

RESEARCH NOTE

Molecular analysis of *Albizia* species using AFLP markers for conservation strategies

SUBBASHREE APARAJITA and GYANA RANJAN ROUT*

Department of Agricultural Biotechnology, College of Agriculture, OUAT, Bhubaneswar 751 003, India

Introduction

Improving commercial and ecological utilization of the perennial tree legume, genus *Albizia*, requires developing approaches that can accurately characterize genetic variation in its gene pool. Amplified fragment length polymorphism (AFLP) markers, in combination with morphological traits, have been used to ascertain the extent of genetic diversity and relatedness among the nine *Albizia* species. The variations observed among the *Albizia* species for seven morphological traits were summarized by means of discriminant analysis. Four significant variables, accounting for about 96.1% of total variance, were mainly correlated with plant height, number of pinnae, number of leaflet per pinnae and leaflet size. The morphological variations appeared to be related to the molecular variations. AFLP analysis of nine *Albizia* species based on 12 primer pair combinations revealed a total of 724 polymorphic-amplified DNA fragments. The genetic similarities estimated among the nine *Albizia* species, based on both shared and unique amplification products, ranged from 0.51 to 0.73 across all species. Both morphological and molecular data will be an important input to determine the appropriate management strategies for conservation and improvement of the breeding programme for this genus.

The genus *Albizia* belongs to subfamily Mimoseae of family Leguminosae and is highly valued multipurpose tree legume. The legume tree species have been severely diminished by logging, immense human pressure, over-exploitation and land clearance. Many *Albizia* species are endemic to Indian subcontinent. The Indian species *A. thomsonii* are classified as vulnerable (Nayar and Sastry 1990). *Albizia* species are socially significant for producing high quality timber and as a valuable resource for gum yield. *Albizia julibrissin*, *A. lebeck*, *A. procera* and *A. amara* are important in ayurvedic medicine. Seeds are

regarded as astringent, and used in the treatment of piles, diarrhea and gonorrhoea (Anonymous 1989). It has been recently demonstrated that three saponin extracts from *A. julibrissin* acts as antitumorals by the induction of apoptosis in certain cell types (Zheng *et al.* 2006) and butanol extract from the bark of *A. julibrissin* has cytotoxic effect on human acute leukemia junket T-cells (Won *et al.* 2006). Some *Albizia* species are regarded as a potential fodder resource (Stewart and Dunsdon 2000). It is also a plant of choice for silviculture and secondary plantation because of thick foliage and quick growing nature. The *Albizia* species like *A. lebeck* and *A. procera* have shown high potential in soil redevelopment process during early phase of mine spoil restoration in dry tropical environment (Singh *et al.* 2004).

As the genus has high ecological, economical and medicinal value and wide distribution, an accurate assessment of genetic diversity and relatedness will be helpful for efficient management of this genus. For evaluation of species diversity, it is essential that individuals can be classified accurately. The identification of taxonomic units and endangered species, whose genetic constitution is distinct from their more abundant relatives, is important in the development of appropriate conservation and breeding strategies. Since morphological traits are very plastic, any recorded variation should not necessarily be interpreted as genetic variation and results cannot be relied upon. Nowadays, molecular markers are used because of their ease and simplicity. There are highly polymorphic dominant markers that cover a larger proportion of the whole genome (Mueller and Wolfenbarger 1999) randomly accessing both coding (rather conservative) and non-coding (not necessarily conservative) regions. These genome-wide datasets may provide high power in testing specific phylogenetic relationships (Rokas *et al.* 2003). Thus, they may provide many informative markers to complement morphological markers. Here, we report a phylogenetic analysis of the genus *Albizia* generated by means of multivariate analysis of morphological traits and AFLP markers.

*For correspondence. E-mail: grout@rediffmail.com.

Keywords. AFLP; multivariate analysis; genetic diversity; *Albizia*.

Materials and methods

Albizia species: *A. amara*, *A. lebbeck*, *A. odoratissima*, *A. procera*, *A. stipulata*, *A. thomsonii*, *A. kalkora*, *A. lucida* and *A. orissensis* from different geographical locations of India were used in this study (see table 1 in electronic supplementary material at <http://www.ias.ernet.in/jgenet/>). Ten individuals of each species except *A. orissensis* (five individuals), was randomly selected for morphological and AFLP marker analyses. Identification of the species was confirmed by the taxonomist and kept in our central herbarium as a voucher specimen. For each species, the following characters were examined: plant height, breadth at chest height, leaf length, number of pinnae per leaf, number of leaflet per pinnae, leaflet length, capitulum's diameter, pod length and number of seeds per pod. Twenty accessions per species across nine species data were scored. Data from seven traits were analysed by univariate and multivariate analyses of variance based on the multiple discriminant analysis. Canonical scores from the significant canonical variables were included in a hierarchical cluster analysis procedure with the average linkage method based on the average Euclidean distance among species (Sokal and Sneath 1963). Principal component analysis (PCA) with a varimax normalized procedure was used to detect those characters contributing most significantly to the variation, and to select a maximum of three characters from those with the highest weight in each factor. All the analyses were done by software Statistix 1.7 (Sydney, Australia).

The leaf samples were used for DNA isolation. Total DNA was extracted from semi-mature bipinnate leaves by using the protocol of Doyle and Doyle (1990). Genomic DNA was subsequently purified by RNase, proteinase-K and phenol: chloroform: isoamyl (25: 24: 1) to get highly-purified DNA free from any type of contamination. DNA concentration was determined electrophoretically against a known amount of lambda DNA as standard. Genomic-bulked-DNA solutions were prepared by mixing equal quantities (i.e. 500 ng per plant) of DNA from each of the 10 individuals (except *A. orissensis*; five individuals) representing each of the nine species. The AFLP reactions were performed as described by Vos *et al.* (1995) with slight modification. From each species 250 ng of bulked genomic DNA solution were digested simultaneously with 10 units of each *MseI* and *EcoRI* at 37°C for 2 h. Following digestion, *EcoRI* and *MseI* adapters (Invitrogen, California, USA) were ligated to the ends of the restricted fragments at 20°C for 2 h and digested fragments were preamplified with primers pair displaying one selective nucleotide, namely *Eco*-primer + A (E-A) and *Mse*-primer + G (M-G) using 20 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min according to the manufacturer's instructions. The pre-amplified DNA of the individual samples from each of the nine species was bulked and this bulked DNA served as the template for selective AFLP amplification. Five μL of the diluted (1:50), bulked pre-amplified solution were used as template for the

selective amplification with 26 *EcoRI* and *MseI* primer pairs using a 'Touchdown' cycle (Don *et al.* 1991) programmed as follows: 12 cycles of 94°C for 30 s 65°C (–0.7°C per cycle) for 30 s; and 72°C for 1 min, until reaching an optimal annealing temperature of 56°C, followed by 24 more cycles of 94°C for 30 s; 56°C for 30 s and 72°C for 1 min. All reactions were carried out in a PTC-100 Thermal Cycler (MJ Research, Waltham, USA).

Five μL of each final AFLP amplification product was combined with 5 μL of AFLP gel loading dye (98% formamide, 10 mM EDTA and 0.25% v/v each of bromophenol blue and xylene cyanol FF). Solutions were denatured at 95°C for 4 min, quenched on ice and 6 μL was then loaded on a 7.5% (w/v) polyacrylamide gel in 1× TBE electrophoresis buffer. The 20-bp DNA ladder (Bangalore Genei, Bangalore, India) was loaded in the flanking lane. Gels were run at constant voltage (100 V) until the xylene cyanol was about two-thirds down the length of the gel. Subsequently, the gel was silver stained following the protocol by Bassam *et al.* (1991). For each of the primer pairs, the number of polymorphic fragments was determined by visually evaluating the gel photograph scanned by Quantity One software in Gel Documentation system (Bio-Rad, California, USA). Only the clearly defined bands were scored (1 for present and 0 for absent) and entered into a data matrix. Monomorphic fragments were excluded from data analysis. Fragment size was determined by Quantity One software by comparing the bands against a 20-bp DNA ruler (Bangalore Genei, Bangalore, India). Fragments were considered to be different when separated by at least +/– 5 bp. Binary matrix was used to estimate the genetic similarities between pairs, by employing the simple matching coefficient $SM_{ij} = M_{ij}/N_{ij}$, where SM_{ij} is the measure of genetic similarity between the *i*th and *j*th species, M_{ij} is the number of matches, N_{ij} is the total sum of matches and 'unmatches'. To cluster the data, the unweighted pair grouping method of arithmetic averages (UPGMA) was used (Sneath and Sokal 1973). The statistical analysis was carried out using NTSYS-PC software, version 2.10 (Rohlf 2001).

Results and discussion

Morphologically, *Albizia* species showed remarkable variability with regard to plant architecture, leaf size, number of pinnae, number of leaflet per pinnae, leaflet number and size, flower diameter and colour, pod colour, size and shape, seed number per pod and size. The mean value of six quantitative traits is reported in figure 1. The discriminate analysis procedure extracted seven canonical variables. The first four factors were highly significant ($P < 0.001$), accounting for 45.6, 25.9, 13.9 and 10.6% of the total variance respectively; thus, altogether they explained about 96.1% of the total variance. The canonical variables were correlated with plant height and leaflet size and were negatively correlated with number of pinnae and number of leaflet per pinnae. The *Albizia* species differed mainly along the second axis

Molecular analysis of *Albizia* species

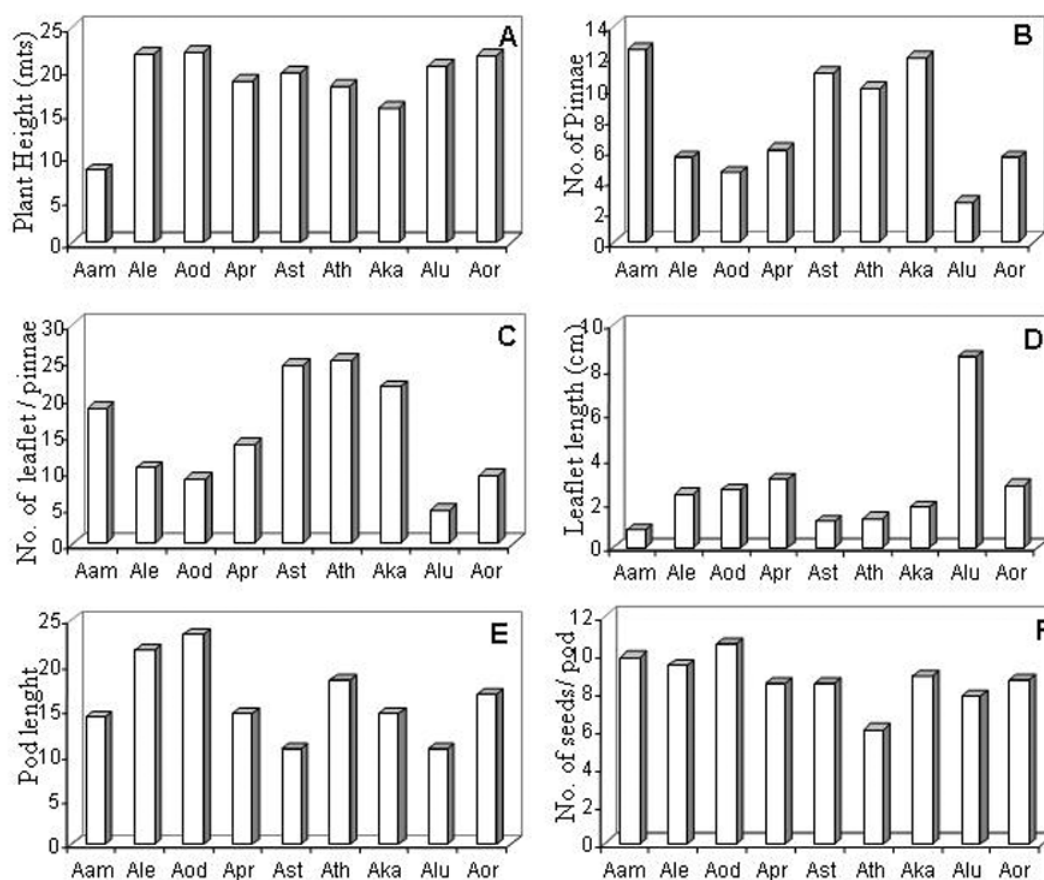


Figure 1. Mean value of six morphological traits in nine *Albizia* species. Aam, *A. amara*; Ale, *A. lebbeck*; Aod, *A. odoratissima*; Apr, *A. procera*; Ast, *A. atipulata*; Ath, *A. thomsonii*; Aka, *A. kalkora*; Alu, *A. lucida*; Aor, *A. orissensis*.

showing three groups. *Albizia amara* and *A. lucida* remained ungrouped whereas *A. amara* and *A. thomsonii* are isolated. The result indicated that a small number of specific morphological traits are enough to detect variation and adequately define plant morphology in aspects such as plant height, number of pinnae, number of leaflet per pinnae, leaflet size and pod length. Since morphological traits are very plastic, any recorded variation should not necessarily be interpreted as genetic variation. The morphological markers combined to molecular markers could be beneficial and of much practical utility.

The AFLP analysis is very useful for assessing the genetic relationships among a collection of accessions that represented species of the *Albizia* genus. The ability to determine genetic variation among the accessions and species at the molecular level is directly related to the number of polymorphisms detected and their reproducibility. The AFLP technique has emerged as a new powerful tool for genomic analysis (Vos *et al.* 1995). This technique has been applied to determine genetic relationships and diversity among the species (Nguyen *et al.* 2004) and populations (Cardoso *et al.* 2004) of legume forest trees. Twelve AFLP primer

pairs were used to study the genetic diversity of *Albizia* species. The repeatability of AFLP fragments exceeded 98% for each of the nine species based on the two independent restriction–ligation reactions of each species that were amplified using two selective primer pairs. The results indicate that DNA bulks were reproducibly amplified and that most polymorphisms represented true genetic differences among the species. The frequency of ‘species’ fragment was within the 2% margin of error detected in the repeatability assay. A total of 724 reproducible and clearly scorable bands, produced from 12 primer combinations, were assessed across the entire collection of *Albizia* species. Of these, 14 were monomorphic and 710 were polymorphic. The number of polymorphic fragments generated per primer pair ranged from 36 to 73 with an average of 59.2 bands. Polymorphism rates ranged from 96.15% to 100% with a mean of 98.06%. The molecular weights of fragments generated ranged from approximately 38 bp to 1548 bp. Polymorphism varied remarkably among primer combination. An example of the pattern of amplification by using two AFLP primers E-AAC/M-CAA and E-AAC/M-CTC have been shown in figure 2, a&b, respectively. The most informative primer combination was

the E-AGG/M-CTA pair, which produced the largest number of amplified bands (76). Two other primer pairs, E-ACT/M-CTA and E-ACG/M-CTT, also detected more than 70 AFLPs each for an additional 142 markers. A total of 106 AFLP markers were classified as species specific because they occurred in only a single species. A maximum of 21 identical fragments were identified in *A. lucida* alone.

Genetic similarity values based on pairwise comparisons among the nine *Albizia* species ranged from a minimum of 0.51 between *A. kalkora* and *A. procera* to a maximum of

0.73 between *A. odoratissima* and *A. orissensis*. The dendrogram constructed by using UPGMA method showed two main clusters between the nine species at 56% similarity level (figure 3): Cluster I containing five species and cluster II containing four species. In cluster I, *A. lucida* has isolated itself forming a single group from the rest four species at 64% similarity level. The species *A. lebeck*, *A. procera*, *A. odoratissima* and *A. orissensis* grouped together whereas *A. amara*, *A. thomsonii*, *A. kalkora* and *A. stipulata* have grouped together, while the monotypic species *A. lucida* is

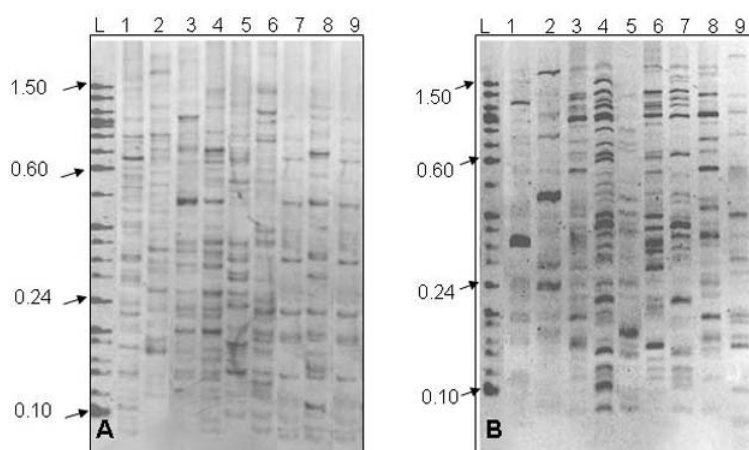


Figure 2. Banding pattern in nine species of *Albizia* using AFLP primer E-AAC/M-CAA (A) and E-AAC/M-CTC (B). L, 20-bp ladder; 1, *A. amara*; 2, *A. lebeck*; 3, *A. odoratissima*; 4, *A. procera*; 5, *A. stipulata*; 6, *A. thomsonii*; 7, *A. kalkora*; 8, *A. lucida*; 9, *A. orissensis*.

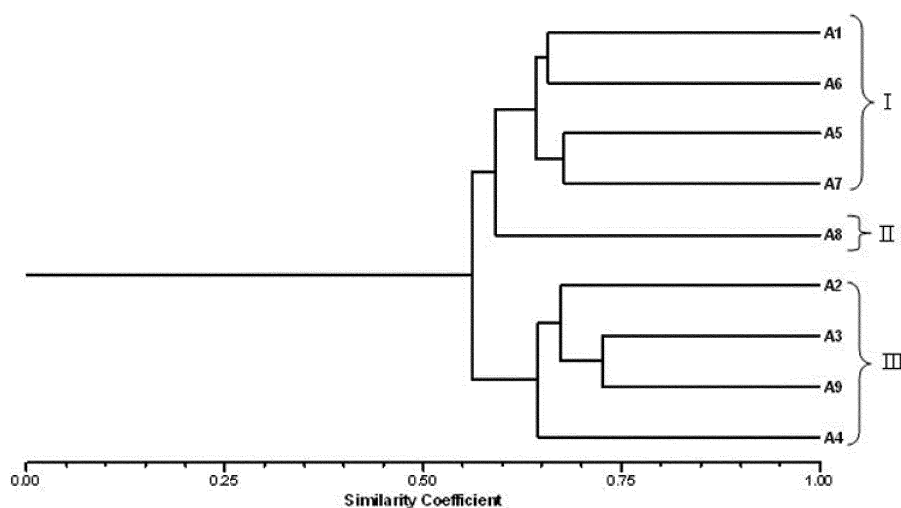


Figure 3. Dendrogram drawn from the UPGMA cluster analysis estimates of genetic similarity are based on AFLP analysis with 12 primer combinations. The nine species were clustered into five major groups (A1, *A. amara*; A2, *A. lebeck*; A3, *A. odoratissima*; A4, *A. procera*; A5, *A. stipulata*; A6, *A. thomsonii*; A7, *A. kalkora*; A8, *A. lucida*; A9, *A. orissensis*).

clearly separated from the group. In general, the clustering of *Albizia* species was in accordance to their morphology. Species having similar physio-chemical, physiological and structural similarities are often a result of convergent evolution (Yanney-Ewusine 1980). AFLP marker has also been used to study the evolutionary relationships at the species or genus level (Koopman *et al.* 2001; Beardsley *et al.* 2003; Brouat *et al.* 2004) and is considered to be able to resolve phylogenetic relationships congruent with analyses based on morphological characters (Stefenon *et al.* 2003). Indeed, AFLP marker proved to be very informative to monitor the genetic diversity of *Albizia* species. It will also be an important input in determining appropriate management strategies for conservation of tree legumes.

Acknowledgements

The authors wish to acknowledge Council of Scientific and Industrial Research, New Delhi, India, for their financial assistance under grant no. 09/795 (007)2K8- EMR-I.

References

- Anonymous 1989 *The wealth of India: raw materials*, vol. A-B. Council of Scientific Industrial Research, New Delhi, India.
- Beardsley P. M., Yen A. and Olmstead R. G. 2003 AFLP phylogeny of *Mimulus* section *Erytranthe* and the evolution of hummingbird pollination. *Evolution* **57**, 1397–1410.
- Bassam B. J., Caetano-Anolles G. and Gresshoff P. M. 1991 Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* **196**, 80–83.
- Brouat C., Mckey D. and Douzery J. P. 2004 Differentiation in a geographical mosaic of plants coevolving with ants: phylogeny of the *Leonardoxa africana* complex (Fabaceae: Caesalpinioideae) using amplified fragment length polymorphism markers. *Mol. Ecol.* **13**, 1157–1171.
- Cardoso S. R. S., Provan J., Lira C. D. F., Pereira L. D. O. R., Ferreira P. C. G. and Cardoso M. A. 2004 High levels of genetic structuring as a result of population fragmentation in the tropical tree species *Caesalpinia echinata* Lam. *Biodiversity Cons.* **10**, 1–11.
- Don R. H., Cox P. T., Wainwright B. J. and Mattick J. S. 1991 Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**, 4008.
- Doyle J. J. and Doyle J. L. 1990 Isolation of plant DNA from fresh tissue. *Focus* **12**, 13–15.
- Koopman W. J. M., Zevenbergen M. J. and Vander Berg R. G. 2001 Species relationship in *Lactuca* s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprints. *Am. J. Bot.* **88**, 1881–1887.
- Mueller M. G. and Wolfenbarger L. L. 1999 AFLP genotyping and fingerprinting. *Trends Ecol. Evol.* **14**, 389–394.
- Nayar M. P. and Sastry A. R. K. (ed.) 1990 *Red data book of Indian plants*, vol 3, pp. 129–130. The Director Botanical Survey of India, Calcutta, India.
- Nguyen T. T., Taylor P. W. J., Redden R. J. and Ford R. 2004 Genetic diversity estimates in *Cicer* using AFLP analysis. *Plant Breeding* **123**, 173–179.
- Rokas A., Williams B. L., King N. and Carroll S. B. 2003 Genome-scale approaches to resolving incongruence in molecular phylogenies. *Nature* **425**, 798–804.
- Rolhf F. J. 2001 NTSYS-pc numerical taxonomy and multivariate analysis system, version 2.1. Exeter, New York, USA.
- Sneath P. H. A. and Sokal R. R. 1973 *Numerical taxonomy: the principles and practice of numerical classification*. Freeman, San Francisco, USA.
- Sokal R. R. and Sneath P. H. A. 1963 *Principles of numerical taxonomy*. Freeman, San Francisco, USA.
- Singh A. N., Raghubansi A. S. and Singh J. S. 2004 Comparative performance and restoration potential of two *Albizia* species planted on mine spoil in a dry tropical region, India. *Ecol. Eng.* **22**, 123–140.
- Stefenon V. M., Nodari R. O. and Ries M. S. 2003 Marcadores moleculares no melhoramento genético de araucaria. *Biotecnologia Ciencia e Desenvolvimento* **31**, 95–99.
- Stewart J. L. and Dunsdon A. L. 2000 The potential of some neotropical *Albizia* species and close relatives as fodder resources. *Agroforestry Systems* **49**, 17–30.
- Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T. and Hornes M. 1995 AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**, 4407–4414.
- Won H. J., Han C. H., Kim Y. H., Kwon H. J., Kim B. W., Choi J. S. and Kim K. H. 2006 Induction of apoptosis in Human acute leukemia Jurkat T cells by *Albizia julibrissin* extract is mediated via mitochondria-dependent caspase-3 activation. *J. Ethnopharmacol.* **19**, 383–389.
- Yanney-Ewusine J. 1980 *Elements of tropical ecology*. Heinemann Educational Books, New Hampshire, UK.
- Zheng L., Zheng J., Zhao Y., Wang B., Lijun W. and Liang H. 2006 Three anti-tumor saponins from *Albizia julibrissin*. *Bioorg. Med. Chem. Lett.* **16**, 2765–2768.

Received 22 April 2009, in final revised form 25 July 2009; accepted 14 September 2009

Published on the Web: 3 March 2010