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A genetic linkage map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance

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Abstract A genetic linkage map of the tetraploid water yam (*Dioscorea alata* L.) genome was constructed based on 469 co-dominantly scored amplified fragment length polymorphism (AFLP) markers segregating in an intraspecific F_1 cross. The F_1 was obtained by crossing two improved breeding lines, TDa 95/00328 as female parent and TDa 87/01091 as male parent. Since the mapping population was an F_1 cross between presumed heterozygous parents, marker segregation data from both parents were initially split into maternal and paternal data sets, and separate genetic linkage maps were constructed. Later, data analysis showed that this was not necessary and thus the combined markers from both parents were used to construct a genetic linkage map. The 469 markers were mapped on 20 linkage groups with a total map length of 1,233 cM and a mean marker spacing of 2.62 cM. The markers segregated like a diploid cross-pollinator population suggesting that the water yam genome is allo-tetraploid ($2n = 4x = 40$). QTL mapping revealed one AFLP marker E-14/M52-307 located on linkage group 2 that was associated with anthracnose resistance, explaining 10% of the total phenotypic variance. This map covers 65% of the yam genome and is the first linkage map reported for *D. alata*. The map provides a tool for further genetic analysis of traits of agronomic importance and for using marker-assisted selection in *D. alata* breeding programmes. QTL mapping opens new avenues for accumulating anthracnose resistance genes in preferred *D. alata* cultivars.

Keywords Yam · *Dioscorea alata* · Genetic mapping · Tetraploid · QTL · Anthracnose

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Introduction

Water or greater yam (*Dioscorea alata*) belongs to the family Dioscoreaceae, order Dioscoreales. It is monocotyledonous and constitutes an important staple food crop for millions of people in the humid and subhumid tropics. *D. alata* is superior to most edible yam species in terms of factors such as yield potential (especially under low to average soil fertility), ease of propagation (production of bulbils and reliability of sprouting), early vigour for weed suppression and storability of tubers. The development of *D. alata* varieties with improved food quality, and resistance to pests and diseases, would encourage greater widespread cultivation and significant increases in production.

In recent years, very limited progress has been made in using morphological traits (Cruz et al. 1999), as well as isozyme (Lebot et al. 1998) and molecular markers (Asemota et al. 1996) for *D. alata* germplasm characterisation. With a basic chromosome number of 10, most cultivars of the species are highly heterozygous tetraploids, hexaploids or octoploids ($2n = 4x$ to $2n = 8x$), but not diploids (Gamiette et al. 1999). It remains unclear, however, if water yam is an allo- or auto-polyploid. Genetic improvement programmes at IITA (Nigeria) and at the Central Tuber Crops Research Institute (CTCRI, India) have been developing high yielding *D. alata* varieties with resistance to pests and diseases to meet farmers' requirements. Apart from recent studies on the inheritance of resistance to anthracnose disease (Mignouna et al. 2001a), little is known concerning the genetics of resistance to the major pests and diseases of water yam.

Anthracnose disease, caused by *Colletotrichum gloeosporioides* (Abang et al. 2002), is a serious constraint to the sustainable cultivation of *D. alata* worldwide (Winch et al. 1984; Nwankiti et al. 1987; Macdonald et al. 1998). Resistance to the moderately virulent fast-growing salmon (FGS) strain of *C. gloeosporioides* in the tetraploid breeding line TDa 95/00328 was found to be strain-specific and appeared to be controlled by a single major dominant locus; however, results suggested the presence of an additional resistance gene (s) (Mignouna et al. 2001a). The FGS strain is presently the predominant virulence phenotype in Nigeria and represents a genetically heterogeneous population (Thottappilly et al. 1999; Abang et al. 2001, 2002). In view of the high genetic diversity of *C. gloeosporioides* from yam, an obvious strategy is to combine resistance to the FGS strain with resistance to strains such as the highly virulent slow-growing grey (SGG) strain, for which sources of resistance were recently identified (Mignouna et al. 2001a). Anthracnose resistance breeding is a considerably slow and cumbersome process, owing to the biological constraints of a heterozygous, vegetatively propagated crop (Tanksley and Nelson 1996; Asiedu et al. 1998). It is, therefore, important to develop varieties carrying as many different genes for resistance as possible in order to provide stable resistance against a broad spectrum of the fungal pathogen.

Amplified fragment length polymorphism (AFLP) markers are an excellent source of polymorphisms in eucaryotic genomes and have been shown to be well-suited for genotyping and map construction in several plant species, including yam (Mackill et al. 1996; Mignouna et al. 1998; Mignouna and Asiedu 1999; Terauchi and Kahl 1999; Mignouna et al. 2001b). Terauchi and Kahl (1999) produced a genetic linkage map of *Dioscorea tokoro* Makino, a diploid wild yam species classified in the botanical section Stenophora that is phylogenetically distant from the cultivated yams in the Section Enantiophyllum. A molecular map of *Dioscorea rotundata* has been constructed which, when saturated, will greatly facilitate the genetic improvement of white Guinea yam (Mignouna et al., 2002a). No such linkage map exists for water yam, the most-widely cultivated species globally. The availability of a high-density genetic linkage map of *D. alata* will greatly improve the prospects for gene mapping and marker-assisted selection, as has been demonstrated in common bean (Adam-Blondon et al. 1994; Mendoza-Herrera et al. 1999; Geffroy et al. 2000) and potato (Hämäläinen et al. 1998; Van der Voort et al. 1999; Solomon-Blackburn and Barker 2001a, 2001b).

The objective of the present study was to produce the first comprehensive molecular linkage map of tetraploid *D. alata* and to identify quantitative trait loci (QTLs) controlling resistance to yam anthracnose disease.

Materials and methods

Mapping population and anthracnose resistance screening

Two tetraploid *D. alata* genotypes that discriminate the FGS and SGG strains of *C. gloeosporioides*, and carry different anthracnose resistance genes (Mignouna et al. 2001a), were chosen for the development of a mapping population. The female parent (TDa 95/00328) and male parent (TDa 87/01091) were cross-pollinated in the field. Both parental accessions were breeding lines. TDa 95/00328 originated from a controlled cross between the susceptible landrace TDa 92-2 and the resistant breeding line TDa 85/00257, while TDa 87/01091 was obtained from open pollination of female line A19-165-445. The F_1 population used in the present study consisted of 176 individuals. This population served as the source of individuals for anthracnose resistance screening and for marker segregation analyses. The *C. gloeosporioides* isolate (Cg33), disease resistance screening and evaluation were as reported previously (Mignouna et al. 2001a). The percentage leaf area affected by anthracnose was scored on a 0–6 scale. Genotypes with a mean leaf-area damage of 0–17.5% (corresponding to scores 0–4) were considered to be resistant, while those with a mean leaf-area damage of >17.5% (corresponding to scores 5 and 6) were considered susceptible (Mignouna et al. 2001a). Chi-square tests for goodness-of-fit of the segregation ratios of resistant to susceptible (R:S) genotypes were carried out using the SAS statistics software (SAS Institute Inc. 1989). The test for the normal distribution of the frequency of the disease severity scores was performed by the Shapiro-Wilks W-statistic test, using the program STATISTICA v. 6.0.

Table 1 The primer combinations (PCs) used to screen the mapping population, PC nomenclature and the number of scored markers per PC

Primer Combination	Extension	Number of scored markers
P12/M15	AC/CA	23
P12/M19	AC/GA	13
P13/M16	AG/CC	18
P14/M15	AT/CA	9
P14/M20	AT/GC	14
P14/M21	AT/GG	8
P15/M20	CA/GC	10
P16/M16	CC/CC	15
P16/M20	CC/GC	16
P17/M15	CG/CA	14
P17/M19	CG/GA	6
E11/M55	AA/CGA	37
E11/M58	AA/CGT	45
E12/M48	AC/CAC	35
E12/M49	AC/CAG	31
E12/M50	AC/CAT	41
E13/M48	AG/CAC	30
E13/M52	AG/CCC	30
E14/M52	AT/CCC	43
E14/M54	AT/CCT	40
E14/M58	AT/CGT	30
Total		508

DNA extraction

DNA was extracted from 100 mg of lyophilised or freshly harvested leaves of the 176 F_1 individuals and the two parental lines according to a modified CTAB procedure (Mignouna et al. 1998). DNA quality was visually assessed on a 1% agarose gel following electrophoresis, and the concentration was determined using a Beckman spectrophotometer model DU 520.

AFLP markers

AFLP analysis was carried out as described by Vos et al. (1995) using the enzyme combinations *Eco*RI/*Mse*I (E/M) and *Pst*I/*Mse*I (P/M). A total of 10 E/M and 11 P/M primer combinations (PCs) were selected (Table 1) based on a pre-screening of 64 E/M and 48 P/M PCs, respectively. The number, density and complexity of

polymorphic AFLP fragments were used as selection criteria. The adaptor and primer sequences employed were based on the core primer design as described by Vos et al. (1995). Gel images were electronically scanned and AFLP markers co-dominantly scored using the proprietary technology developed by Keygene N.V., Wageningen, The Netherlands (Vuylsteke et al. 1999; Park et al. 2000). Each polymorphic AFLP fragment was identified by a code referring to the primer combination (PC), *Eco*RI/*Mse*I (E/M) and *Pst*I/*Mse*I (P/M), followed by the estimated size of the DNA fragment in nucleotides. A 10-bp ladder DNA from SequaMark (Research Genetics, Huntsville, Ala., USA) was used as a standard to estimate the size of the fragments. The primer nomenclature of Keygene was used throughout and the primer sequences can be deduced from the marker designations (Table 1).

Mapping analysis

Marker segregation data were obtained by analysing the entire F_1 population with the primer combination E/M, whereas 90 individuals were analysed with the combination P/M. Markers were tested against the expected segregation ratio using a χ^2 goodness-of- fit. A chi-square value below 2.0 was considered to be reliable for this analysis. Linkage analysis was performed with the computer software package JoinMap 2.0 (Stam 1993; Stam and Van Ooijen 1996) and the segregating population was treated as a cross-pollinated one. Kosambi's mapping function (Kosambi 1944) was used to convert the recombination frequency into map distance (cM). The pairwise analysis obtained from JOINMAP was used to assign markers to linkage groups with a LOD ≥3.

QTL mapping

A search for QTL effects for anthracnose resistance was carried out on the entire mapping population with all the segregating markers, using the non-parametric mapping procedure of the software package MapQTL versions 3.0 and 4.0 (Van Ooijen and Maliepaard 1996). The mean leaf area infected (LAI) of the F_1 individuals was used as the phenotypic trait score and significant associations were searched on the *D. alata* linkage map. Because the Kruskal-Wallis rank-sum test which was performed in this non-parametric mapping method used both linked and unlinked markers, a more-stringent significance level ($P = 0.005$) served as the threshold for declaring the likely presence of a QTL near a marker. In addition, association between molecular markers and anthracnose resistance was determined by simple linear regression of LAI data on marker genotype means using the computer package Q-GENE 3.06 running on a G3 Power Macintosh (Nelson 1997). The proportion of the phenotypic variance explained by the marker segregation was determined by the R^2 value. A putative QTL map location was assigned to the linkage group using a LOD threshold of 3.00.

Results

Generation of marker data sets

Two classes of markers were found showing single- and double-dose band intensities indicating an allo-tetraploid status in *D. alata.* As a result, segregation data were treated as for a diploid cross-pollinated population. Due to heterozygosity, segregating marker loci could be followed from both parents. In total, three marker segregation types were found: 187 <AB × AB> markers, 130 <AB × AA> markers and 191 <AA × AB> markers, the first giving information about segregation of both parents, while the latter two types provided information about the maternal and paternal parent, respectively. The number of markers scored per primer combination is presented in Table 1.

Table 2 Mapping data for yam population (TDa 95/00328 × TDa 87/01091) showing the number of markers mapped after 1, 2, or 3 JoinMap-rounds including the mean chi-square value and the distance (cM) per linkage group

Linkage group	JM-round	Mean χ^2	No. of markers	Length (cM)
LG 1	Round 1	0.994	23/27	41.2
	Round 2	0.994	23/27	41.2
	Round 3	1.173	27/27	68.5
LG 2	Round 1	0.754	29/43	133.5
	Round 2	0.754	29/43	133.5
	Round 3	1.167	43/43	102.6
LG 3	Round 1	0.426	23/25	63.8
	Round 2	0.588	24/25	63.3
	Round 3	0.973	25/25	63.3
LG 4	Round 1	1.038	23/26	77.1
	Round 2	1.038	23/26	77.1
	Round 3	1.331	26/26	85.1
LG 5	Round 1	0.676	18/19	46.6
	Round 2	0.676	18/19	46.6
	Round 3	1.381	19/19	40.6
LG 6	round 1	0.477	20/20	51.8
LG 7	Round 1	0.840	31/36	75.8
	Round 2	0.903	33/36	77.2
	Round 3	1.286	36/36	79.3
LG 8	Round 1	1.086	18/22	42.3
	Round 2	1.086	18/22	42.3
	Round 3	1.486	22/22	41.5
LG 9	Round 1	0.611	21/25	58.9
	Round 2	0.611	21/25	58.9
	Round 3	0.656	25/25	58.5
LG 10	Round 1	0.615	28/38	64.3
	Round 2	0.615	28/38	64.3
	Round 3	0.648	38/38	65.2
LG 11	Round 1	0.688	13/17	58.4
	Round 2	0.627	14/17	58.1
	Round 3	0.894	17/17	58.0
LG 12	Round 1	1.024	23/31	64.8
	Round 2	1.002	25/31	64.4
	Round 3	1.134	30/31	70.8
LG 13	Round 1	0.136	10/13	43.7
	Round 2	0.113	13/13	43.5
LG 14	Round 1	0.555	25/26	77.1
	Round 2	0.642	26/26	87.3
LG 15	Round 1	0.537	27/27	75.8
LG 16	Round 1	0.524	18/25	44.3
	Round 2	0.647	20/25	61.2
	Round 3	0.683	25/25	54.0
LG 17	Round 1	0.031	9/9	6.5
LG 18	Round 1	0.507	10/10	58.4
LG 19	Round 1	1.076	14/16	51.3
	Round 2	1.076	14/16	51.3
	Round 3	1.675	16/16	49.3
LG 20	Round 1	0.561	14/15	72.3
	Round 2	0.561	14/15	72.3
	Round 3	0.563	15/15	72.3
Totalising across	Round 2		409 (87%)	1,235.6
linkage groups:	Round 3		469 (100%)	1,232.9

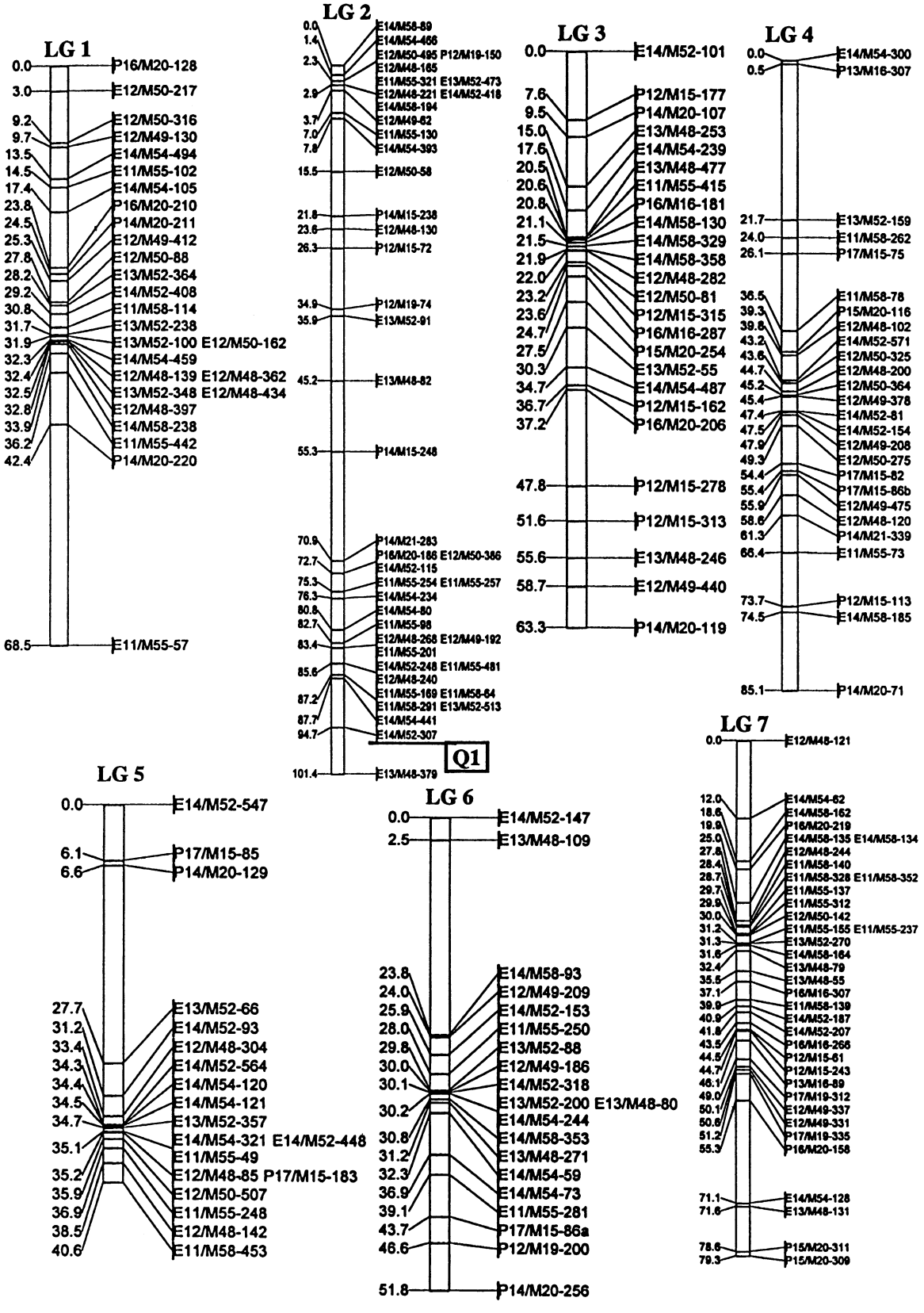

Fig. 1 Genetic linkage map of water yam (*D. alata*) based on F_1 progeny from maternal line (TDa 95/00328) × paternal line (TDa 87/01091). Distances (on the left of the linkage groups) represent genetic distances in Kosambi centiMorgans (cM). Locus names are on the right side of the linkage groups. The loci are named with (1) the code referring to the corresponding primer combination (see Table 1), followed by (2) the estimated size of the DNA fragment in nucleotides. One marker associated with a minor QTL for anthracnose resistance (explaining 10% of the phenotypic variance) is marked Q1. LG = linkage group

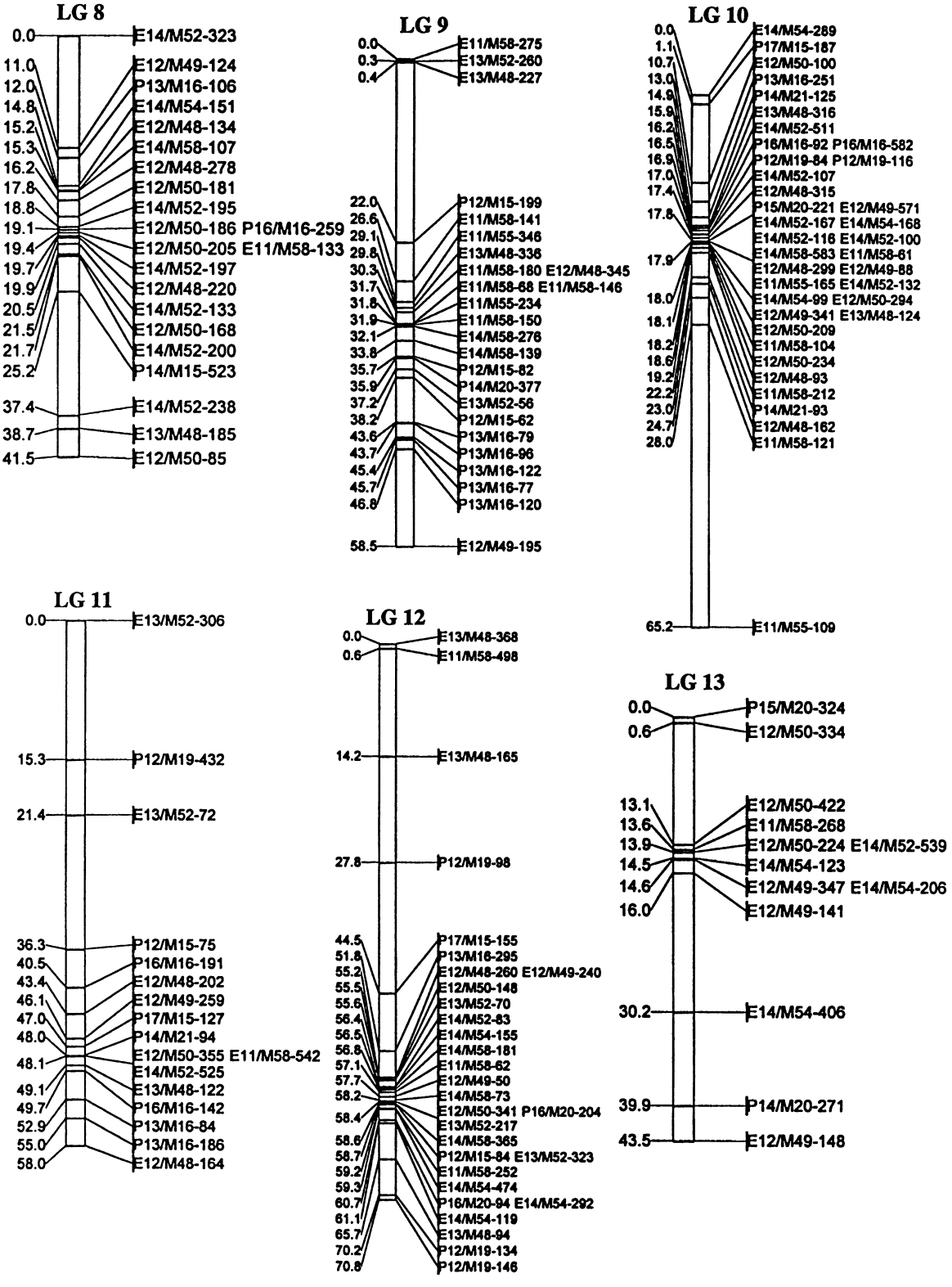

Fig. 1 (continued)

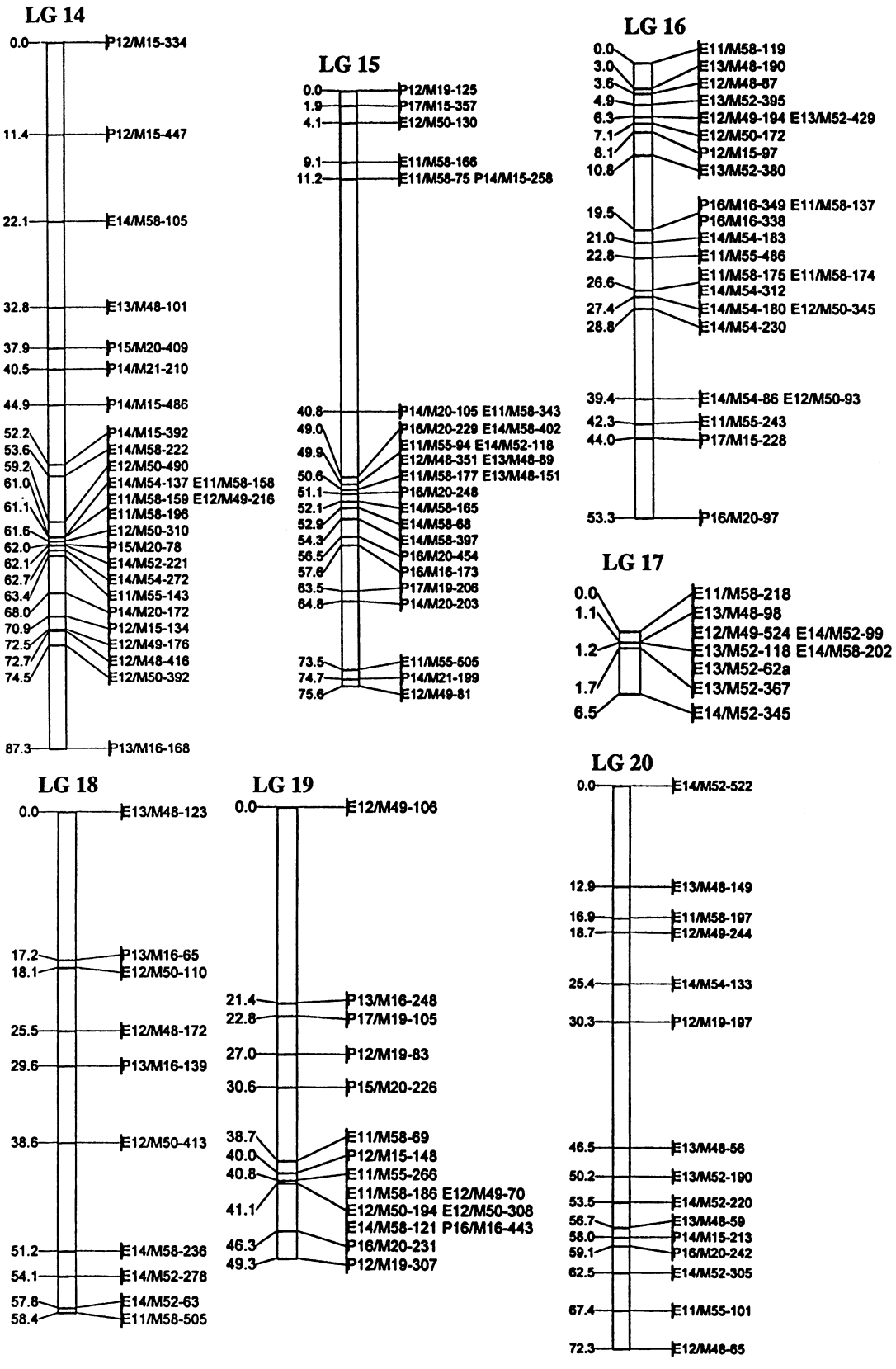

Fig. 1 (continued)

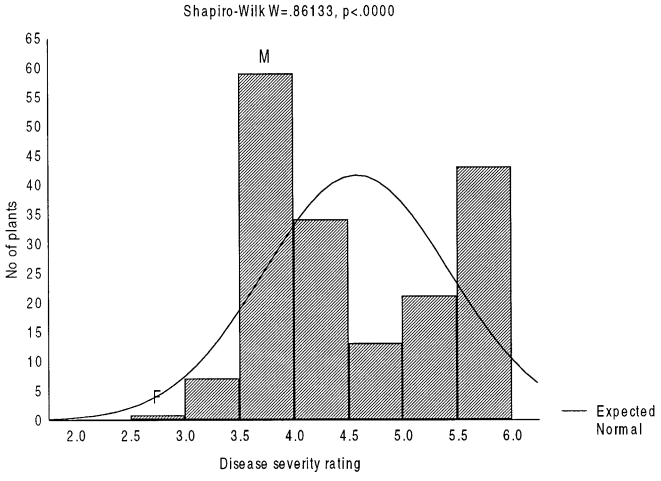

Fig. 2 Distribution of the mean disease severity rating for the parents and the 176 F_1 individuals of the cross TDa 95/00328 (F) × TDa 87/01091 (M) following evaluation of anthracnose resistance using isolate Cg33. Plants with a mean leaf area damage of 0–17.5% (corresponding to scores 0–4) were considered to be resistant, while those with a mean leaf area damage of >17.5% (corresponding to scores 5 and 6) were considered susceptible (Mignouna et al. 2001a)

Mapping the TDa 95/00328 × TDa 87/01091 population

The map was constructed using both parental markers. Of the total of 508 markers, 494 were assigned to 20 linkage groups (LOD score 4.5), while 14 markers could not be fitted into any of these linkage groups. Markers that caused a large increase in the chi-square value were removed from the data set. Finally, 469 markers were mapped in 20 linkage groups with a total length of 1,233 cM. Chi-square values remained low when all the 469 markers were eventually mapped (Table 2). The genetic map is presented in Fig. 1. The average distance between markers was estimated at 2.62 cM.

Segregation and QTL analysis for anthracnose resistance

Although isolate Cg33 attacked both parental accessions, the severity of symptoms was slightly higher on TDa 87/01091 (disease severity score of 3.75) compared with TDa 95/00328 (severity score 2.67). None of the F_1 individuals remained symptomless. The distribution of disease scores obtained for the 176 F_1 individuals and their parents is shown as a frequency histogram (Fig. 2). Anthracnose resistance scores showed a continuous distribution from resistant to highly susceptible. The distribution of the disease severity rating deviated from normality in the F_1 population and was close to bimodal. The Shapiro-Wilks test for normality rejected the hypothesis of normal distribution (Fig. 2). After inoculation of the 176 F_1 progeny with isolate Cg33, 113 resistant and 63 susceptible genotypes could be identified. The observed segregation pattern fits almost perfectly a 5:3 (R:S) ratio ($\chi^2 = 0.110$, $P = 0.741$), which might be expected from the segregation of two independent R genes (Ra and Rb) in the progeny and a dominant suppressor of the Ra gene (Ordonez et al. 1997).

The non-parametric method of QTL mapping was used since the population was considered to behave like a diploid cross-pollinator and the phase type of the markers was not known. The most-significant QTL with effect on anthracnose resistance (associated with marker E14/M52-307) was identified on linkage group 2. Three regions on linkage group 12 could be identified but at a much lower significance level ($P = 0.05$). These three additional regions with effect on anthracnose resistance could not be declared as valid QTLs from the stringent threshold set in this study in order to avoid Type-I errors (Dudley 1993). The results of single-marker regression analysis of LAI values were similar to those obtained by the Kruskall Wallis rank-sum test.

Discussion

Density and distribution of *Pst*I/*Mse*I versus *Eco*RI/*Mse*I markers

A comparison of the *Eco*RI/*Mse*I and the *Pst*I/*Mse*I data sets showed that the latter contained fewer markers per primer combination. Also, while the overall distribution of the AFLP markers appeared satisfactory some E/M markers clustered in specific chromosomal regions. Clustering of E/M markers was also observed in *D. rotundata* (Mignouna et al. 2002a). If the rather low level of heterozygosity observed in *D. rotundata* (Zoundjihékpon et al. 1994) also applies to *D. alata*, that may explain the relatively low level of polymorphic markers detected by *Pst*I.

Genome structure of water yam

It was previously unknown whether the yam parental lines TDa 95/00328 and TDa 87/01091 were auto- or allo-tetraploid. Segregation of AFLP markers in this study supported a disomic inheritance that revealed an allo-tetraploid structure for *D. alata*. These results confirm the possible allo-tetraploid origin of water yam, although there are no known diploid wild relatives of water yam. It is believed that water yam is a true cultigen, derived from wild species through domestication, although there is no data to support this claim (Hahn 1995). Barrau (1965) postulated that *D. alata* might have been domesticated in Indo-China from two ancestral species, *Dioscorea hamiltonii* and *Dioscorea persimilis*.

Using flow cytometry and chromosome counting, Gamiette et al. (1999) found three levels of ploidy in *D. alata* germplasm (4*x*, 6*x*, 8*x*) and concluded that these species are allotetraploid; however, they did not identify any diploid varieties. The allopolyploid nature of *D. alata* as revealed in this study is also supported by the observation of 20 bivalents during the meiosis of a tetraploid *D. alata* (Ramachandran 1968). The number of

linkage groups reported in the present study is exactly the expected number of 20 (assuming disomic inheritance), which corresponds to the 20 gametic chromosomes of diploid (2n = 2*x* = 20) yam species such as *D. tokoro* and *Dioscorea gracillima*.

Since the diploid ancestors are not known, and following the hypothesis of Hahn (1995) for *D. rotundata*, one could also hypothesize that *D. alata* has a genome constitution of HHPP. This might have resulted from hybridisation between a putative wild diploid with a genome constitution HH (*D. hamiltonii*) and another one with a genome constitution PP (*D. persimilis*) to produce a diploid hybrid with the genome constitution HP. Subsequently, the hybrid chromosome number was doubled under natural conditions to produce the wild form *D. alata* (HHPP), an allotetrapoid, which was then domesticated thousands of years ago in Indo-China and/or Papua New Guinea and Solomon Island.

The total tetraploid map length of 1,233 cM covered at least 77% of the yam genome based on a known total diploid *D. tokoro* map length of 800 cM (Terauchi and Kahl 1999). If the yam genome is estimated to be 1,894 cM, as inferred from the AFLP markers from this study, the 494 loci of the current map covered 1,233/1,894 cM (about 65% of the yam genome). These estimates are the minimum genome coverage, and as more markers are added to the linkage map the unmapped markers from the present study could be fitted in the genetic linkage map. Considering the haploid nuclear DNA content of *D. alata* to be 550 Mbp/1C the physical distance per map unit could be estimated as 550 Mpb/1,894 cM = 290 kb per cM, making map-based gene cloning a feasible strategy in water yam.

Use of the genetic linkage map in marker-assisted selection

Segregation of the F_1 progeny towards a resistance response, the continuous distribution of anthracnose resistance scores, and the observed 5:3 resistant:susceptible segregation ratio confirmed earlier studies, which showed that resistance to yam anthracnose is dominantly but quantitatively inherited (Mignouna et al. 2001a).

We detected one AFLP marker associated with a QTL that explained 10% of the phenotypic variance in the F_1 population, indicating the likely presence of a minor QTL. This would seem to be at variance with the mostly major dominant nature of resistance in water yam to anthracnose (Mignouna et al. 2001a). Specific inferences are limited, however, to the population examined and the single environment in which resistance screening was conducted (Staub et al. 1996). The evidence produced so far is based on a segregating F_1 population derived from medium resistant or susceptible parental genotypes. Future genetic analysis from crosses between highly resistant and susceptible parents may detect QTLs with larger effects (Crouzillat et al. 2000). It is likely that, if different strains of *C. gloeosporioides* were used to screen the mapping population, additional regions of the yam genome involved in anthracnose resistance would have been identified. The stringent threshold chosen (P = 0.005) while reducing the chance of Type-I errors (false positive), may have also led to a higher frequency of Type-II errors (not detecting valid QTLs) (Dudley 1993).

Resistance in *D. alata* to anthracnose may be strain-specific or non-specific (Mignouna et al. 2001a). In the present study, only one *C. gloeosporioides* strain was evaluated; further QTL mapping involving more strains of *C. gloeosporioides* will be necessary to see if the QTL detected in this study is strain-specific. QTL studies in late blight of potato (Leonards-Schippers et al. 1994), rice blast (Wang et al. 1994) and anthracnose of common bean (Geffroy et al. 2000) have indicated the presence of strain-specific components in partial resistance. For instance, Geffroy et al. (2000) used two strains of *Colletotrichum lindemuthianum* and found that eight of ten QTLs detected displayed isolate specificity. QTLs specific for resistance in leaves, stems and petioles of common bean to anthracnose were also detected (Geffroy et al. 2000). *C. gloeosporioides* attacks the vines, petioles and leaves of water yam (Winch et al. 1984). Methods of assessing quantitative resistance in vines and petioles are urgently needed, in addition to the current leaf-based assays (Green et al. 2000; Mignouna et al. 2001a), to allow detailed genetic analysis of the components of anthracnose resistance.

This study has proven that AFLP markers are adequate for map construction and QTL analysis in *D alata*, which agrees with the findings of a recent study on *D. rotundata* (Mignouna et al. 2002a). The present molecular map of *D. alata*, though incomplete, opens new avenues for marker-based selection in the breeding of this important tropical staple food crop. Additional markers (SSRs, RFLPs, AFLPs, etc.) will ensure a better coverage of the yam genome in preparation for marker-assisted selection. The identification of genes involved in anthracnose resistance can be used to understand the molecular and genetic bases of *D. alata* disease resistance. Such a strategy could be greatly facilitated by the candidate gene approach (Geffroy et al. 1998; Pflieger et al. 1999).

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