Cross-Species Amplification of Shorea Microsatellite DNA Markers in Parashorea malaanonan (Dipterocarpaceae)

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Sixteen (16) microsatellite DNA markers previously developed for two Bornean species of Shorea (Dipterocarpaceae), viz. 5 from Shorea curtisii (Shc) and 11 from Shorea leprosula (Sle), were tested on Philippine Parashorea malaanonan for cross-species amplification. Initially, these loci were tested in Parashorea malaanonan from the Makiling Forest Reserve on Luzon Island. All five Shc loci had amplification products, three (3) were polymorphic and two (2) were non-polymorphic. Of the 11 Sle loci, five (5) were polymorphic, three (3) were non-polymorphic, two (2) did not have amplification products, and one (1) had multiple bands. Four loci were further tested on individuals of Parashorea malaanonan from a plantation on Leyte Island. High levels of genetic variability were observed. Cross-transferability was 75%. Expected heterozygosity (Hₑ) ranged from 0.39 to 0.82. Results indicate potential use of these markers derived from Shorea in studies of genetic diversity assessments in Parashorea malaanonan.

Key Words: cross transferability, Dipterocarpaceae, genetic diversity, Parashorea malaanonan, Shorea, SSR

INTRODUCTION

Parashorea malaanonan (Blanco) Merr. is one of about 45 species of the timber family Dipterocarpaceae thus far recorded from the Philippines (Ashton 1982; Newman et al. 1996). It is known from at least 10 of the larger islands in the Philippine archipelago and also from the northeast of Borneo (Newman et al. 1996; Ashton 2004). The species is common in lowland forests and occurs up to 1300 m alt. One recent study has characterized the mating system and assessed the genetic diversity of this species using isozymes (Gamboa-Lapitan and Hyun 2005).

In most dipterocarps, genetic variation within species is largely unknown. Isozymes have been used in assessing population variation in Stemonoporus oblongifolius Thw. by Murawski and Bawa (1994), Lee et al. (2000a) in Shorea leprosula Miq., and Lee et al. (2000b) in Dryobalanops aromatica Gaertn.f. Cao et al. (2006) used amplified fragment length polymorphism (AFLP) to determine genetic diversity and differentiation among Indonesian populations of Shorea leprosula and Shorea parvifolia Dyer. Microsatellite DNA markers have previously been used by Konuma et al. (2000) and Nagamitsu et al. (2001) in analyses of dipterocarp breeding systems and genetic diversity assessments.

In order to aid in the assessment of genetic diversity in Parashorea malaanonan we investigated the potential use of microsatellite DNA markers. These markers,
also known as simple sequence repeats (SSRs), have the potential to resolve genetic relationships at all levels of population structure (Jarne and Lagoda 1996). The development of new microsatellite markers, however, is often time-consuming and expensive owing to the need for DNA sequence data of these short tandem repeats and their flanking regions from the genome of the target species. One alternative approach is to exploit the available information by cross-species amplifications among a range of phylogenetically related species. There is increasing number of studies on plants that report some cross-species amplification of SSR loci, with occasional informative transferability of loci to divergent taxa (e.g. Dayanandan et al. 1997; Zucchi et al. 2002; Aldrich et al. 2003; Yasodha et al. 2005).

Ujino et al. (1998) developed eight microsatellite loci for *Shorea curtisii* Dyer ex King, five of which were transferable to other Bornean species belonging to the following genera of the family Dipterocarpaceae, viz. *Anisoptera, Hopea, Neobalanocarpus, Parashorea, Vatica* and *Upuna*. Lee et al. (2004) also characterized 20 microsatellite loci for *Shorea leprosula* and 16 of these loci were cross-transferable to *Shorea parvifolia*.

In this study we investigated the transferability of these two sets of microsatellite loci characterized for two Bornean species of *Shorea* to *Parashorea malaanonan* from the Philippines.

**MATERIALS AND METHODS**

**Leaf samples and DNA extraction**

We obtained leaf material from four individuals of *Parashorea malaanonan* from separate populations and different elevations in the Mt. Makiling Forest Reserve on Luzon Island, Philippines for the initial screening of cross-amplification. The collected leaves were tagged, placed in resealable plastic bags and stored in a styrofoam container with ice while in the field, and immediately transferred to a biofreezer in the laboratory until DNA extraction. Since the main objective of this initial study was to assess the transferability of SSRs rather than polymorphism, the number of individuals tested is considered satisfactory. In addition, leaf material of this species was also obtained from nine individuals in a tree plantation in the Cienfuegos–San Vicente Forests near Baybay on Leyte Island. This was done because of the potential presence of unique alleles in distant populations, especially in an archipelagic setting such as the Philippines.

DNA extraction was done from frozen (-80°C) mature leaf material using the protocol of Tsumura et al. (1996) with modifications to improve the quality and quantity of the DNA obtained. Using this protocol, a viscous and brownish pellet was obtained indicating that polyphenols and tannins precipitated with the DNA. Sodium bisulfite was used in place of beta mercaptoethanol as antioxidant. Incubation period was extended to 1 hour at a higher temperature of 65°C to allow for the efficient breakage of the cells considering that mature leaves already developed rigid cell walls. Both RNAse A and proteinase K treatments were extended to 1 hour each to allow for sufficient degradation of RNA and other proteins.

Phenol:chloroform:octanol (25:24:1) was effective in removing impurities of DNA extracted from mature leaves that were not degraded by the enzymes RNAse and proteinase. Phenol and chloroform are organic solvents and they effectively trapped hydrophobic substances such as polysaccharides and other PCR-inhibiting substances, leaving the hydrophilic DNA in the aqueous phase. Chloroform:octanol (24:1) purification removed the cloudiness of the solution brought about by PVP (Porebski et al. 1997). Precipitation of the DNA by absolute ethanol and sodium acetate increased its yield.

The amount of DNA extracted was determined by comparing it with a standard uncut lambda (λ) phage DNA marker. Two μL of resuspended DNA was mixed with 18 μL of 1x blue juice (0.04% bromphenol blue, 6.67% sucrose in water). The mixture was loaded on a 1% agarose gel run in 1x TBE buffer (90 mM Tris borate, 2mM EDTA) at 100V for 30 minutes. DNA was visualized by staining with ethidium bromide (10mg/μL) and viewed under ultraviolet light (410-720 nm) using Biorad Gel Documentation System (Biorad Laboratories, Segrate Milan, Italy). DNA working stock (5ng/μL) was prepared from the visual quantification of DNA per sample.

**PCR and Electrophoresis**

Sixteen (16) microsatellite DNA markers or simple sequence repeats (SSRs) developed for two Bornean species of *Shorea* (Dipterocarpaceae), viz. *Shorea curtisii* and *Shorea leprosula*, were used in this study (Table 1). Five of the SSRs were previously developed for *Shorea curtisii* (*Schn*) (Ujino et al. 1998) and 11 for *Shorea leprosula* (*Sle*) (Lee et al. 2004). These primers were chosen based on the high (>0.60) expected heterozygosity (*H*). The oligonucleotide primers used in this study were synthesized by Invitrogen Philippines. These sixteen (16) loci were screened using individuals of *Parashorea malaanonan* from four different sites within the Mt. Makiling Forest Reserve. Annealing temperatures were adjusted to obtain the best amplification product as applied
to *Parashorea malaanonan*. Successful amplification was obtained at a recommended or slightly lower annealing temperature for the Shc loci and at a recommended or slightly higher temperature for the Sle loci (Table 1). PCR amplifications were performed in 10 µL reaction mixture which consisted of the following: 1x PCR buffer (50 mM KCl and 20 mM Tris HCl, pH 8.0), 1.5 mM MgCl₂, 0.2mM dNTPs, 0.2 µM forward and reverse primers, 0.5 U of Taq polymerase and 10 ng of DNA.

The reaction mix was overlaid with mineral oil to prevent evaporation during PCR amplification. The Eppendorf tubes containing the reaction mix were loaded in PTC-100™ Programmable Thermal Controller, MJ Research, Inc. PCR amplification was carried out using the following profile: 1 cycle of initial denaturation step of 94°C for 3 minutes, 40 cycles of denaturation step of 94°C for 1 minute, annealing step of 30 seconds at the optimized annealing temperature for each primer, and extension step of 72°C for 30 seconds and 1 cycle of final extension of 72°C for 3 minutes. The PCR products were kept at -20°C until electrophoresis.

PCR products were resolved using a polyacrylamide gel electrophoresis. Based on an established criterion, successful amplification was determined by the presence of one or two sharp bands as amplification products around the expected size range (Huang et al. 1998). A fragment is considered outside the expected size range if it is >100 bp (base pair) larger or smaller than the original sequence (Arnold et al. 2002). Where multiple bands were generated around the expected size range, they are considered stutter bands and were not included in the study.

For polymorphic loci that yielded amplification products and at the same time, exhibited polymorphism across the individuals tested from the Mt. Makiling Forest Reserve, cross amplification was further assessed by a survey of nine individuals from the Leyte plantation. The other polymorphic loci were considered in a subsequent study.

### Data analysis

Fragment sizes were scored by visually comparing it with standard DNA molecular weight marker (Marker VIII, Roche Diagnostics). Tools for Population Genetic Analyses (TFPGA; Miller 1997) was used to calculate...
descriptive statistics (observed heterozygosity, $H_o$, expected heterozygosity, $H_e$ following Nei 1978). PopGene (Yeh et al. 1997) was used to calculate $n_o$, (expected number of alleles, according to Kimura and Crow 1963) while $n_e$ (number of alleles) was scored by visual inspection of the gel. Polymorphic information content (PIC) (Powell et al. 1996) was also computed.

RESULTS AND DISCUSSION

Transferability of Shorea SSR markers to Parashorea malaanonan

All of the primers developed for Shorea curtisii (Shc01, Shc04, Shc07, Shc09 and Shc11) yielded amplification products when initially screened with four individuals from the Mt. Makiling Forest Reserve. Nine of the primers (82%) developed for Shorea leprosula had amplification products (Table 1; Figure 1). The other three primer pairs did not amplify scorable bands. Ujino et al. (1998) were able to obtain amplification products when they tested the Shc loci with other dipterocarp species. They have noted the possible transfer of these loci even in phylogenetically distant species like Anisoptera, Dipterocarpus and Vatica where at least four out of the five loci exhibited strong amplification.

Figure 1. Banding patterns in Parashorea malaanonan from Mt. Makiling Forest Reserve, Philippines using different primer pairs developed for Shorea curtisii (Ujino et al. 1998) and Shorea leprosula (Lee et al. 2004). A. Shc, Shorea curtisii. B. Sle, Shorea leprosula. Numbers represent gel lanes for source materials of Parashorea malaanonan. 1 – Maralas, 2 – Flatrocks, 3 – Mudspring, 4 – Station 13

Peakall et al. (1998) and Arnold et al. (2002) pointed out the importance of sequencing the PCR products obtained in cross amplification due to size homoplasy. This is one limitation of this study. However, Konuma et al. (2000) and Nagamitsu et al. (2001) were able to use Shc07 and Shc09 without sequencing the amplification products in their study of paternity analysis in Neobalanocarpus heimi (King) Ashton and breeding systems in Shorea leprosula, respectively, and obtained good results.

Various authors recognize that cross amplification success is attributed to the sequence conservation at the flanking regions of the SSRs (Kijas et al. 1995; Byrne et al. 1996; Dayanandan et al. 1997). Sequence conservation depends on the evolutionary relationship between the source species (species for which the SSR locus is developed) and the target species (the species to which cross amplification has been made). The more divergent the taxa, the lesser is the success of cross amplification. For example, Hornero et al. (2001) obtained 54% cross amplification success in SSRs of Quercus myrsinifolia Blume transferred to Quercus suber L., while Yasodha et al. (2005) obtained 30% success in cross amplification of Eucalyptus SSRs to Casuarinaceae.

Because Shorea and Parashorea have close affinity (Tsumura et al. 1996; Kamiya et al. 2005), cross species amplification was obtained with high success. It is interesting to note however, that in the study of Ujino et al. (1998) it was not possible to obtain amplification products with Shc04 when tested with Parashorea lucida, a species congeneric with Parashorea malaanonan. The phylogenetic relationship between these two species might be worth exploring in the future.

Polymorphic loci included Shc04, Shc07 and Shc11, Sle074, Sle216, Sle290, Sle392, and Sle562 when tested with individuals from the Mt. Makiling Forest Reserve. These loci produced sharp scorable bands. The non-polymorphic loci (Shc01, Shc09, Sle111a, Sle280 and Sle303a) should also be considered carefully in future studies. It is possible that they will show polymorphism when tried with other material, i.e. Parashorea malaanonan of a different provenance. For example, Sle290 which showed monomorphic bands with the Leyte population was polymorphic in another population (Abasolo et al. in preparation). In addition, they may be useful in molecular studies of other closely related species of dipterocarp.

Thus, to test the usefulness of these primers to ecological and diversity studies, initial analysis of four polymorphic primers (Shc04, Shc07, Shc11, and Sle290) were used to survey individuals from the Leyte population.
SSR polymorphism of *Parashorea malaanonan* in a plantation

Three of the four tested loci (*Shc*04, *Shc*07, and *Shc*11) were polymorphic when tested on individuals from a plantation while *Sle*290 had non-polymorphic bands, thus, polymorphic loci is 75% (Table 2). Expected heterozygosity (H_e) ranged from 0.39 in *Shc*07 to 0.82 in *Shc*04, with an average of 0.46. Polymorphic information content (PIC) (Powell et al. 1996) obtained for the nine individuals are also high in each of the microsatellites: *Shc*04 yielded 0.81, 0.47 for *Shc*07 and 0.43 with *Shc*11. The mean number of alleles per locus is 3.75.

### Table 2. Characterization of the subset of four polymorphic SSR markers applied to individuals of *Parashorea malaanonan* from a plantation on Leyte Island, Philippines

<table>
<thead>
<tr>
<th>Locus</th>
<th>N_a</th>
<th>n_a</th>
<th>n_b</th>
<th>PIC</th>
<th>H_e</th>
<th>H_o</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shc</em>04</td>
<td>9</td>
<td>6</td>
<td>4.38</td>
<td>0.81</td>
<td>0.33</td>
<td>0.82</td>
</tr>
<tr>
<td><em>Shc</em>07</td>
<td>9</td>
<td>3</td>
<td>1.59</td>
<td>0.47</td>
<td>0.44</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Shc</em>11</td>
<td>8</td>
<td>5</td>
<td>3.56</td>
<td>0.43</td>
<td>0.25</td>
<td>0.65</td>
</tr>
<tr>
<td><em>Sle</em>290</td>
<td>9</td>
<td>1</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>3.75</td>
<td>2.63</td>
<td>0.26</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

% polymorphic loci (PPL) – 75

The individuals of *Parashorea malaanonan* tested here were from a plantation and the microsatellites used were able to detect high heterozygosity. Wickneswari and Seng (2003) obtained average heterozygosity value of 0.64 in individuals from a seed production area using four combined primers, *Shc*02, *Shc*03, *Shc*09 and *Shc*11, comparable with heterozygosity obtained in this study. Since heterozygosity is based on the genotypic frequencies, a more homogenous population like that in a plantation tends to have lower heterozygosity compared to natural populations. In a subsequent study (Abasolo et al. in preparation), higher PIC estimtes were obtained from materials in a natural population of *Parashorea malaanonan*.

Thus, the high heterozygosity and high estimates of PIC basically demonstrate the utility of these markers in the assessment of variation even in more homogenous populations of *Parashorea malaanonan*. This may find practical application in the assessment of clones for micropropagation and for the identification of timber or planting stock origin. Closely related individuals can be properly identified by the use of these highly polymorphic loci.

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