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ORIGINAL ARTICLE

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Identification of a male-specific AFLP marker in a functionally dioecious fig, *Ficus fulva* Reinw. ex Bl. (Moraceae)

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Abstract A male-specific amplified fragment length polymorphism (AFLP) marker was identified in the functionally dioecious fig species, *Ficus fulva*. A total of 89 polymorphic fragments from three primer combinations were produced, of which one (246 bp) was present in all males (n=23) and absent in all females (n=24) of two populations. This strong association suggests a tight chromosomal linkage between the AFLP marker and the sex-controlling locus. Further analysis indicated that the marker segregated in open-pollinated progenies from natural populations in a 1:1 ratio (n=156), implying that males are the heterogametic sex. Chromosome preparations showed no evidence for morphologically distinct sex chromosomes. The low frequencies of associated markers argue against a morphologically cryptic non-recombining sex chromosome. The sex-locus is therefore likely to be autosomal. The male-specific AFLP marker was sequenced and converted into a sequence characterised amplified region (SCAR) marker. This SCAR marker produced a fragment of equal size in males and females, suggesting that sequence divergence between male- and female-specific chromosomal regions is low.

Keywords Sex determination · Functional dioecy · *Ficus* · Heterogametic sex · Linkage disequilibrium

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Introduction

Plants exhibit a wide variety of breeding systems ranging from hermaphroditism, through various forms of monoecy, to dioecy (Sakai and Weller 1999). Hermaphroditism is the most common form of breeding system in plants (ca. 72% of species) (Yampolsky and Yampolsky 1922). Four percent of species are truly dioecious, 7% are either gynodioecious or androdioecious and 75% of plant families have some dioecious members (Yampolsky and Yampolsky 1922). Bawa (1980) even argues for much higher figures for dioecy, given higher rates of dioecy in tropical floras and their relatively unstudied status.

The sexual phenotype in plants can be determined by genetic and environmental factors (Grant 1999). The diverse independent phylogenetic incidence of dioecy implies that this breeding system has evolved numerous times (Charlesworth 2001), which explains the great diversity of sex determination systems in plants. According to Grant (1999), genetic determination systems can be controlled by (1) a single locus, (2) a group of tightly linked loci on an autosome, (3) a number of unlinked loci on autosomes, or (4) loci on heteromorphic sex-chromosomes (e.g. *Silene* and *Rumex* spp.).

The genus *Ficus* exhibits two breeding systems, either monoecy (ca. 400 species) or gynodioecy (ca. 350 species) (Berg 1989). Gynodioecious species are functionally dioecious because hermaphrodites function as males as explained below (Janzen 1979; Anstett et al. 1997). Functional dioecy is restricted to the subgenus *Ficus* (distributed in the Palaeotropics with Malaysia as centre of diversity). The *Ficus* breeding system is particularly interesting because of the tight pollination mutualism with Agaonidae wasps. A trade-off exists between seed production and wasp reproduction as wasps also utilise short-styled female flowers for larvae development. *Ficus* flowers are unisexual and arranged in an urn-shaped receptacle (syconium). In monoecious species, male and female flowers exist a single syconium with female flowers being divided between seed and wasp production. In functionally dioecious species, female trees produce syconia containing only long-styled female

flowers that only produce seed. The long style lengths in female plants exceed wasp ovipositor length. Upon entering the syconium the female wasp usually cannot leave and therefore provides only pollination services to the plant and does not reproduce. In this character, functionally dioecious *Ficus* is unique in the plant kingdom (Valdeyron and Lloyd 1979). Male trees, while being functionally male (produce anthers), also produce short-styled female flowers that are almost exclusively used for wasp reproduction.

Breeding studies in the cultivated fig *Ficus carica* L. (the edible fig) led Storey (1955, 1975) to hypothesise that sex in *Ficus* is genetically determined by a supergene consisting of two very tightly linked genes. The first gene controls the style length of female flowers (the *G*-allele for short styles is dominant over the *g*-allele for long styles) and the second the formation of male flowers (the *A*-allele for male flowers is dominant over the *a*-allele for absence of male flowers). His experiments indicated that males are the heterogametic (*GA*/*ga*) and females the homogametic sex (*ga*/*ga*) (Storey 1955). Homogametic males (*GA*/*GA*) formed through male/male crosses are fully fertile (males of dioecious *Ficus* retain female flowers and, in *F. carica*, some ability to produce seed; Storey 1975; Valdeyron and Lloyd 1979). Further, there is no evidence for morphologically distinct sex chromosomes in this genus ($2n$=26 is the common somatic chromosome number in *Ficus*) (Condit 1964).

Knowledge of the genetic control of sex-determination in *Ficus* is important for the understanding of evolution functional dioecy in the genus. So far all knowledge of the genetic control of sex in *Ficus* is based on the breeding studies in *F. carica*. This species, however, is rather exceptional within the genus, having a long cultivation history and growing in rather temperate climates (Valdeyron and Lloyd 1979). It is therefore important to know if the genetic control of dioecy is similar in wild tropical species. 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 successfully in a considerable number of dioecious plant species (e.g. *Pistacia vera*, Hormaza et al. 1994; *Salix viminalis*, Alstrom-Rapaport et al. 1998; *Atriplex garrettii*, Ruas et al. 1998; *Silene latifolia*, Mulcahy et al. 1992; Zang et al. 1998; *Cannabis sativa*, Mandolino et al. 1999; *Carica papaya*, Deputy et al. 2002). Here we report on a sex-specific AFLP (amplified fragment length polymorphism; Vos et al., 1995) marker in *Ficus fulva* Reinw. ex Bl (subgenus *Ficus*, section *Ficus*; Corner 1965) a small, early successional, functionally dioecious tree distributed widely throughout South-East Asia and India. Our results suggest that sex in this fig species is under the control of a single autosomal locus, and that functional males are the heterogametic sex, corroborating Storey's results in *F. carica*.

Materials and methods

Materials

Leaves and fruits of adult trees of *F. fulva* were collected on three Krakatau islands (Rakata, Panjang and Anak Krakatau) in Indonesia for population studies. Trees were sexed according to the presence or absence of male flowers (anthers around the ostiolar opening in the syconium) or to the development of seeds. In total, 23 male and 24 female trees were used for genetic analysis (8 males and 8 females each from Rakata and Panjang, and 7 males and 8 females from Anak Krakatau). Leaf samples for DNA analysis were preserved in a NaCl/CTAB solution (Rogstad 1992). Seeds were sampled from five females (a total of eight fruits per island) from both Rakata and Anak Krakatau. These were later germinated on wet filter paper (12 h day, at 27°C day/25°C night) and then transferred to 4:1 peat:sand mix.

DNA extraction and AFLP procedures

DNA was extracted from both adult and seedling leaf material following the hot CTAB method of Rogstad (1992). Two slight modifications were made to this protocol; small quantities of leaf material (2×2 cm^2) were mixed in 600 µl extraction buffer and incubated at 65°C for approximately 2 h. The rest of the protocol was scaled according to this volume.

The AFLP protocol followed was similar to that of Vos et al. (1995) using the restriction enzymes *Eco*RI and *Mse*I. AFLP primers were screened for population studies. Different combinations of primer selective-nucleotide numbers were tested in order to optimise band number and ease of gel reading. Initial screening of AFLP primers using three selective nucleotides for both the *Eco*RI and *Mse*I primers produced a low number of bands. Therefore, further studies were carried out using two selective nucleotides for the *Mse*I primer. A total of nine primer combinations were screened before three were chosen for population studies. As the sex of all individual plants was known, populations were subsequently examined for markers that amplified in only one sex and not the other.

AFLP band isolation and sequencing

A sex-specific fragment was excised from a dried sequencing gel and eluted in water at 65°C for several hours. PCR was performed on 5 µl aliquots using the same AFLP primer combination used to generate the marker and 15 µl PCR reaction mix (as used for first round AFLP amplification, above). Thermal cycles were performed as follows: denaturing at 94°C 2 min, then 1 min at 9°C, 1 min at 56°C, and 1 min 30 s at 72°C for 30 cycles. PCR products were purified using the Qiagen PCR purification kit and resuspended in 30 µl ddH_2O. The PCR fragment was ligated into a 'T' overhang vector using the P Gem-T Easy cloning kit (Stratagene). Further procedures were as described in Sambrook et al. (1989). Minipreps were further purified using the Qiagen miniprep kit. Two of the clones were then sequenced using T7 and SP6 primers by the University of Leeds Biomolecular Sciences DNA sequencing facility (Leeds, UK).

Primer design for direct PCR of the fragment

The sequences obtained for the two clones were identical in length. In order to design a more direct PCR method for the amplification of the male-specific fragment, forward and reverse PCR primers were designed against the sequence data and used in a PCR reaction containing 0.2 mM dNTPs, 3 mM MgCl, 5 pmol each primer, 50 ng genomic DNA, and 0.4 U *Taq* polymerase (Promega) in a final volume of 20 µl. Thermal cycles were performed as follows: denaturing at 94°C 4 min, then 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C for 35 cycles, followed by an extension step of 2 min

Table 1 Amplified fragment length polymorphism (AFLP) selection sequences used in population studies and screened for male-specific markers. Primer combination 2 produced a male-specific marker. The number of polymorphic and fixed bands that were generated is listed

Primer no.	*Eco* extension	*Mse* extension	Number of polymorphic bands	Number of fixed bands
1	5′-AGA-3′	5′-CT-3′	36	26
2	5′-AGG-3′	5′-CA-3′	27	37
3	5′-ATC-3′	5′-CA-3′	26	36
		Total number of bands	89	99

at 72°C and cooling of 2 min at 25°C. PCR products were visualised on a 1% agarose gel.

Cytological investigations

For chromosome counts, young root tips were pre-treated in a 1% aqueous solution of 8-hydroxyquinoline at 4°C for 6 h and fixed and stored in a 1:3 mixture of acetic acid:ethanol (Carnoy) at −20°C. Chromosome spread preparations were prepared according to the techniques described in Pijnacker and Ferwerda (1984). Chromosomes were visualised with DAPI (4′,6 diamidino-propyl-indole) and counted under a fluorescence microscope. The genome size of *F. fulva* was determined by flow cytometry (Ploidy Analyzer, Partec, Münster, Germany) using DAPI as the fluorescent stain (Tas and Van Dijk 1999). Leaf samples were taken from ten seedlings grown in the greenhouse. A sample of *Brassica oleracea* nuclei was used as an internal standard (genome size: 1.37 pg/2C; Arumuganathan and Earle 1991).

Results

AFLP marker identification

The three primer combinations used produced 89 readily scorable polymorphic bands in three populations on the Krakatau island material (Table 1). One primer combination (*Eco*-AGG, *Mse*-CA primer # 2, Table 1) produced a fragment of 246 bp that was present in all males (n=23, from three populations) but absent in all females (n=24, from three populations) (Fig. 1). The probability that such a sex association could arise by chance in this sample size is extremely small (Fisher's exact test: $P=0.62\times10^{-14}$). Given the dominant nature of AFLP fragments, males could be genotypically *11* or *10* and females *00* for the presence (*1*) or absence (*0*) of the 246 bp fragment.

If sex is determined by a single locus with one of the sexes heterogametic (*10*), a 1:1 ratio of male and females among the offspring of either controlled or open-pollinated crosses would be expected. To test the likelihood of Mendelian inheritance, the frequency of the male-specific marker was examined in an array of open-pollinated seedlings. Seedlings were raised from five open-pollinated mothers from each of two populations (Rakata and Anak Krakatau). Seedling samples were pooled for each population, as within-fruit seedling sample sizes were too small for individual statistical analyses. When both populations were pooled, the segregation ratio did not differ significantly from a 1:1 ratio (78 individuals with the marker present from 156), expected for homogametic *00* females and heterogametic *10* males (χ^2=0.00, P=1.00, Table 2).

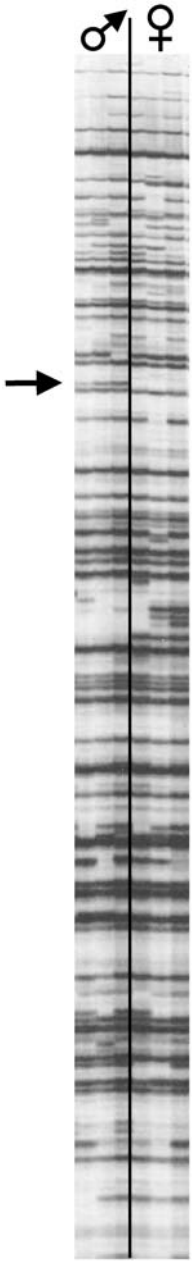

Fig. 1 An amplified fragment length polymorphism (AFLP) profile showing the 246 bp male-specific band (*arrow*) in a population sample of three males and three females

Table 2 Segregation of the marker in 16 fruits (approximately 10 seeds per fruit) from five mothers from each of two populations, Anak Krakatau and Rakata. Also indicated is a chi-square test for deviations from the expected 1:1 sex ratio among the progenies

Population	Number of female trees	Number of fruits	Number of progeny	Male marker		X^2 (df=1)	P
				present	absent		
Rakata	5	8	77	40	37	0.117	0.73
Anak	5	8	79	38	41	0.114	0.74
Total	10	16	156	78	78	0.000	1.00

Sex-marker sequence and primer design

The sequence of the cloned sex-marker is deposited with GenBank (accession number AY382781). A Blastn search against all GenBank and EMBL sequences identified no homologous sequences (all E values >0.09).

In order to develop a direct PCR technique to amplify male-specific DNA, a primer set based on the cloned sequence was designed: 5′ TAA CAT CAT ACT TG 3′ F1 (forward) and 5′ CAG GAA TTA GCA TAA CCG 3′ R (reverse). Primer amplification was tested on DNA from three male and four female *F. fulva*. However, amplification products of the same size were observed in both sexes, thus indicating that there is considerable sequence homology between males and females.

Cytology

Flow cytometry gave a fluorescence peak for *F. fulva* at position 0.61 relative to the 2C *Brassica oleracea* standard peak, corresponding to a 2C genome size of *F. fulva* of 0.84 pg DNA. No variation was observed between the ten seedlings analysed.

Figure 2 shows well-spread metaphase chromosomes of root-tip mitosis. Most chromosomes are small, about 1–2 μm. Either 26 or 28 somatic chromosomes are visible, depending on the cytological interpretation. In the case of 2n=26, there are two larger meso-centric chromosomes (Fig. 2, chromosomes A and B). In the case of 2n=28, these two meso-centric chromosomes are in fact four chromosomes. One satellite chromosome is clearly visible (chromosome C) in Fig. 2.

Six seedlings were cytogenetically investigated. Although the sex of the seedlings was not known, the probability that at least one of them was male, assuming a 1:1 sex ratio, is greater than 0.98. In none of the six seedlings could a pair of heteromorphic chromosomes be observed. However, with the exception of the meso-centric chromosomes, the chromosomes are too small to identify morphological distinct sex-chromosomes.

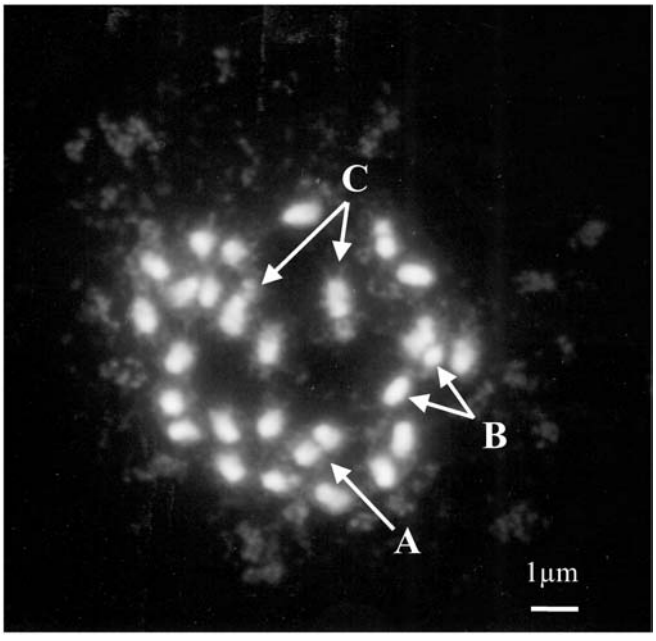

Fig. 2 Mitotic chromosomes of root tip preparations stained with 4′,6 diamidino-propyl-indole (DAPI). *A*, *B* Mesocentric chromosomes, *C* satellite chromosome. For further explanation see text. *Bar* 1 μm

Discussion

This study identifies a male-specific AFLP marker in the functionally dioecious fig species, *F. fulva*. To our knowledge, this is the first report of a sex-specific molecular marker in *Ficus*.

Chromosome numbers and morphology in the genus *Ficus* have been studied mainly by Condit (1928, 1934, 1964). Although Condit's papers report on almost 100 fig species, *F. fulva* is not included. Condit states that the chromosomes of the various fig species are similar to one another in appearance, and are generally very small, ranging from less than 1 μm to a little over 2 μm. Most chromosomes are acrocentric, some meso-centric. Occasionally, satellite-chromosomes are distinguishable. For 99 of the 100 species, Condit gives n=13 as the basic chromosome number. Krause (1930) reported besides n=13 also one species with n=14 and two species with possibly n=14. However, for two of these species Condit (1934) counted n=13, leaving one uncertain case of n=14. Our cytological observations in *F. fulva* largely corroborate Condit's observations. We believe that the 2n=26 interpretation of the chromosomal configuration in Fig. 2, with two larger meso-centric chromosomes, is the most likely.

Flow cytometry indicated that the genome size of *F. fulva* is small, less than three times that of *Arabidopsis*. Slightly larger genome sizes have been reported by Ohri and Khoshoo (1987) for other *Ficus* species, including *F. carica* (2C: 1.41 pg), using Feulgen staining and micro-

densitometry. The small genome size explains why AFLP with three selective nucleotides generated relatively few fragments. Because of the small size of the chromosomes it is hard to distinguish sex-specific morphological chromosomes. No sex-specific peculiarities in chromosome morphology have been reported for other dioecious *Ficus* species (Condit 1964). In species with large sex-chromosomes, like *Silene* species (Costich et al. 1991), it is possible to distinguish sex by flow cytometry. This is definitely not possible in *Ficus fulva* and *F. carica*.

In most studies where sex-specific molecular markers have been isolated, crosses that segregated for sex were used (examples cited in the introduction). The present association between the AFLP marker and the male sex in *F. fulva* was not established in a cross, but was detected in natural populations. Close chromosomal linkage between the marker and the sex-determining locus is the most likely explanation for this linkage disequilibrium (LD). In general, the amount of LD between closely linked markers found in outcrossing populations is low and decays rapidly with increasing inter-locus distances (Remington et al. 2001; Nordborg and Tavar 2002). Founder effects have been shown to increase LD dramatically (e.g. in *Arabidopsis thaliana*, Nordborg et al. 2002). Perhaps the founder effects and genetic drift that could have occurred during the re-colonisation of the Krakatau islands have affected LD in *F. fulva*. It is generally assumed that the enormous volcanic eruption in 1883 completely sterilised the Krakatau islands. However, signatures of founder effects other than LD, like loss of rare alleles and loss of heterozygosity, are not obvious in the present AFLP material (T.L. Parrish, unpublished results). *F. fulva* plants were first collected in 1896 and 1897 and the species was already common by 1919 (Docters van Leeuwen 1936). The present population size in the Krakatau islands has been estimated as more than 35,000 individuals (N. Mawdsley, personal communication). Clearly the population has expanded rapidly in the last 100 years, which will have caused a rapid decay in LD, especially in an outcrossing plant species such as *F. fulva*.

Strong LD is to be expected if sex is controlled by a heterogametic non-recombining sex chromosome system. As stated above, there is no evidence for morphologically distinct sex chromosomes in *Ficus*. The association between maleness and one AFLP fragment in the studied material was perfect. Out of 89 polymorphic fragments, 1 was strictly associated with maleness. Assuming a random genomic distribution of polymorphic AFLP markers, the likelihood of a non-recombining morphologically cryptic sex-chromosome can be estimated. Assuming that the two meso-centric chromosomes have twice the size of the other chromosomes, on average 5.93 AFLP markers are expected on the 11 small chromosomes and 11.87 on the two long chromosomes. Since only one sex-associated marker was found, it is unlikely that there is a cryptic non-recombining small sex chromosome (P=0.018; assuming a Poisson distribution for the distribution of the AFLP markers) or a long chromosome (P=0.00009). It is therefore likely that the sex locus in *Ficus* is autosomal. Given the strong LD, it may be that recombination is suppressed around this autosomal sex-locus.

The identification of a sex-specific marker strongly suggests that sex determination in *F. fulva* is, as hypothesised for *F. carica* (Storey 1955), under simple genetic control, possibly by a single gene or a group of very tightly linked genes. If sex-determination was under the control of multiple, unlinked genes, recombination would prevent the identification of a single male-specific marker in *F. fulva*. Given the dominant nature of AFLP markers our results concur with the observations of Storey (1955), i.e. that females in *Ficus* are the homogametic sex in *F. carica*. The 1:1 segregation for presence and absence of the fragment in the open-pollinated progenies implies that males have to be the heterogametic sex.

In *F. carica* males can occasionally produce some functional female flowers and produce some seeds (Storey 1975). This means that homozygous *GA/GA* males could be formed. Such males would be homozygous *11* for the AFLP sex-marker in *F. fulva*. Also, recombination between the AFLP marker and the sex locus or mutations can generate *11* males. The strict 1:1 band segregation in the open-pollinated material suggests that *11* males are absent or uncommon in *F. fulva*, because homogametic *11* pollen donors would have inflated the number of *11* offspring.

The primers designed from the male-specific AFLP fragment produced amplification products of the same size in both male and female DNA samples. This suggests that there is considerable sequence homology between males and females at this chromosomal region. No sequences homologous to the fragment identified in this study were found in other species when the sequence databases were searched. This result may be expected since AFLPs detect random genomic variation, most of which will be non-coding DNA.

Markers for early sexing in *F. carica* are economically important (Condit 1928; Story 1975), but chromosome morphology and flow cytometry cannot be used for this purpose. Our study suggests that sex-specific AFLP-markers could likely also be found in *F. carica*. Sex markers can also be used to determine the sex of pre-adult and non-flowering individuals in natural populations, which is useful, for example, in studies on sex ratio distortion where the distortion level varies with the life stage (Conn and Blum 1981; Purrington and Smith 1995). Studies in *Ficus* (including *F. fulva* on Krakatau) have so far found that adult sex-ratios do not differ significantly from equality (Compton et al. 1994; Harrison and Yamamura 2003). However, the number of these studies is rather limited (Harrison and Yamamura 2003).

Phylogenies based on rDNA sequences and morphology suggest that monoecy is the ancestral state in the genus *Ficus* and that dioecy has evolved twice (Weiblen 2000). The *Ficus* section *Ficus*, to which both *F. carica* and *F. fulva* belong, forms a single dioecious clade (Weiblen 2000). Therefore dioecy in these two species is most likely to share a common ancestry, explaining the similarities in genetic control of functional dioecy. In the *Ficus* section *Ficus* clade no reversals to monoecy have occurred.

Weiblen (2000) suggested that the tight linkage between the genes for style-length and male flower production could prevent a reversal to monoecy. Without tight linkage, recombinants between the style length and the male flower locus, e.g. *gA*/*ga* would produce pollen, but would still be functionally female, because no offspring pollinator wasps would be produced. However, a wasp with a mutation for a longer ovipositor could reproduce in such recombinant figs, which could then result in a reversal to monoecy in the figs. In the second dioecious clade, two reversals to monoecy occurred. Perhaps these reversals were possible because the genes that determine functional sex in this clade are not so closely linked as in the *Ficus* section *Ficus* clade. To test this idea it would be of great interest to study the genetic control of sex in *Ficus* species belonging to the second dioecious clade.

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