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# Possibilities of breeding teak (*Tectona grandis* L.f.) in Costa Rica assisted by AFLP markers

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**Abstract**

Teak tree (*Tectona grandis* L.f.) is a tropical tree specie naturally distributed in Southeast Asia, where it is also widely planted. It is planted as exotic specie in Africa, South and Central America. Tree improvement activities have been initiated in several countries including Costa Rica, which it has become the largest teak developer in Latin America. Teak sprouts were collected in a 4-year-old progeny test from the best two and the worst two families for volume as well as for stem quality traits. A DNA extraction protocol described before was modified in order to yield high quality DNA. AFLP reactions were performed as described previously. Seven selective primer combinations (E-ACG + M-CCG, E-ACT +M-CCG, E-AGC + M-CCG, E-ACG + M-CTC, E-ACT + M-CTC, E-AGC + M-CTC, E-AGG + M-CCA) resulted in 330 scoreable markers. A large number of markers were obtained when families where analyzed separately from its performance. Genetic distances based on AFLP markers frequencies were used to construct a dendrogram among families. The best quality performer families grouped widely separated from the worst performers, sharing 61.63% of their genetic elements. In volume, worst performer families grouped sharing 45.85% of their genetic elements.

**Key words:** AFLP, Breeding, Gene marker, Progeny test, Genetic distance, *Tectona grandis*, Costa Rica

**Resumen**

**Posibilidades de reproducción de teca (*Tectona grandis* L.f.) en Costa Rica asistida por marcadores AFLP**. La teca (*Tectona grandis* L.f.) es una especie tropical distribuida naturalmente en el Sudeste de Asia, donde también es ampliamente plantada. Así mismo, es plantada como especie exótica en Africa, América Central y del Sur. Actividades de mejoramiento genético han sido iniciadas en varios países incluyendo a Costa Rica, país que se ha convertido en uno de los mayores desarrolladores de teca en América Latina. Se colectaron brotes tiernos de teca de un ensayo de progenie de 4 años de edad, tratando de investigar el ADN de familias contrastantes en cuanto a su producción volumétrica, así como en su calidad. Se tomaron muestras de las dos mejores y de las dos peores familias con respecto a su producción volumétrica e índice de calidad. Un protocolo de ADN descrito previamente fue modificado para obtener ADN de alta calidad. Para las reacciones de AFLP se utilizaron siete combinaciones selectivas de imprimadores (E-ACG + M-CCG, E-ACT +M-CCG, E-AGC + M-CCG, E-ACG + M-CTC, E-ACT + M-CTC, E-AGC + M-CTC, E-

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AGG + MCCA), lo cual resultó en 330 marcadores. Se obtuvo un gran número de marcadores cuando las familias fueron analizadas separadamente por su desempeño. Las distancias genéticas basadas en los marcadores AFLP fueron usadas para construir un dendrograma. Para la variable calidad, las mejores familias fueron agrupadas y compartieron un 61,63% de sus elementos genéticos, separándose de las peores familias. Para el volumen, las peores familias se agruparon compartiendo un 45,85% de sus elementos genéticos.

**Palabras clave:** AFLP, Mejoramiento genético, Marcador genético, Ensayo de progenie, Distancia genética, *Tectona grandis*, Costa Rica.

**INTRODUCTION**

Teak (*Tectona grandis* L.f.) is a tropical tree species widely distributed in Southeast Asia, growing in countries such as India, Burma, Thailand and Indonesia (Kaosa-ard, 1981; Schubert, 1974). Teak is used for many purposes, but the most important end use is timber, preferably for fine furniture and other high valuable products (Kjær and Foster, 1996).

The species is distributed over an area with large variation in edaphic and climatic condition. Today it is widely planted in South East Asia as well as in Africa, South and Central America (Ball *et al*, 1999). These new plantation areas out of Asia, have offered teak further variation in growth conditions. Continuing selection of individuals adapted to local climates and soils may have formed 'landraces' in these new areas, each with its own distinctive characteristics (Kjær and Foster, 1996). Teak has been used for decades in plantation establishment either as an indigenous or an exotic species. The species is easily established in plantations. This makes teak one of the most promising species for plantations in the tropics, although the soil requirement is rather specific (Keogh, 1996). Tree improvement activities have been initiated in several countries including Costa Rica (Murillo and Meza, 1992; Murillo and Badilla, 2003; Leandro *et al*, 2003; Murillo and Badilla, 2004). It has become the largest teak developer in Latin America. Teak is interesting from a tree improvement point of view because it is used on a large scale, the timber is of high value and it is prevailingly regenerated artificially, which it allows introduction of improved genetic material (Kjær and Foster, 1996).

During the last 20 years, advances have emerged in the technologies available for assessing genetic diversity at the molecular level. Genetic markers (isozymes, RFLPs, AFLPs, RAPDs, and more recently microsatellites) have been developed and applied for genome mapping, population genetics and marker assisted breeding (Young *et al*., 2000; Grattapaglia *et al*., 2000). These technologies are becoming in an important tool for forest tree breeding and conservation genetic studies. The genetic diversity of teak from India, Thailand and Indonesia has been determinated by isozyme variation of provenances (Kertadikara and Prat, 1995). Kjær and Siegismund (1996) evaluated allozyme diversity and genetic distance for two Tanzanian and two Nicaraguan landraces of teak. A previous work in the Java Island of Indonesia was developed with teak, trying to achieve a highly reliable clone management of teak plus trees by using RAPD (Watanabe *et al*, 2004).
Early selection and testing of superior teak trees in breeding activities is important because of long term testing. Genetic markers may play a significant role in future teak breeding programs. Therefore, this study aims to explore the possibilities of AFLPs as an important teak breeding tool in Costa Rica.

## MATERIALS AND METHODS

### Plant material

Leaves were collected in a 4-year-old progeny test from the best two and worst two families for volume as well as for quality traits (Table 1). The progeny test belongs to an active teak breeding program the dry Pacific of Costa Rica. The test is conformed by 30 families established in a 6-half-sib progenies by 6 blocks in a complete randomized block design. Families were ranked based on its volume and its quality traits. Family volume was obtained as the average volume of its progeny, based on the regular volume function:

$v = dbh^2 * p/4 * 0.7 * h$,

where dbh stands for diameter at breast height; h stands for total height (in m).

Family quality was determined as an index from 1 to 4 (1 the best locking trees, 2 trees with light defects for solid wood production, 3 trees with severe defects but still productive and 4 the worst or non merchantable ones), obtained as an average of its progeny (Murillo and Badilla, 2004).

**Table 1.** Families selected in this study for quality and volume traits.

| Trait | Families selected | |
|---|---|---|
| | Best performer | Worst performer |
| Quality | 27, 14 | 19, 1 |
| Volume | 2, 20 | 1, 35 |

*DNA extraction*

A protocol described by Muhammad *et al* (1994) has been modified in order to yield high quality DNA. Tissue was ground with liquid nitrogen before extraction. Approximately 50-100 mg of tissue was put in a 1.5 mL tube. The tissue were ground with 500 µL of the buffer extraction (20 mM sodium EDTA and 100 mM Tris-HCl, adjust pH to 8.0 with HCl, 1.4 M NaCl, 2.0% (w/v) CTAB, 2% (w/v) PVP and 0.2 % of beta-mercaptoethanol) and additional 250 µL were added later. The tubes were incubated at 60°C for 20 minutes. After incubation 700 µL of chloroform:octanol (24:1) were added and tubes mixed gently by inverting 20 times.

The mixture was centrifuged at 6000 rpm for 15 minutes at 4°C and 400 µL of the aqueous phase was transferred into a new 1.5 mL tube. A second chloroform:octanol extraction was performed when the aqueous phase was brown-colored or turbid. In order to precipitate DNA, 0.5 volume of 5M NaCl and two volumes of cold 95% ethanol were added and refrigerate for 1 hour at -20°. Alternatively, DNA can be precipitated at 4-6°C overnight.

The solution was centrifuged at 10 000 rpm for 10 minutes at 4°C. The supernatant was pour off and the pellet kept at the bottom of the tube. The pellet was washed with 70% ethanol and the ethanol removed without drying the DNA. The DNA was dried at 37°C for 1 hour and then dissolved in 100 µL TE (Tris-HCl 10mM and EDTA 1mM). For RNA treatment 1 µL RNase was added to each tube and incubated at 37°C for 1 hour. After extraction, DNA was cleaned using a DNeasy Plant Mini Kit from QiaGen Inc. DNA was kept at -20°C until its use. Extracted DNA quality was determinated running samples in an agarose gel (0,8%). In addition, extracted DNA was subjected to two restriction enzyme digestions (EcoR I and Hind III) for 1 hour and also subjected to agarose gel (1%) electrophoresis with undigested DNA as control.

**AFLP reactions**

AFLP reactions were performed as described by Vos *et al*. (1995) with commercially available products and kits (Applied Biosystems AFLP™ Plant Mapping). All AFLP reactions were performed at Keim Genetics Laboratory at Northern Arizona University, Flagstaff, Arizona. Pre-amplification step was performed with primers complementary to the EcoR I and MseI adaptors. The PCR reactions were performed in a MJ Research (Gradient Cycler) thermocycler using the following PCR conditions: 72ºC for 2 minutes, 94ºC for 20 seconds (Step 2), 56ºC for 30 seconds, 72ºC for 2 minutes, Go to Step2 for 19 more times, 60ºC for 30 minutes and 4ºC forever.

Initially, nine primer combinations were evaluated by combining EcoR I selective primers with MseI selective primers: ACG, ACT, AGC combined with CCG and CTC; AGG, ACA, ACC combined with CCA. PCR reactions were performed in a MJ Research (Gradient Cycler) thermocycler with the steps as follows: 94ºC for 2 min, 94ºC for 20 sec, 66ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 65ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 64ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 63ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 62ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 61ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 60ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 59ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 58ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec, 57ºC for 30 sec, 72ºC for 2 min, 94ºC for 20 sec (Step 32), 56ºC for 30 sec, 72ºC for 2 min, Go to step 32 for 20 more times, 60ºC for 30 min and 4ºC forever. After amplification, the samples were denatured adding 1 µL of form amide and heating for 5 minutes at 95 ºC; then, tubes were immediately placed on ice. Products of selective amplifications were analyzed in an ABI PRISM ® 3100 Genetic Analyzer.

**Data analysis**

Data obtained from the ABI PRISM ® 3100 Genetic Analyzer were initially screened with the ABI PRISM® GeneScan® Analysis Software (Version 3.7) and then analyzed with the ABI PRISM® Genotyper® 3.7 NT Software. Each marker was coded as present (1) or absent (0) for each individual (Figure 1), thus creating a binary data matrix. Information from primer combinations was analyzed by using PopGen software (Version 1.32). This software was also used to construct a dendrogram in order to group families.

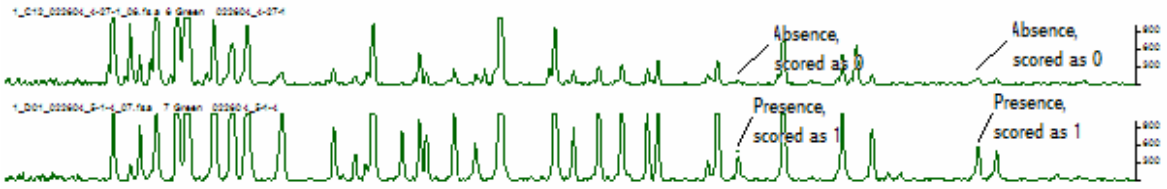


Figure 1. Data obtained from the ABI PRISM ® 3100 Genetic Analyzer. Each marker was coded as present (1) or absent (0) for each individual.

**RESULTS AND DISCUSSION**

**DNA extraction**

The CTAB based method for DNA extraction evaluated yields high quality DNA. Degradation of DNA was not observed when samples were run in a 0.8% agarose gel. Differential solubility in the presence of CTAB allows the separation of polysaccharides from nucleic acids (Rogers and Bendich, 1988). CTAB binds strongly to DNA, removes proteins and prevents DNA degradation

(Valadez and Kahl, 2000). In addition, the methodology evaluated was low time-consuming. DNA from sixty samples can be extracted in a working-day when tissue was previously ground with liquid nitrogen. However, it has been observed that the same amount of samples can be handled with tree species having softer leaves than teak.

Results of DNA restrictions with EcoR I and Hind III showed complete digestion. DNA was extracted with a modified protocol from Muhammad *et al* (1994), as described before. These authors report a complete digestion with three endonucleases (EcoR I, Eco RV and Hind III) working with *Vitis* and *Ampelopsis* species. Also it has been observed that the extraction protocol evaluated yields high quality DNA working with different tree species. A complete DNA restriction has been observed when was used EcoR I and Hind III.

**AFLP reactions**

In order to evaluate the suitability of *Teak* DNA for its use in AFLP analysis, reactions were performed as described before. Initially, nine primer combinations were tested on sixteen samples from different families. Two primer combinations were not used in further analyses because these resulted in non-scoreable markers in most of the samples. The following seven primer combinations were finally retained for analyses: ACG, ACT, AGC combined with CCG and CTC; AGG combined with CCA. The number of markers scored for samples are shown in Table 2.

**Table 2**. Number of markers scored for seven primer combination evaluated in *Tectona grandis*

| Primer combination | Number of markers scored |
|---|---|
| E-ACG + M-CCG | 51 |
| E-ACT +M-CCG | 44 |
| E-AGC + M-CCG | 64 |
| E-ACG + M-CTC | 47 |
| E-ACT + M-CTC | 49 |
| E-AGC + M-CTC | 25 |
| E-AGG + M-CCA | 50 |
| Total scoreable markers | 330 |

**Family information obtained from AFLP reactions**

The application of the seven primer combinations on seven families resulted in 330 scoreable markers (Table 2), of which 311 were polymorphic. Further analyses were developed separately, in order to obtain information for families categorized as best progeny test performer and worst progeny test performer. Quality and volume traits were considered for analyses. The number of polymorphic markers and the percentage of polymorphism loci per family are shown in Table 3.

**Table 3.** Number of polymorphic loci and percentage of polymorphic loci for quality and volume traits in two teak family categories. Best and worst progeny test performer data were analyzed separately for each family category and for each variable studied.

| | Family Category | | | |
|---|---|---|---|---|
| | Best performer | | Worst performer | |
| Trait | Number of polymorphic markers | % of polymorphic loci | Number of polymorphic markers | % of polymorphic loci |
| **Quality** | 187 | 56,66 | 166 | 50,45 |
| **Volume** | 168 | 50,91 | 154 | 46,66 |

The AFLP reactions produce many bands from a single assay. From the complex genomes of trees, the basic AFLP assay produces too many bands to analyze (Young *et al*, 2000). As shown in Table 2, more than 330 bands were generated by using seven primer combinations. Stenefon *et al* (2003), tested seven primer combinations on *Araucaria angustifolia* and obtained 625 markers. In a study in *Quercus petraea* eight primer combinations were evaluated obtaining 374 scoreable markers (Coart *et al*., 2002). The fact AFLP technique produces many bands, analysis has been found to be more informative than RAPD and RFLP analysis (Savelkoul *et al*, 1999). A critical characteristic of genetic markers is its reproducibility. Since relatively small amounts of DNA are digested and detection of AFLP fragments does not depend on hybridization, partial digestion and faint patterns, which are sources of irreproducibility with restriction fragment length polymorphism (RFLP) genotyping, can easily be avoided. Furthermore, the possibility of using stringent PCR annealing temperatures renders the AFLP analysis method more reproducible and robust than RAPD analysis (Savelkoul *et al*, 1999).

**AFLP technique as a potential tool for teak breeding**

Genetic distances based on AFLP markers frequencies for volume and quality traits were used to construct a dendrogram (UPGMA) among families. The best quality performer families (27 and 14) were grouped widely separated from the worst performers, sharing 61.63% of their genetic elements. The worst performer families did not grouped but they shared 89,53% of their genetic elements (Figure 2). In volume, worst performer families grouped sharing 45.85% of their genetic elements. Best performer families did not group clearly, but they shared 70.74% of their genetic elements.

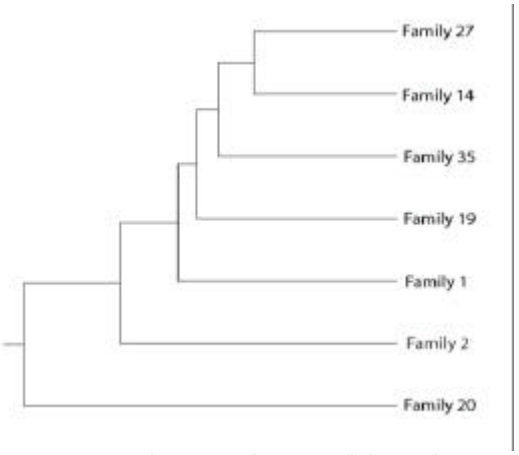


**Figure 2**. Dendrogram (UPGMA) based on AFLP genetic distances for tree stem quality trait among best and worst teak performer families.

The ranking of families was based on a 4-year-old data, which could be very early for traits like volume. However, the average dbh of the test was 16 cm, close to the mid rotation dbh of 20 cm. For the integrated tree quality trait, as an index based on more than 8 individual stem and branch quality traits, most of them usually exhibit a strong early expression and genetic control (Zobel and van Buijtenen, 1989). Therefore, family ranking based on a quality assessment value at this age, is completely consistent. In the case of the volume trait, even though the ranking information for families is based on relatively early data, results are promising in the context of finding specific DNA elements, registered only in the best performing families, as well as, in the worst performer families. Next approaches must include the following ranking families, in order to broad the putative superior and worst entries, and try to verify the degree of uniqueness of the DNA material registered in any of the contrasting collections. New measurements (older trees) could also help in terms of a more consistent volume ranking assessment of families in the genetic test. Oversites data analysis will be also performed in order to obtain better ranking estimates of families.

In this study it has been obtained a large number of markers when testing seven primer combinations. A previous work based on RAPD was developed with teak, trying to achieve a highly reliable clone management of teak plus trees. It is reported only 26 fragments when 24 primers were evaluated (Watanabe *et al*., 2004). These results suggest that information from AFLP can be used for clone management in teak breeding programs, more accurately than RAPD markers.

## CONCLUSIONS

A large number of primer combinations for high resolution in teak were screened. Best two and worst two performer families, for volume and tree quality, in a 4-year-old progeny test, were found associated to specific DNA regions. Ranking of families at this age, based on trait quality, is completely consistent and with a high degree of reliability. However, family ranking based on volume trait must be considered with cautious. Future approaches must utilize older data for volume and preferably, oversites family information. The inclusion of the next following ranking families, could also improve the consistency of DNA regions uniqueness for the best and worst entries.

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