RESEARCH ARTICLE

The first genetic linkage map of *Eucommia ulmoides*

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Abstract

In accordance with pseudo-testcross strategy, the first genetic linkage map of *Eucommia ulmoides* Oliv. was constructed by an F_1 population of 122 plants using amplified fragment length polymorphism (AFLP) markers. A total of 22 AFLP primer combinations generated 363 polymorphic markers. We selected 289 markers segregating as 1:1 and used them for constructing the parent-specific linkage maps. Among the candidate markers, 127 markers were placed on the maternal map LF and 108 markers on the paternal map Q1. The maternal map LF spanned 1116.1 cM in 14 linkage groups with a mean map distance of 8.78 cM; the paternal map Q1 spanned 929.6 cM in 12 linkage groups with an average spacing of 8.61 cM. The estimated coverage of the genome through two methods was 78.5 and 73.9% for LF, and 76.8 and 71.2% for Q1, respectively. This map is the first linkage map of *E. ulmoides* and provides a basis for mapping quantitative-trait loci and breeding applications.

[Wang D., Li Y., Li L., Wei Y. and Li Z. 2014 The first genetic linkage map of *Eucommia ulmoides*. *J. Genet*. **93**, 13–20]

Introduction

Eucommia ulmoides Oliv., also called Du-Zhong, is the single extant species of the genus *Eucommia* (Eucommiaceae), an economically important tree endemic to China. The bark of *Eucommia* has long been used as an antihypertensive medicine (Kawasaki and Uezono 2000). Its leaves and bark are rich in polyphenolics, flavonoids and triterpenoids. Extracts from the barks and leaves have several pharmacological actions such as antifungal (Xiang *et al.* 2004), inhibiting adipogenic differentiation (Lee *et al.* 2004), antihypertensive (Kwan *et al.* 2004), antioxidation (Hsieh and Yen 2000), low-density lipoprotein oxidative modification (Yen and Hsieh 2002), and antiinflammatory. Gutta-percha is an industrial gum obtained from the seeds, barks and leaves of *E. ulmoides* (Tangparkdee *et al.* 1997).

The prospects of exploitation and utilization are very extensive, but the breeding programmes of *E. ulmoides* are constrained by its narrow genetic base. Therefore, in the near future, genetic improvement will be one of the most important subjects in *E. ulmoides* research. To ultimately selectively improve *Eucommia*, a coordinated breeding scheme that includes phenotypic selection, family selection and marker-assisted selection based on quantitative trait loci (QTL) needs to be implemented. A key step in such a genetic improvement scheme is genetic mapping.

Genetic maps are essential tools for genomic studies, and high-density genetic maps are prerequisite for marker-aided selection to improve the selection efficiency and to accelerate breeding process. This is particularly significant for long generation species, such as forest trees. Genetic linkage mapping has become a necessary tool for genome analysis and molecular genetic breeding in forest trees (Grattapaglia and Sederoff 1994). In the last decade, genetic linkage maps have been constructed for many commercial forest tree species, including *Pinus* (Yin *et al.* 2003; Zhou *et al.* 2003), *Populus* (Cervera *et al.* 2001; Yin *et al.* 2002, 2004), *Eucalyptus* (Thamarus *et al.* 2002; Freeman *et al.* 2006), rubber tree (Lespinasse *et al.* 2000a), cacao (Pugh *et al.* 2004), white birches (Jiang *et al.* 2011). These genetic linkage maps facilitate the analysis of QTL that control commercially important traits such as disease resistance (Lespinasse *et al.* 2000b; Junghaus *et al.* 2003; Devey *et al.* 2004; Butcher 2004), wood quality (Kumar *et al.* 2000; Brown *et al.* 2003; Thamarus *et al.* 2004), growth (Weng *et al.* 2002), leaf oil composition (Shepherd *et al.* 1999), vegetative propagation (Marques *et al.* 1999, 2002), frost tolerance (Hurme *et al.* 2000), and drought tolerance (Rönnberg-Wästljung *et al.* 2005).

In this study, we constructed the first genetic linkage map for *E. ulmoides* based on 122 F_1 progeny from the cross between clone LF and variety Q1 using AFLP markers and pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994). The availability of the genetic map can

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Keywords. linkage map; AFLP; pseudo-testcross; *Eucommia ulmoides*.

provide more genomic information on *Eucommia* and will be used for the detection of loci contributing to quantitative-trait expression.

Materials and methods

Development of mapping population

Using 12 selected varieties (clones) as the hybrid parents, a total of 35 crossing combinations were hybridized according to the analysis of mating design. Based on the variation analysis of field phenotypic characteristics and detecting the levels of polymorphisms between parents using AFLPs, higher genetic variability was found in clone LF (maternal, originating from Lingbao of Henan province) and variety Q1 (paternal, originating from Yangling of Shaanxi province) of *E. ulmoides*. These two trees have been selected as the parent for permanent mapping population for the construction of the first genetic linkage map in *E. ulmoides* Oliv. (Li *et al.* 2012). LF had characteristics of strong adaptability and early frutification, high and stable yield with high gutta-percha content. Q1 had characteristics of high gutta-percha and medicinal components content, strong drought resistance and fast growing.

Controlled pollinations between the two parental lines were carried out in April 2009 at the farm of TianDi Science and Technology Ltd in Henan province, China. Generated hybrid seeds were planted in the greenhouse and the developed young seedlings were transplanted into field at the farm of Northwest A & F University in the spring of 2010. One hundred and twenty-two F_1 individuals were used as the mapping population in the study.

DNA extraction

Genomic DNA was isolated from the leaves of the parents and from each individual tree according to CTAB method (Wang *et al.* 2010). All genomic DNA were kept frozen at −20°C for digestion and subsequent analysis of AFLPs.

AFLP analysis

Genomic DNA (500 ng) from two parents and 122 individuals was digested for 6 h at 37°C with 10 U *Eco*RI (New England BioLabs, Ipswich, USA), 10 U *Mse*I (New England BioLabs), 2.5 μL NEBuffer 4, 0.25 μL 100× BSA, in a 25 μL reaction volume adjusted with distilled water. In the same tube, 10 μL ligation mixture was added consisting of 1 μL *Eco*RI adapter (5 pmol/μL), 1 μL *Mse*I adapter (50 pmol/μL), 3.5 μL T_4 DNA ligase buffer, 400 U T_4 DNA ligase (New England BioLabs) and 3.5 μL distilled water. The reaction was incubated for 3 h at 16°C and stopped by heating at 65°C for 10 min. The ligated DNA was stored at −20°C until preamplification.

For preamplification, a mix was prepared composed of 1 μL of ligated DNA, 2.5 μL 10× *Taq* DNA polymerase buffer, 0.5 μL of 2 mM dNTPs (Sangon, China), 0.6 μL *Eco*RI preamplification primer (50 ng/μL), 0.6 μL *Mse*I preamplification primer (50 ng/μL), 1.5 μL of 25 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Fermentas, Ottawa, USA) and distilled water to a volume of 25 μL. The following cycling parameters were used for preamplification: 30 cycles of 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C and 5 min 72°C for final extension. The PCR (polymerase chain reaction) products were stored at −20°C until selective amplification.

A total of 64 *Eco*R1/*Mse*1 primer combinations were used for the selective amplification (table 1). The components for each 25 μL amplification reaction were 1 μL diluted (1:30) preamplified products, 2.5 μL 10× *Taq* DNA polymerase buffer, 0.5 μL of 2 mM dNTPs (Sangon), 1 μL of *Eco*RI + 3 primer (50 ng/μL), 1 μL *Mse*I + 3 primer (50 ng/μL), 1.5 μL of 25 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Fermentas) and distilled water. The AFLP products were amplified using touchdown PCR. An initial denaturation consisted of 1 cycle of 30 s at 94°C, 30 s at 65°C and 60 s at 72°C. The annealing temperature was then lowered by 0.7°C per cycle during the first 13 cycles and then 23 cycles were performed at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. Finally, selective amplification products were separated on 6% denaturing polyacrylamide gel and visualized by silver nitrate staining (Wang *et al.* 2011).

Scoring of AFLP markers and genetic linkage map construction

The polymorphic markers were analysed by a chi-square test for goodness of fit to the expected Mendelian segregation ratio of a dominant locus in an F_1 population ($P < 0.05$). Only the markers with 1:1 segregation were used to generate the linkage maps following the two way pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994).

Two parental linkage maps were constructed with JoinMap 3.0 (Ooijen and Voorrips 2001). Markers were placed into linkage groups with the 'LOD groupings' command using the Kosambi map function (Vinod 2011). Calculation parameters were set for a minimum LOD threshold of 5.0. Map construction was performed using the Kosambi mapping function with JoinMap parameter settings as follows: Rec = 0.3, LOD = 5.0 and Jump = 5. Map distances were calculated with the Kosambi mapping algorithm. AFLP markers were designated with the primer combination code followed by the fragment size in base pairs (e.g. E1M5-180).

Genome lengths and map coverages

The observed genome length G_o was estimated according to the method of Nelson *et al.* (1994), which takes all markers into account. The expected genome length G_e was estimated under the assumption of random marker distribution described by Hulbert *et al.* (1988) and Chakravarti *et al.* (1991). The observed map coverage C_o was evaluated in two ways: the ratio of the observed genome length G_o

Table 1. Adapter and primer sequences used in the AFLP analysis of *E. ulmoides* Oliv.

Restriction enzyme	*Mse*I	*Eco*RI
Adapters	5′-GACGATGAGTCCTGAG-3′	5′-CTCGTAGACTGCGTACC-3′
	3′-TACTCAGGACTCAT-5′	3′-CTGACGCATGGTTAA-5′
Preamplification primers	5′-GATGAGTCCTGAGTAAC-3′	5′-GACTGCGTACCAATTCA-3′
Selective amplification primers	M1:M-CAA:5′-GATGAGTCCTGAGTAACAA-3′	E1:E-AAC:5′-GACTGCGTACCAATTCAAC-3′
	M2:M-CAC:5′-GATGAGTCCTGAGTAACAC-3′	E2:E-AAG:5′-GACTGCGTACCAATTCAAG-3′
	M3:M-CAG:5′-GATGAGTCCTGAGTAACAG-3′	E3:E-ACA:5′-GACTGCGTACCAATTCACA-3′
	M4:M-CAT: 5′-GATGAGTCCTGAGTAACAT-3′	E4:E-ACT: 5′-GACTGCGTACCAATTCACT-3′
	M5:M-CTA:5′-GATGAGTCCTGAGTAACTA-3′	E5:E-ACC: 5′-GACTGCGTACCAATTCACC-3′
	M6:M-CTC: 5′-GATGAGTCCTGAGTAACTC-3′	E6:E-ACG:5′-GACTGCGTACCAATTCACG-3′
	M7:M-CTG:5′-GATGAGTCCTGAGTAACTG-3′	E7:E-AGC: 5′-GACTGCGTACCAATTCAGC-3′
	M8:M-CTT: 5′-GATGAGTCCTGAGTAACTT-3′	E8:E-AGG:5′-GACTGCGTACCAATTCAGG-3′

on the estimated genome length G_e (Tani *et al.* 2003); or the ratio of the total length of the map G_f on the estimated genome length G_e (Cervera *et al.* 2001).

Results

Polymorphism of AFLP markers

Out of 64 *Eco*RI/*Mse*I AFLP primer combinations screened, 22 primer combinations were further used and they produced a total of 363 polymorphic markers (table 2). An average of 16.5 polymorphic markers was produced from one AFLP primer combination. Chi-square analysis indicated that 289 (79.7%) polymorphic markers gave a compatible fit to the expected 1:1 segregation ratio and 74 (20.3%) showed a significant segregation distortion from the expected 1:1 ratio (P = 0.05). Among the 289 polymorphic markers, 155 (53.6%) were segregating through female and 134 (46.4%) through male.

Construction of genetic linkage maps

Based on 289 segregating 1:1 markers, two parent-specific linkage maps were constructed using 262 markers while 27 markers were unlinked (table 3).

For the maternal LF map, a total of 127 markers linked into 14 linkage groups with four or more markers for each linkage group (figure 1; table 3), five linkage groups with two markers each, and 18 markers without a group. The 14 linkage groups ranged in length from 41.8 to 108.6 cM. The number of markers per linkage group ranged from four (LFLG3 and LFLG13) to 20 (LFLG6). The linked markers covered 1116.1 cM and the distance between two adjacent markers ranged from 0.6 to 35.2 cM with an average distance of 8.78 cM. The density of markers varied within each linkage group, LFLG6 was the most dense (4.5 cM per locus), while LFLG13 was the least dense (19.7 cM per locus) (table 3). The longest gap (35.2 cM) was between markers E7M6-420 and E8M5-470 on LFLG11 and three pairs of markers were found to completely cosegregate on LFLG1, LFLG6 and LFLG12, respectively.

For the paternal Q1 map, there were 108 markers linked into 12 linkage groups with four or more markers for each linkage group (figure 2; table 3). One linkage group had three markers; seven linkage groups had two markers each; and nine markers could not be placed in any group. The length of each group varied from 34.7 to 114.8 cM, and the number of markers per linkage group ranged from four (Q1LG4 and Q1LG5) to 18 (Q1LG2). The paternal map Q1 covered 929.6 cM, and ranged from 0.8 to 22.6 cM with an average of 8.61 cM between adjacent markers. Among 12 linkage groups, Q1LG2 was the most dense (5.4 cM per locus) while Q1LG4 and Q1LG12 were least dense (11.4 cM per locus) (table 3). The longest gap (22.6 cM) was between markers E2M5-350 and E2M6-410 on Q1LG8, and two pairs of markers were found to completely cosegregate on Q1LG1 and Q1LG3.

Genome length and coverage

The total lengths (G_f) of the maternal (LF) and paternal (Q1) maps were 1116.1 and 929.6 cM, respectively (table 4). According to the method of Nelson *et al.* (1994), the observed genome length G_o of all the linkage groups detected in this study were 1185 and 997 cM. The expected genome lengths G_e were 1511 and 1299 cM with the assumption of random marker distribution. On the basis of the expected genome lengths, genome coverage for the LF and Q1 framework maps had been estimated. The observed map coverage C_o through the two methods described by Tani *et al.* (2003) and Cervera *et al.* (2001) were 78.5 and 73.9% for LF and 76.8 and 71.2% for Q1, respectively (table 4).

Discussion

AFLP markers have the potential to efficiently and rapidly construct high-resolution maps and to identify and isolate those markers that are tightly linked without the requirement of any prior knowledge of sequence information (Beedanagari *et al.* 2005; Ukrainetz *et al.* 2008). The high level of DNA polymorphism detected by AFLP technique

Table 2. Number of AFLP amplification products generated with different primer combinations.

Primer combinations	*Eco*RI+3	*Mse*I+3	Number of polymorphic markers	Range of marker size	Number of markers segregating as 1:1	Number of markers showing segregation ratio distortion
E1M1	E-AAC	M-CAA	07	250–550	7	0
E1M2	E-AAC	M-CAC	16	160–610	11	5
E1M5	E-AAC	M-CTA	19	39–590	16	3
E1M8	E-AAC	M-CTT	15	100–530	13	2
E2M5	E-AAG	M-CTA	16	120–650	11	5
E2M6	E-AAG	M-CTC	15	80–600	13	2
E3M4	E-ACA	M-CAT	18	80–700	16	2
E3M6	E-ACA	M-CTC	14	220–630	8	6
E3M8	E-ACA	M-CTT	19	60–500	15	4
E4M2	E-ACT	M-CAC	12	50–500	12	0
E4M4	E-ACT	M-CAT	20	70–480	14	6
E4M8	E-ACT	M-CTT	15	140–700	10	5
E5M4	E-ACC	M-CAT	20	90–700	18	2
E5M6	E-ACC	M-CTC	15	50–500	12	3
E6M4	E-ACG	M-CAT	17	50–510	15	2
E6M6	E-ACG	M-CTC	8	80–470	8	0
E7M4	E-AGC	M-CAT	25	60–600	15	10
E7M6	E-AGC	M-CTC	16	50–610	16	0
E7M8	E-AGC	M-CTT	24	90–580	14	10
E8M4	E-AGG	M-CAT	16	49–560	16	0
E8M5	E-AGG	M-CTA	13	20–520	13	0
E8M8	E-AGG	M-CTT	23	60–550	16	7
Total			363		289	74
Per cent (%)			100		79.7	20.3

in *Eucommia* allowed us to construct its genetic linkage map using F_1 progeny. In this paper, the maternal and paternal genetic linkage maps in *E. ulmoides* were constructed based on AFLP marker and pseudo-testcross strategy. To our knowledge, this is the first report of a genetic linkage map in the genus *Eucommia*.

Genetic linkage maps in plants are usually constructed by using segregating populations obtained from crosses between inbred lines. Such populations are generally not available for forest trees because of the long generation constraints (Grattapaglia and Sederoff 1994). We have not been able to generate F_2 hybrid or backcross family in *E. ulmoides* in the short term as yet, so a F_1 mapping population was created instead of the backcross F_2 progenies in the crop to avoid inbreeding depression of long growth-cycle trees. The pseudo-testcross strategy has been successfully applied to various tree species such as apple (Maliepaard *et al.* 1998), cashew (Cavalcanti and Wilkinson 2007), eucalyptus

Table 3. Summary of the linkage maps of LF and Q1.

	LF				Q1			
Linkage group	Marker number	Max. (min) distance (cM)	Total distance (cM)	Average distance (cM)	Marker number	Max. (min) distance (cM)	Total distance (cM)	Average distance (cM)
1	13	30.8 (0.7)	108.57	8.3	11	13.7 (1.8)	92.52	8.4
2	15	15.6 (2.7)	102.44	6.8	18	16.1 (1.6)	97.65	5.4
3	4	18.1 (6.6)	41.73	10.2	16	20.3 (0.8)	92.68	5.7
4	5	17.3 (4.8)	54.28	10.8	4	21.6 (9.5)	45.73	11.4
5	11	18.5(2.1)	94.31	8.5	4	13.5 (9.1)	34.75	8.7
6	20	10.7 (1.7)	90.36	4.5	6	15.5 (4.6)	57.13	9.5
7	7	19.8 (10.5)	88.93	12.7	8	20.2 (2.9)	87.21	10.9
8	6	21.4 (2.6)	55.81	9.3	12	22.6 (0.8)	114.82	9.6
9	8	15.6(1.8)	68.37	8.5	7	15.3 (4.5)	69.61	9.9
10	11	16.1(2.2)	97.35	8.8	5	18.6 (1.6)	49.71	9.9
11	5	35.2 (0.9)	67.03	13.4	9	17.4 (2.5)	96.61	10.7
12	10	27.6 (0.5)	80.90	8.1	8	18.1 (4.7)	91.15	11.4
13	4	28.7 (21.5)	78.68	19.7				
14	8	23.3 (2.7)	87.32	10.8				
Total	127		1116.08		108		929.57	
Average	9.1			8.78	9			8.61

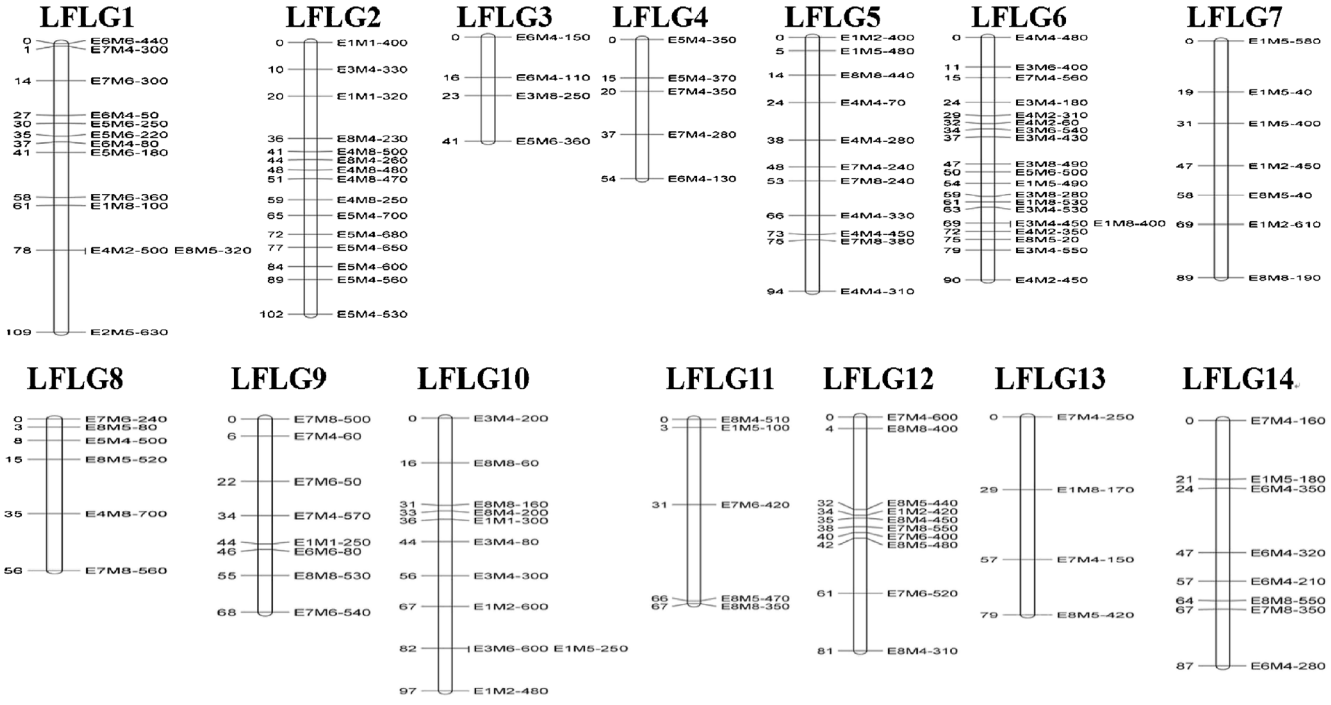

Figure 1. Maternal genetic linkage maps of LF based on 14 linkage groups. The map was constructed based on AFLP markers, obtained through the analysis of 122 F_1 progeny generated from the cross between the parental lines. The Kosambi map distances (cM) and marker names are indicated on left and right sides, respectively of each linkage group. The numbers in each marker name represent the code used in our lab for primer combination along with size of the band.

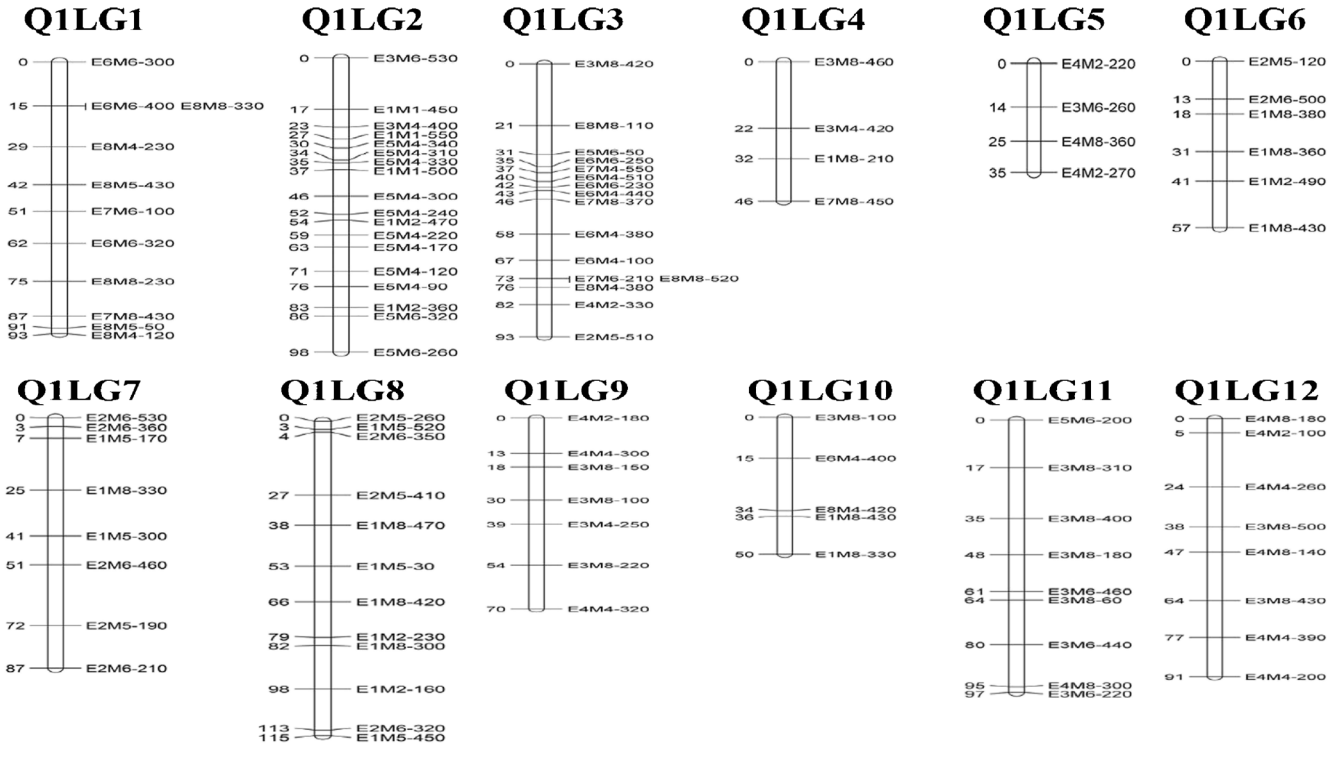

Figure 2. Paternal genetic linkage maps of Q1 based on 12 linkage groups. The map was constructed based on AFLP markers, obtained through the analysis of 122 F_1 progeny generated from the cross between the parental lines. The Kosambi map distances (cM) and marker names are indicated on left and right sides, respectively of each linkage group. The numbers in each marker name represent the code used in our lab for primer combination along with size of the band.

Table 4. Summary of the length and genome coverage of the linkage maps

Item	LF	Q1
Linkage map length (G_f)	1116 cM	930 cM
The observed genome length (G_o)	1185 cM	997 cM
The expected genome length (G_e)	1511 cM	1299 cM
The observed map coverage $(C_o)^C = G_{f/}G_e$	73.9%	71.2%
The observed map coverage $(C_o)^T = G_{o/}G_e$	78.5%	76.8%

(Verhaegen and Plomion 1996), oak (Barreneche *et al.* 1998), larch (Arcade *et al.* 2000) and white birches (Jiang *et al.* 2007). In each case, marker data were analysed as a pseudo-testcross and a map was constructed separately for each parent.

We observed that a total of 22 primer combinations generated 363 polymorphic markers in *E. ulmoides* (16.5 per primer combination). Of the 363 segregating loci scored, 74 loci (20.3%) showed distorted segregation at the $P < 0.05$ level. Segregation distortion can be caused by many factors including low progeny population size, statistical bias, genotyping and scoring errors (Plomion *et al.* 1995) or biological reasons such as genetic drive, chromosome loss, viability per lethal genes, genetic isolation mechanisms and genetic load (Bradshaw and Stettler 1994; Liebhard *et al.* 2003). The percentage of significantly distorted markers we detected (20.3%) was similar to that observed in linkage analyses of willow (18%; Hanley *et al.* 2002), *Betula platyphylla* (20%; Wei *et al.* 2010) and *Pinus koraiensis* (25%; Chen *et al.* 2010). Because the order of markers on linkage groups may be affected by segregation distortion (Lorieux *et al.* 1995), we decided to eliminate the distorted markers for linkage map construction (Venkateswarlu *et al.* 2006).

It is well known that, AFLP markers in linkage analysis tend to cluster together and the level of clustering increases with the marker number (Liu *et al.* 2003). Two female and two male linkage groups presented slight clusters in the maternal and paternal genetic linkage maps of *E. ulmoides*, respectively. In the genetic linkage maps of other tree species, including pecan (Beedanagari *et al.* 2005) and cashew (Cavalcanti and Wilkinson 2007), the AFLP markers in linkage groups also showed clustering. Nevertheless, clustered AFLP markers can reduce the usefulness of genetic linkage maps. Adding other types of markers to the maps could make the marker distribution more even.

Including all the triplets and doublets, the number of linkage groups was 19 in the maternal map and 20 in the paternal map, respectively, which was higher than the haploid chromosome number ($n = 17$) of *E. ulmoides* (Li and Xu 1986). For a complete genetic linkage map, the number of two parental linkage groups is supposed to be equal to the haploid chromosome number. It is obvious that some linkage groups belong to the same chromosome in this study, indicating that these two maps were not saturated. Filling the gaps on the linkage groups will require a larger mapping population and more markers, either dominant such as AFLP or codominant such as SSR, should be placed on this map, which could ultimately make the number of linkage groups correspond to the chromosome number.

The mean map distance between markers is an important factor for the detection of quantitative loci associated with a trait of interest (Tanksley *et al.* 1992). In this paper, the average distance between two adjacent markers is 8.78 and 8.61 cM on the female and male maps, respectively. In comparison with other maps, these averages represent good marker coverage within the portion of the genome spanned by both maps. For example, in other tree species the average distance between markers was found to be 8.7 and 9.0 cM in European chestnut (Casasoli *et al.* 2001), 15.7 and 17.4 cM in *Pinus* (Shepherd *et al.* 2003), 7.8 and 8.0 cM in willow (Hanley *et al.* 2002), 6.7 and 10.8 cM in eucalyptus (Myburg *et al.* 2003), 14.3 and 14.1 cM in birch (Jiang *et al.* 2011). According to the method of Chakravarti *et al.* (1991), the result showing that the female and male maps provide 73.9 and 71.2% coverage of the genome, respectively. Although these figures leave some scope for QTLs falling outside the mapped region of the genome, they nevertheless provide sufficient coverage to have utility for preliminary genetic characterization for traits of agronomic interest. It is still necessary, however, to construct a highly saturated genetic linkage map for accurate QTL mapping.

In the present study, a preliminary genetic linkage map was constructed in *E. ulmoides*, a species that was relatively lacking the genetic information. The genetic map will prove useful in QTL analysis, molecular marker assisted selection, and map-based cloning.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30771751).We would like to thank Mr. Pengtao Gong (Hainan Institute of Tropical Agricultural Resources, Sanya, Hainan Province, China) for instructing on use of map software.

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Received 20 August 2013, accepted 3 September 2013
Published on the Web: 26 February 2014