BIOLOGIA PLANTARUM 47 (3): 445-448, 2003/4

BRIEF COMMUNICATION

Molecular characterization of three *Heritiera* species using AFLP markers

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Abstract

In the present study three species of *Heritiera* Aiton (*Sterculiaceae*) were characterized using 9 amplified fragment length polymorphism (AFLP) primer combinations and the genetic relationship among these three species was assessed. Nine AFLP primer combinations yielded 445 bands out of which 210 were monomorphic and 235 were polymorphic. Out of the 235 polymorphic bands 79 were present only in a single species. Among the total amplified bands 255 were shared between *H. fomes* and *H. littoralis*, 225 were shared between *H. fomes* and *H. macrophylla* and 306 bands were shared between *H. littoralis* and *H. macrophylla*. The cluster analysis showed *H. littoralis* is closer to *H. macrophylla* than *H. fomes*. The similarity between *H. fomes* and *H. littoralis* was higher than that of *H. fomes* and *H. macrophylla*. The present study indicates that *H. littoralis* is better classified as mangrove associate or back mangal than a true mangrove.

Additional key words: cluster analysis, mangroves, monomorphic and polymorphic bands.

The genus *Heritiera* Aiton (*Sterculiaceae*) is one of the main components of rain forest trees with a distribution from eastern tropical Africa (2 species) and the remainder from India to the Pacific, with *H. littoralis* introduced further east in Polynesia and Hawaii for its valuable timber (Kostermans 1959). Inspite of their immense ecological importance to the coastal belt the high economical value of these plants have made them endangered trees due to over exploitation. Out of the three species *H. fomes* and *H. littoralis* are reported from Sunderban mangrove forest of West Bengal, Bhitarkanika and Mahanadi delta of Orissa, India (Banerjee and Rao 1990). The *H. fomes* is a true mangrove with tolerance to saline conditions (Santisuk 1983, Naskar and Mandal 1999) but the status of *H. littoralis* as mangrove is controversial. The species *H. littoralis* has been described by most of the authors as fresh water loving back mangal or mangrove associate (Kartawinata *et al.* 1979, Tomlinson 1986) whereas a few workers have described it as true mangrove (Santisuk 1983, Das *et al.* 1994, Parani *et al.* 1998) and *H. macrophylla* is reported from hills of Assam and Andamans and Nicobar Islands.

For proper conservation programme it is essential to characterise these plants genetically. Number of molecular markers are being regularly used for studying genetic relations, population genetics and genetic characterisation in different plant groups and crop cultivars (Henry 1997). The molecular markers are not influenced by the external environmental factors unlike that of morphological markers and hence accurately

Received 17 September 2002, *accepted* 28 January 2003.
Abbreviation: AFLP - amplified fragment length polymorphism.
Acknowledgements: Authors are thankful to the Department of Forest and Environment, Government of Orissa, India for financial assistance.
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testify the genetic relationship between and among plant groups. Molecular markers like restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have been used to study inter and intra specific variations in some species (*e.g.* Parani *et al.* 1997, 1998, Ranade *et al.* 2002). Although RAPD analysis is simpler and cheaper it is inadequate due to low reproducibility and reliability (Skroch and Nienhuis 1995, Karp *et al.* 1997). Sometimes false banding pattern due to competition between different DNA fragments for amplification were reported (Hansen *et al.* 1997). On the other hand, RFLP is expensive and time consuming. A recently developed technique amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995) seems to be adequate and highly reproducible due to its more stringent amplification conditions used to produce AFLP bands (Brown 1996, Folkertsma *et al.* 1996). AFLP produces more polymorphic bands than RFLP and RAPD and is reliable (Maughan *et al.* 1996, Ajmone Marsan *et al.* 1998). The AFLP technique has been extensively used to study variability within and between cultivated and wild crops and inter and intra specific variations among different crop and tree species (Lerceteau and Szmidt 1999, Garcia-Mas *et al.* 2000). However the use of AFLP in mangroves is limited to only one report (Maguire *et al.* 2002) which studied intraspecific variations among *Avicennia marina*. But there is no report on use of AFLP markers for studying the genetic relationships among the species of *Heritiera*. Hence, the present research was conducted with the objectives of characterizing and studying genetic relations among three species of *Heritiera* using AFLP markers.

Seeds from 10 randomly selected plants of *H. fomes* and *H. littoralis* were collected from mangrove forest of Bhitarkanika, Mahanadi delta of Orissa and *H. macrophylla* from tropical evergreen forest of Assam, India. They were grown in the mangrove nursery of Regional Plant Resource Centre (RPRC), Bhubaneswar, Orissa, India (living samples as well as herbarium specimens are preserved in RPRC garden and herbarium respectively). Young tender healthy leaves from 10 seedlings each representing earlier selected individual plants, were collected for isolation of genomic DNA.

Genomic DNA was isolated from freshly collected leaves using the CTAB method (Saghai-Maroof *et al.* 1984), purified by RNaseA treatment with 60μg RNaseA per 1 cm^3 of crude DNA solution followed by two washes with phenol:chloroform:iso-amyl-alcohol (25:24:1) and three washes with chloroform:iso-amyl-alcohol (24:1). Collected aqueous phase was mixed with $1/10^{th}$ volume of 3 M-sodium acetate (pH 4.8). DNA was precipitated by adding 2.5 volumes of chilled absolute ethanol, pelleted, dried in vacuum and dissolved in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) buffer. Quantification of DNA was accomplished by analyzing the purified DNA in 0.8 % agarose gel alongside diluted uncut lambda DNA as standard. DNA was diluted in TE buffer to the concentration of 100 μg cm^{-3} for AFLP reaction.

AFLP analysis was performed following the modified method of Vos *et al.* (1995) and according to the protocol supplied with the 'AFLP Core Reagent Kit' and the 'AFLP Starter Primer Kit' of *Life Technologies* (New York, USA). The amplified products were analyzed in pre-warmed 5 % acrylamide electrophoresis gels. Gels were run at 55 W for approximately 2 h in 1× TBE (Tris-borate-EDTA) buffer, transferred to *Whatman No. 1* filter paper and then dried under vacuum (*Gel Dryer Model 583, BioRad*, Hercules, USA). AFLP products were revealed by exposure to X-ray films (*Kodak-BioMax MR*). The primer combinations used for the present study are given in the Table 1.

The banding patterns obtained from AFLP auto-radiograms were scored as present (1) or absent (0). All the bands (polymorphic and monomorphic) were taken into account in similarity calculation to avoid over estimation of the distance (Gherardi *et al.* 1998). Jaccard's coefficient of similarity (Jaccard 1908) was measured and a dendrogram based on similarity coefficients was generated by using unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal 1973) and the SAHN clustering. All analyses were done using the computer package *NTSYS-PC-2.02e* (Rohlf 1997).

Table 1. Details of banding pattern in three species of *Heritiera* using 9 AFLP primers (Hf - *H. fomes*, Hl - *H. littoralis* and Hm - *H. macrophylla*).

Primer combinations	Number of bands			Number of bands amplified in			Sharing bands between		
	monomorphic	polymorphic	unique	Hf	Hl	Hm	Hf + Hl	Hf + Hm	Hl + Hm
EACT/MCAA	33	27	15	49	35	54	33	43	35
/MCAC	14	17	2	16	28	30	15	15	27
/MCAG	29	23	3	30	50	49	29	29	49
/MCAT	11	40	13	36	49	14	35	12	13
/MCTA	15	18	13	19	30	19	16	15	19
/MCTG	29	32	13	38	57	43	35	29	42
/MCTT	24	28	8	34	51	36	32	25	35
/MCTC	23	24	5	41	46	45	23	24	41
EAAG/MCAC	32	26	7	33	53	44	37	33	45

Amplified fragment length polymorphism with 9 primer combinations produced a total of 445 bands with an average of 49.44 bands per primer. Out of 445 loci amplified 210 (47.19 %) were monomorphic and 235 (52.81 %) were polymorphic. Out of the 235 polymorphic bands 79 bands were present only in a single species. The highest number of band (61) was amplified by the primer combination *EACT/MCTG* where as the lowest number of bands (31) were amplified with the primer combination *EACT/MCAC* (Table 1). The highest

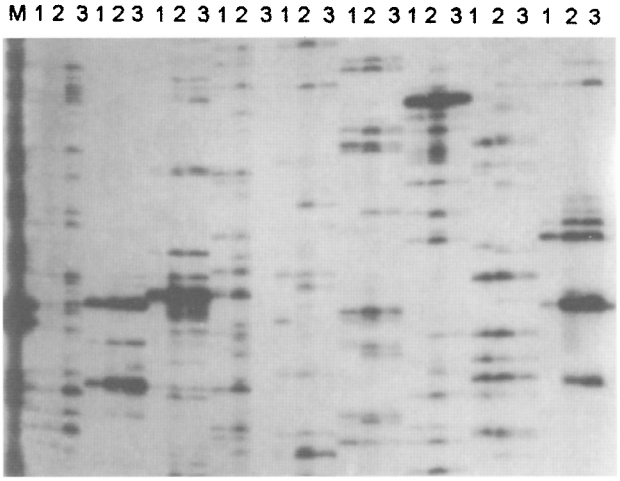

Fig. 1. A part of AFLP autoradiogram of three species of *Heritiera* Aiton using 9 AFLP primer combinations. 1 - *H. fomes*, 2 - *H. littoralis* and 3 - *H. macrophylla* (M = a part of 20 bp ladder, *arrow mark* indicates the sequence of primer combinations used, *i.e. EACT/MCAA, EACT/MCAC, EACT/MCAG, EACT/MCAT, EACT/MCTA, EACT/MCTG, EACT/MCTT, EAAG/MCAC, EACT/MCTC*, respectively).

percentage (78 %) of polymorphic bands was obtained in *EACT/MCAT*. The highest numbers of bands were amplified in *H. littoralis* (399) whereas the lowest was amplified in *H. fomes* (296). In case of all the primer combinations it was observed that the highest number of bands were shared between *H. littoralis* and *H. macrophylla* except in the primer combination *EACT/MCAT* where the band sharing was the highest between *H. fomes* and *H. littoralis* (Table 1). A portion of the AFLP autoradiogram is presented in the Fig. 1.

Cluster analysis using the AFLP banding patterns of all the primer combinations revealed that *H. littoralis* and *H. macrophylla* were grouped under the same sub-cluster (Fig. 2) where as the *H. fomes* came under a completely separate node. The similarity index using Jaccard's similarity coefficient showed 71.19 % similarity between *H. littoralis* and *H. macrophylla*, 60 % between *H. fomes* and *H. littoralis* and 56.93 % between *H. fomes* and *H. macrophylla*.

We introduced the AFLP markers for the first time to study the genetic similarity between mangrove and non-mangrove species and hence confirm the possible status of *H. littoralis* as mangrove, which represents a very complex ecosystem. In-spite of the relatively high expense and need of sophisticated equipment, AFLP is highly reproducible due to its stringent amplification procedure (Folkertsma *et al.* 1996, Brown 1996). AFLP markers were reported to be superior to RAPD when closely related species are studied (Garcia-Mas *et al.* 2000).

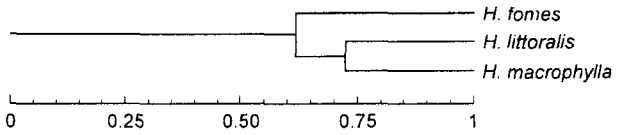

Fig. 2. Dendrogram showing phylogenetic relationship among three species of *Heritiera* using 9 AFLP primer combinations.

In the present study three species of *Heritiera* were characterized using 9 AFLP primer combinations. A total of 445 bands amplified out of which 235 (52.81 %) were polymorphic (Table 1). The higher similarity in banding pattern was observed between *H. littoralis* and *H. macrophylla* sharing a total of 306 bands (Table 1) suggesting that *H. littoralis* is closer *to H. macrophylla* than *H. fomes*. In an earlier study using molecular markers like RAPD and RFLP, Parani *et al.* (1998) confirmed *H. fomes* to be a true mangrove. *H. littoralis* sharing the same node with *H. macrophylla* and being intermediate in term of band sharing as well as similarity values suggest that *H. littoralis* is possibly a back mangal. This observation is in accordance with the earlier reports on the status of *H. littoralis* (Kartawinata *et al.* 1979, Tomlinson 1986). In an earlier report Tomlinson (1986) observed that *H. littoralis* was intolerant of high salinities, did not occur in very exposed or poorly drained sites and also lacked pneumatophore but usually was found in association with the mangrove forest which suggests that probably it is the intermediate form between mangrove and non mangrove sharing the characters of the both. Our observation also confirmed the status of *H. littoralis* as back mangal. Probably the *H. fomes* in the due course of evolutionary process migrated to land and evolved as a new species through *H. littoralis* to finally *H. macrophylla*. However, further investigation using more sophisticated marker like DNA sequences may be helpful in studying the evolutionary process of *H. fomes* to *H. macrophylla*.

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