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Full Length Research Paper

Application of amplified fragment length polymorphism (AFLPs) for detection of sex–specific markers in dioecious *Uapaca kirkiana* Muell. Årg.

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***Uapaca kirkiana* Muell. Årg is a dioecious fruit tree species for priority domestication in Southern Africa. It reaches reproductive maturity in eight to ten years with male plants making up 50% of breeding populations. Early identification of sex of seedlings is a prerequisite for selection and tree improvement. The amplified fragment length polymorphism (AFLP) technique was used to amplify DNA segments of *U. kirkiana* male and female plants to identify sex-specific markers. A total of 84 selective primer combinations were screened using bulked segregant analysis (BSA) for males and females. More than 110 polymorphic markers were obtained but each of the four primer pairs (E-ACT/M-CTG, E-ACA/M-CAA, E41+A/M-CTA and E-AGG/M-CTC) showed one band that was linked to sex. When the four primer pairs were tested in ten individuals from different populations only one primer pair (E-ACT/M-CTG) amplified a 320 bp band in female plants only. It is possible that this marker is linked to a sex-determining locus. The results suggest that the gene that determines sex of *U. kirkiana* is autosomal in nature and this marker may be important during fruit domestication and tree improvement programmes. The marker has been sequenced and sequence characterised amplified region (SCAR) marker will be developed and used for precise and rapid identification of female plants.**

Key words: AFLP, bulked segregant analysis, dioecious, domestication, SCAR, sex determination, *Uapaca kirkiana*.

INTRODUCTION

Uapaca kirkiana Muel. Årg is a dioecous plant belonging to the family Euphorbiaceae. The genus *Uapaca* is stable, apparently devoid of polyploidy with a chromosome number of 2n=26. The range of *U. kirkiana* includes Angola, Burundi, Southern Democratic Republic of Congo, Malawi, Mozambique, Tanzania, Zambia and Zimbabwe (Ngulube et al., 1995). Ethnobotanical studies within the miombo ecological zone in Malawi (Maghembe and Seyani, 1992), Tanzania (Karachi et al., 1991), Zambia (Kwesiga and Chisumpa, 1992) and Zimbabwe (ENDA, 1991) identified *U. kirkiana* as a priority indigenous fruit tree species for conservation and domestication among ten indigenous fruit species. Fruits of *U. kirkiana* are much sought after as a food and they are eaten raw, made into jam and sweetmeats, or used to produce a refreshing drink and a variety of wines. The fruit is highly valued as a source of nutrients and income to rural communities in Malawi (Saka and Msonthi, 1994). Due to the extended period of fruit ripening through the dry season, fruits of *U. kirkiana* are important as a source of main meal particularly in times of famine. Large quantities of seedlings are now required to meet the growing demand for whole fruits in rural and city markets (Ngulube et al., 1998). Through cultivation of indigenous fruit trees on farms, the potential exists to further and sustainably exploit the economic and nutritional potential of *U. kirkiana*. Several organisations in southern Africa in

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partnership with World Agroforestry have begun a programme to domesticate it. This domestication process involves the characterisation, selection, production and adoption of desirable tree germplasm in a process that directly involves farmers' participation. One of the areas earmarked for research work include molecular characterisation to understand the genetic factors controlling dioecy. Currently the fruit domestication challenge lies in the possibility of grafting male and female scions on the same individual tree and this requires identifying the sex of the tree at the seedling or juvenile phase of growth (Akinnifesi et al., 2004).

The sex of *U. kirkiana* plants cannot be determined from morphological characteristics until flowering and fruiting. There is no mention of any distinguishing cytological and vegetative features to identify sex of young plants. The inability to determine the sex of *U. kirkiana* at an early stage creates problems in tree improvement and domestication programme. Plants of *U. kirkiana* reach reproductive maturity in 8-10 years with male plants making up 50% of breeding populations (Akinnifesi et al., 2004; Ngulube et al., 1998). Early growth data in planted stands indicate slow growth (Maghembe and Seyani, 1992), but fruiting has been reported to occur within 10 years following establishment (Malaya, 1992). Reproductive information is mainly limited to its general flowering, fruiting and insect visitors and pollinators. In populations where fruit selec-tions are desired the requirement to grow seedlings to maturity before eliminating the excess males represents a considerable cost in plant maintenance. As only about 5% of male plants are required for pollination (Ngulube et al., 1998), there is need to weed out excess males which would lead to wastage of resources. Knowledge of the genetic control of sex determination is important in early identification of female and male plants. The identification of male and female seedlings has a great scientific interest and molecular markers such as amplified fragment length polymorphisms (AFLPs) are eminently suited for such a purpose. Molecular markers have been used in breeding to diagnose and select a genotype, long before the phenotype is apparent. It is important that AFLP sex-specific markers be used identify male and female plants before reproductive age. A method of identifying sex of seed-lings at an earlier stage would be useful for conservation, selection and fruit tree improvement. Early identification of sex would also increase the efficiency of a domestication programme by allowing proper arrangement of male and female seedlings in fruit orchards and allow early elimination of unwanted plants. It is vital that fast and reliable techniques be developed that can be used to identify male and female *U. kirkiana* plants regardless of the reproductive age. If sex determination is under the control of a single gene or a group of tightly linked genes then it should be possible to identify sex-specific DNA markers. Sex-related markers have been identified suc-cessfully in a considerable number of dioecious plants such as *Pistacia vera*, (Hormaza et al., 1994), *Carica papaya*, (Deputy et al., 2002), *Mercurialis annua* (Khadka et al., 2002) and *Silene latifolia*, Poiret (Mulcahy et al., 1992). The amplified fragment length polymorphism (AFLP) (Vos et al., 1995) is one of the most dependable molecular marker techniques used in identifying sex specific markers in plant species which are sexually dimorphic. AFLPs have unlimited utility in comparative studies across dioecious plants. AFLP technique has successfully been used to identify sex-linked markers in *Asparagus officinalis* (Reamson-Büttner et al., 1998; *Dioscorea tokoro* (Terauchi and Kahl, 1999) and *Ficus fulva* Reinw (Parrish and Koelewijn, 2004). The objective of the study focussed on use of AFLP technique to evaluate DNA from male and female *U. kirkiana* plants to identify sex-specific molecular markers.

MATERIAL AND METHODS

Leaf samples were collected from adult *U. kirkiana* individuals from miombo woodland of Bunda forest situated 33^0 45' N and 14^0E at University of Malawi in Central Malawi. Mature trees in permanent sample plots were observed for 12 months from flowering to fruit ripening. Female individual trees were identified according to absence of male flowers and development of fruits. Sex of adult *U. kirkiana* trees was further confirmed during flowering period between October 2004 to April 2005 by studying stage of development of flower buds, anthesis, inflorescence structure based on presence and/or absence of anthers and stigmas.

DNA Extraction

Leaf samples from ten male and ten female individuals were collected and preserved in silica gel. Using the bulked segregant analysis (BSA) (Michelmore et al., 1991) samples were obtained by pooling the same weight of silica gel-dried leaf sections from each individual to give 200 mg of leaf samples for DNA extraction. Total genomic DNA extraction from plant leaves followed the protocol of Patterson et al. (1993) with minor modifications. Samples were gently inverted ten times and incubated in a water bath at 65°C for 30 min, with occasional gentle agitation. After incubation the samples were centrifuged for 20 min at 2700 g after which the upper aqueous phase was transferred to a clean tube and the supernatant was extracted twice with one volume of cold chloroform and tubes were inverted for 10 min before being placed on ice. Following addition of 0.6 vol of isopropanol, samples were centrifuged for 20 min at 2700 g after which the upper aqueous phase was transferred to a clean tube, the DNA was precipitated for 3 h at -20°C, then recovered by centrifugation at 3000 g for 15 mins. The DNA pellet was washed with 2 ml of 70% ethanol, allowed to air dry and resuspended in 200 µl sterile TE. Following precipitation of impurities and RNase digestion the isolated DNA was resuspended in 200 µl Tris-EDTA buffer and stored at 4^0C for 24 h. The quality of DNA was evaluated by loading 10 µl from sample on a 1% agarose gel. The DNA concentration of each sample was estimated by visual assessment in relative to λ–phage DNA of different known concentrations on 1% agarose gel (Sambrook et al., 1989).

AFLP analysis

Initial screening of primers involved use of eighty four primer combinations tested in order to maximize band number and clarity.

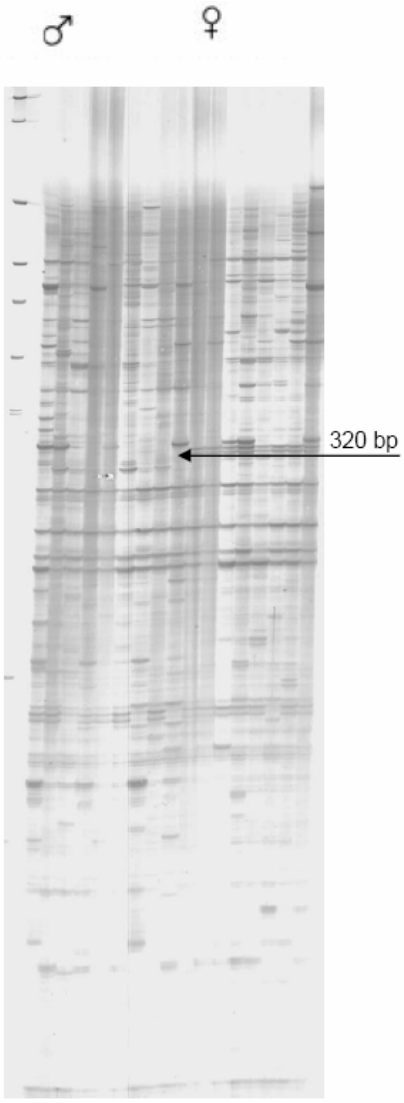

Figure 1. AFLP profiles showing the 320bp (indicated by arrow) female specific band in a population sample of eight male and eight female *Uapaca kirkiana*.

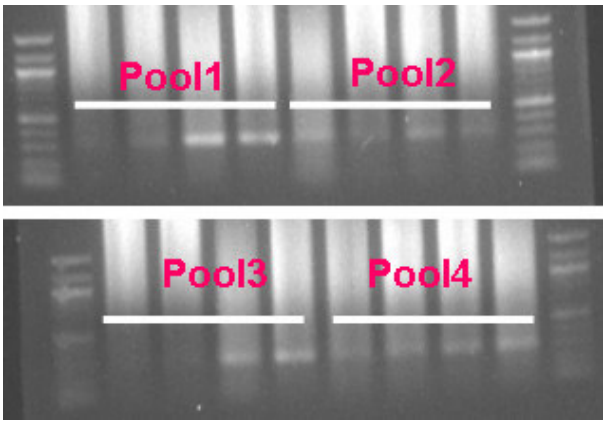

Figure 2. Reamplification of AFLP bands on agarose gel.

Sixty four primer pairs were selected to search for sex–linked markers. Two pairs of bulks each consisting ten plants as males and the other pair of bulk as females were prepared such that bands that appeared in one set of bulk but not in the other of different sex were considered to be putative markers for the sex. Since sex of the plants was known DNA bulks were examined for markers that amplified in only one sex and not the other. AFLP analysis was carried out according to the protocol of Vos et al., (1995) using restriction enzymes *Eco*RI and *Mse*I. Five microlitres of 1:10 diluted pre-amplification was used as a template for selective amplification using primers with three and four selective nucleotides at the 3'end. Two replicates of the PCR reactions from independent DNA extractions were performed to determine the accuracy of the analysis. AFLPs were separated on 5% denaturing polyacrylamide sequencing gels and bands were visualised in sodium carbonate developing solution for 5 min and the silver-stained gels were rinsed in ultra pure water and dried at room temperature in fumehood.

AFLP band isolation and sequencing

The single AFLP sex specific fragment 320 bp (n= 8) was excised from a dried polyacrylmide gel and eluted in 20 µl of TE at 65^0C for 4 h. The excised bands were amplified with the same primer pair used for the amplification of the primary AFLP template in 20 µl PCR reaction containing 8 µl deionised water, 5 µl 10X PCR buffer, 3 µl of 50 mM Mg Cl_2, 1 µl of 10 mM dNTPs, 1.5 µl of 10 pmol of each of the primer E-ACT/ M-CTG, 0.5U Taq DNA polymerase (Amersham Biosciences, UK) and 0.5 µl of eluted DNA. Thermal cycles were performed as follows: denaturing at 94^0 C 2 min, then 1 min at 9^0C, 1 min at 56^0C and 1 min 30s at 72^0C for 30 cycles. The PCR products were run on 1.5% standard agarose gel and the prominent band of 320 bp was excised from each lane and pooled into 4 pools as indicated in Figures 2 and 3. The PCR products were then purified using Qiagen PCR purification kit and resus-pended in 25 µl distilled water. The DNA representing the four pools was separately cloned into TOPO-TA vector (Invitrogen) and the TOPO vector with cloned fragment was digested with *Eco*RI which released the insert. Out of 16 clones, 4 individual clones were analyzed from each pool.

RESULTS AND DISCUSSION

AFLP female specific marker

Among all the 84 primer combinations screened in bulked male and female DNA of *U. kirkiana* four selected primer combinations produced 89 clearly scorable polymorphic bands in eight populations. The four primer combinations resulted in a range of 16 to 23 polymorphic markers with an average of 22 polymorphic markers per primer combi-nation (Table 1). Of the 89 polymorphic markers only 4 segregated in 1:1 ratio. The remaining 85 markers signi-ficantly deviated from Mendelian segregation ratio of 1:1 on Chi square analysis. The four primer pairs (E-ACT/M-CTG, E-ACA/M-CAA, E41+A/M-CTA and E-AGG/M-CTC) that showed sex- linked markers between the two bulks were tested in individual samples from eight populations and three of them were not found to be co-segregating with sex but only one primer pair E-ACT/M-CTG was completely associated with female sex in the individuals studied. The four pri-mers were also used in individuals that were used for bul-

Table 1. Number of sex-specific fragments and polymorphism produced by AFLP primer pairs on bulked samples

Primer name	Primer combination		Fragment Length (bp)	Sex	Total bands	Polymorphic bands	Polymorphism rate (%)
	*Eco*RI	*Mse*I					
E38/M61	ACT	CTG	320	female	77	23	30
E41+A/M59	AGGA	CTA	360	female	73	16	22
E35/M47	ACA	CAA	190	female	75	27	36
E41/M62	AGG	CTC	330	male	59	23	40
Total					284	89	8
Mean					71	22.25	32

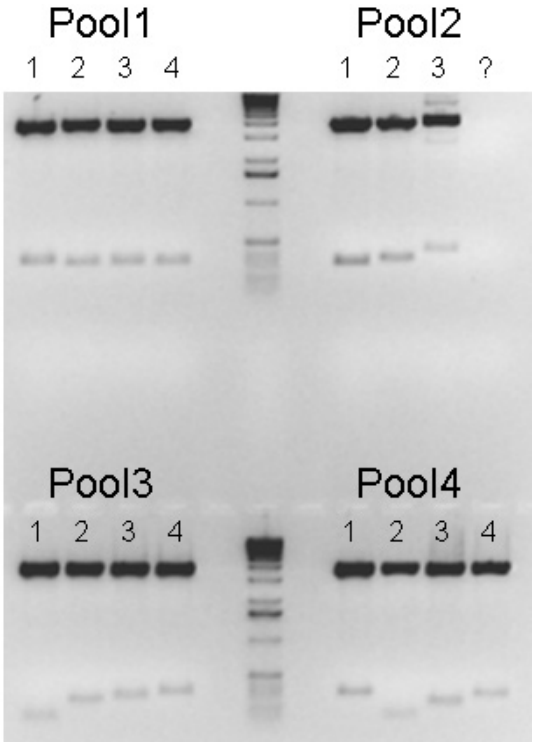

Figure 3. Restriction analyses of the insert sizes in the 15 clones representing the 4 pools. The upper strong band represents the vector (4kb); the lower and weaker band represents the cloned insert fragment, varying in sizes between 150-450 bp.

ked sample preparation. When individual male and female DNA were subjected to PCR amplification using the four primer combinations that revealed sex specific markers they did not display any recombination between marker presence or absence for the individual males and females suggesting that the markers were tightly linked to the region controlling sex.

The primer pair EACT/MCTG showed a band 320 bp expressing 1:0 segregation between female and male individuals and this was found from DNA of 10 females and absent in 10 males (Figure. 1). When this marker was tested for female specificity in 8 female individuals from different populations and 8 male individuals there was no one female that lacked the marker, nor was there any male individual showing presence of the female specific marker. Given the dominant nature of AFLP fragment females could be genotypically 11 or 10 and males 00 for the presence (1) or absence (0) of the 320 bp fragment.

The identification of one female specific AFLP marker of *U. kirkiana* 320 bp is being reported for the first time. This marker was amplified consistently as a female specific marker at the optimized PCR conditions. Testing of the marker on individuals of *U. kirkiana* collected from different populations demonstrated the female specificity without exception. The use of bulked segregant analysis has proved to be effective for tagging qualitative traits that can be classified into two discreet classes and this approach has an advantage in that the likelihood of identifying false positive markers is very small (Michelmore et al., 1991). The resulting gel showed that a band of the expected size was apparent above the background smear in approximately 50% of the reamplifications (Figure 2).

Clone and sequence of the female-specific AFLP band

The cloned 320 bp band produced different transformants. The cloned insert fragment varied in size between 150-450 bp, this was expected as the cloned DNA was not pure bands, but also contained a smear of DNA. The selection for the right fragment was based on the rational assumption that due to the stronger intensity of the correct band over the background, the correct fragment would be expected to have a much higher chance of being cloned than random fragments representing the background smear. Based on two observations that identical fragments in 7 out of 15 clones (47%) was observed and that the fragment corresponds exactly to the expected size of 320 bp it is a clear indication that the correct band has been identified. Thus, the correct fragment turned up several times after sequencing thus confirming that the clone contained the female specific fragment while the remaining 8 clones all represented different DNA fragments (sequences not shown). The DNA sequence of the fragment is shown in Figure 4. A Blast search against all Genbank and EMBL sequences

GATGAGTCCTGAGTAACTGATGGGATCTCAAATCTCTCGTAATGGGGGTT
GAATTTATTTGTAATAATGAATGCCGAATTTTTTGATTGCATAGTTTGGA
TTCCAATTTCTTTTGAAGCTTTATTTTGAATGAAGAATGTCATGGCATCT
AAGAGTGACTCTTTTCATCTCTACAGGGAAGGCCACAGTGATAACTATAG
GAGGTCTTGTAGCTGGTGCTGTTGTGGGATCAGCTGTTGAGAATTGGTTG
CAGGTTGATGTTGTACCATTTCTTGGCATACACTCGCCTGCGACTGTAGT
TAGTGAATTGGTACGCAGTC

Figure 4. Sequence of the seven identical female sex–specific cloned fragments (320 bp;1-1, 1-2, 1-3, 1-4, 3-4, 4-1, 4-4).

revealed homologous sequence to a genomic fragment on rice chromosome 3 with unknown function (gi|73746173|gb|DP000009.1|. The limited identity is interesting in that it is known that DNA from sex chromosomes in different dioecious plants is rich in these types of sequences (Grant et al., 1994). Identical clones are represented by clones; 1-1, 1-2, 1-3, 1-4, 3-4, 4-1, 4-4, which also appear as having the same size of 320 bp.

Strong linkage disequilibrium is expected if sex is controlled by heterogametic non-recombining sex chromosome system. The association between femaleness and one AFLP fragment in the 16 individuals studied was perfect. Since only one sex-associated marker was found, it is unlikely that there is cryptic non-recombining chromosome. Therefore it is very likely that sex in *U. kirkiana* is determined by autosomal locus rather than sex chromosomes. The low frequency of sex-linked marker suggests that the DNA segments involved in sex determination is small and probably involve a single gene or very few genes. The identification of this female specific marker suggests that sex determination in *U. kirkiana* could be as hypothesized in *Carica papaya,* under simple genetic control probably by a single gene or a group or very tightly linked genes. The pattern in *U. kirkiana* is exactly the opposite of that observed in *Dioscorea tokoro* (Terauchi and Kahl, 1999) and *Cannabis sativa* (Mandolino et al., 1999) where the sex-linked marker was present in males and absent in females. In all the cases it could be inferred that the male was the heterogametic sex and the female homogametic. If sex determination was under the control of multiple, unlinked genes it is highly likely that genetic recombination would prevent the identification of a single female specific marker and these results suggest that males of *U. kirkian*a have to be the homogametic sex. Whether the presence of sex-specific DNA markers in *U. kirkiana* suggests the presence of additional sex determining locus or that they arise from non-genic sequences could be an open question at the moment. The identified marker will be useful in distinguishing male plants from females. The study suggests that sex-specific AFLP marker can be used to determine the sex of pre-adults and seedlings of *U. kirkiana*. This marker would be of immense importance in domestication and breeding of *U. kirkiana* for directly selecting female plants at the seedling stage. Therefore, a desired ratio of male to female plants to be transplanted could be achieved and this could save resources by committing most of the resources to female plants. The success of sex identification of plants from different populations demonstrates that the marker could have considerable practical application in reducing costs of plant maintenance.

While AFLP technique is efficient for tagging of traits the markers may not amenable for routine and quick screening as such for broader application there is need to convert AFLP to simple sequence characterised amplified region (SCAR) markers by designing suitable pair of primers based upon the nucleotide of the original AFLP markers. SCAR markers being sequence specific are not biased to minor variables in experimental condition and they are simple, highly reproducible, reliable, simple to use and suitable for multiplex PCR analysis (Manoj et al., 2005).

Conclusion

The AFLP and bulk segregant analysis have proved as good techniques for finding molecular markers closely linked to sex in *U. kirkiana*. There has been no chance of early sex determination in *U. kirkiana* and this marker is apparently useful for early screening of plants to be planted out in orchards. Future research work shall aim at designing sequence characterised amplified region (SCAR) primers to amplify female specific markers that shall prove the reproducibility of the 0/1 pattern and test for specificity of the markers with many *U. kirkiana* individuals from different populations. In most studies where sex specific molecular markers have been isolated crosses that segregate for sex are used hence there is need to establish the association between AFLP marker and the female sex in a cross. Efforts are in place to screen more bulked DNA samples to identify additional markers associated with sex determination in *U. kirkiana* and characterize genes that determine sex.

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