostic fragments were identified from the DNA profiles of *E. zwageri* and *P. melagangai* individuals generated by using M13 universal primer. Those diagnostic fragments were extracted from the agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN) and subsequently, cloned into the pGem®-T Easy Vector (Promega). Three clones were selected for plasmid extraction using Wizard® Plus SV Minipreps DNA Purification System (Promega) sequenced by an automated ABI 3130*xl* Genetic Analyzer using Big Dye (dRhodamine) terminator cycle sequencing-ready reaction kit (Applied Biosystems). Based on the nucleotide sequences of the cloned diagnostic fragments, pairs of oligonucleotide primers ranging from 17 to 30 bases in length were designed using the Primer Premier 5.0 software. The newly designed primers were validated using eight *E. zwageri* and one *P. melagangai* samples. Reaction components were as described above, with the following exception: 20 ng of template DNA and the annealing temperature was increased to 65.5°C. This assay was replicated three times to test for the reproducibility of the amplified diagnostic fragment. Amplified

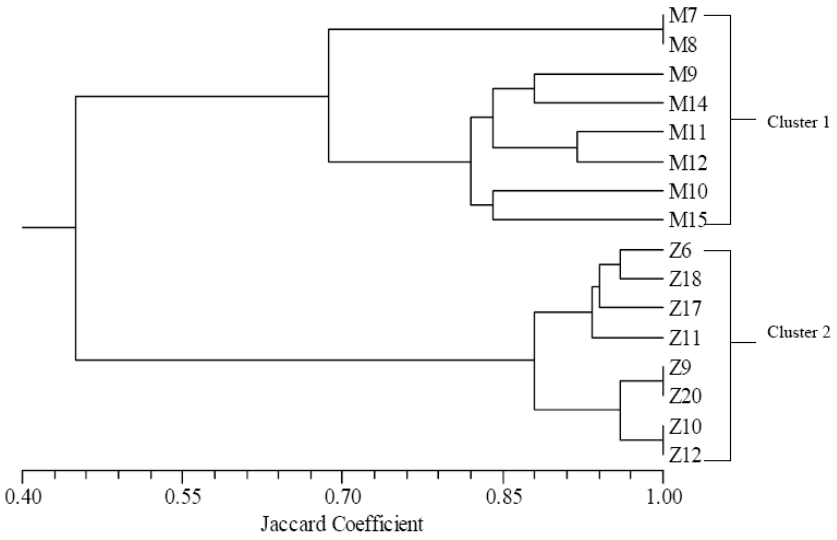

Figure 2: Dendrogram represents the cluster analysis of Jaccard similarities among the Belian individuals, i.e. *P. melagangai* (Cluster 1) and *E. zwageri* (Cluster 2)

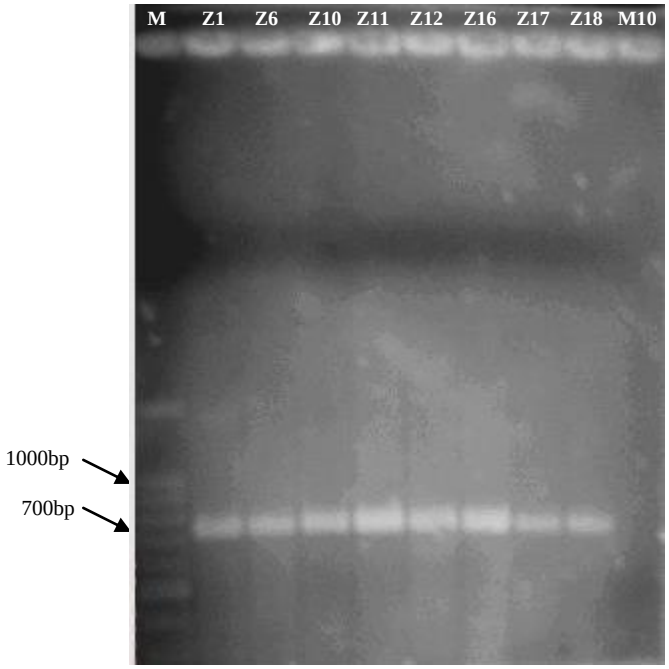

Figure 3: Banding pattern of SCAR primer pair amplifying a single and reproducible band specifically for *E. zwageri*. M is 100 bp ladder and Z1, Z6, Z10, Z11, Z12, Z16, Z17, and Z18 are *E. zwageri* samples. M10 is *P. melagangai* sample

products were resolved using 1.5% agarose gel electrophoresis.

Results and Discussion

The total genomic DNA of all 16 samples of *E. zwageri* and *P. melagangai* were isolated using the modified CTAB extraction method (Doyle and Doyle, 1987). The DNA quality of *P. melagangai* was relatively better than *E. zwageri* due to less polysaccharides and secondary metabolites. The presence of these contaminants will cause the DNA solution become very viscous and difficult to extract, especially in *E. zwageri* samples. The A_{260}/A_{280} absorbance ratios were 1.28 - 2.08 with DNA concentrations ranged from 10ng/µl to 3µg/µl in both species. Figure 1 shows the multiple banding patterns (25 bands) generated by M13 universal primer in *P. melagangai* and *E. zwageri*. The constructed dendrogram based on

Jaccard similarity matrix (Table 1) had grouped Borneo Ironwood into 2 major clusters that composed of *P. melagangai* (Cluster 1) and *E. zwageri* (Cluster 2) (Figure 2). The mean genetic similarity within individuals in Cluster 1 was 0.78 and 0.91 for Cluster 2, meanwhile the mean genetic similarity between Cluster 1 and Cluster 2 was 0.45. This suggests that *P. melagangai* and *E. zwageri* are two genetically distantly related species. Two common bands were also found in all the individuals of *E. zwageri* and *P. melagangai* (Figure 1), indicating that both *E. zwageri* and *P. melagangai* were derived from the same ancestral but had evolved over the years for survival. This may explain why both *E. zwageri* and *P. melagangai* have a very close similarity in their vegetative characters (Browne, 1955).

One distinct and highly reproducible diagnostic band was identified from the 25 loci generated by M13 universal primer, i.e. locus M13-16 from *E. zwageri*. This diagnostic band was then cloned and sent for sequencing (Figure 1). Sequence data from this diagnostic band was submitted to GenBank (accession number FJ624400) and no significant sequence similarities were found with NCBI GenBank non-redundant database. Analysis of sequence data was performed using BLAST search. SCAR marker was created by sequencing a single diagnostic band and designing a specific primer pair to amplify the band of specific size. The SCAR primer pair is designated as Zg16-F (5'-AGGGTCGCTATTGGTGGA- 3') and Zg16-R (5'–AGCCTCTTTCAGTCGGTCA- 3'). A single and distinct band at approximately 720 bp was observed in all the eight *E. zwageri* samples while no band was detected in *P. melagangai* sample (Figure 3). Differentiating this species by SCAR markers is very useful for screening large numbers of Borneo Ironwood seedlings collected from natural populations.

To the best of our knowledge, this is the first report on the development of SCAR marker in determining Borneo Ironwood using species specific marker developed from M13 universal primer. We hope this approach could pave the way for the unambiguous identification of Borneo Ironwood. However, more samples need to be used to reconfirm the validity of the designed SCAR marker. Once the confirmation is done, the other three selected diagnostic bands also can be converted to SCAR markers and subsequently, multiplex PCR can be developed using these markers. Multiplex PCR would save time and money by using only one reaction per sample instead of several reactions, one for each primer (Scheef et al. 2003). The identification of individuals at species level constitutes one of the first basics in any effective conservation programme. In fact, the Borneo Ironwood has been counted as one of the endangered species in Sarawak.

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