RESEARCH NOTE

Quantitative trait loci mapping for stomatal traits in interspecific hybrids of *Eucalyptus*

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Introduction

Eucalyptus is grown in tropics mostly for pulpwood production. Globally, more than 21Mha of eucalypt plantations are available (Midgley 2013) and produce 17.5% of world's paper pulp (Hart and Santos 2015). Next to Brazil, India occupies the second position in growing eucalypts with more than 4.4Mha plantations (Xie 2015). The major species grown in India are *E. camaldulensis* and *E. tereticornis*, due to their adaptability to drought and suitability for paper pulp production. *E. grandis* is a subtropical species targeted for the breeding programs worldwide because of its high pulp productivity. It is closely related to *E. tereticornis* and production of interspecific hybrids displaying hybrid vigour in terms of pulp yield and drought tolerance is possible (Madhibha *et al*. 2013). Despite of India being a major grower of eucalypts, studies on hybrid breeding and applied molecular genetics are very limited.

Physiological traits, important for plant growth responses, are often quantitative, suggesting that they are influenced by multiple genes. Patterning of stomata and their function in angiosperms are considered key determinants of growth rate and water balance in plants. This requires a better understanding of molecular mechanisms involved in stomatal distribution. Stomatal parameters like density and pore size have been reported to be correlated to rate of photosynthesis and transpiration, drought, salinity, yield, elevated CO_2 in woody and crop species (Gailing *et al.* 2008; Liu *et al.* 2017). Considerable progress has been made in detection of stomatal traits related quantitative trait loci (QTL) for salt and drought tolerance in crop species (Liu *et al.* 2017; Shahinnia *et al.* 2016). In eucalypts, genetic mapping and QTL analysis for various traits including growth and form, wood properties, adventitious rooting, flowering time, biotic and abiotic stresses and secondary metabolism were reported (Henry and Kole 2014). Currently, only limited information is available on QTL governing stomatal characteristics and such QTL can pave way for production of high yielding trees with abiotic stress tolerance, especially when combined with QTL for growth and wood properties**.** Hence, the objectives of the study were to develop a consensus linkage map for the interspecific cross *E. tereticornis* and *E. grandis* using simple sequence repeats (SSRs), inter simple sequence repeats (ISSRs) and sequence related amplified polymorphism (SRAP) markers and identify QTL associated with stomatal traits.

Material and Methods

Plant materials

The mapping population used in this study consisted of the parents of the cross, *Eucalyptus tereticornis* (Et86) × *Eucalyptus grandis* (Eg9) and their F1 individuals. Et86 is a selection from a seed production area (Pudukottai, 10º 23' N, 78º 51'E), while Eg9 is a selection from the provenance trail (13017, Lorne) located at Osamund (11º 26' N, 76º 37'E). Controlled pollination experiments were carried out during May 2010 and F1 were field planted in 2011 at Panampally (10°47'N; 76°45'E), Kerala.

Marker genotyping

Two parents (Et86 and Eg9) and 98 F1 individuals were genotyped with 114 SSR loci (Supplementary Table 1), 13 ISSR primers (Supplementary Table 2) and 13 SRAP primer combinations (Supplementary Table 3)'.

PCR amplifications and electrophoresis for SSR, ISSR and SRAP markers were carried out as reported by Arumugasundaram *et al.* (2012), Balasaravanan *et al.* (2005) and Li and Quiros (2001), respectively.

Generation of genetic linkage maps

ISSR and SRAP markers were named after the primer serial number and the approximate fragment size. Chi-square (χ^2) test was performed and markers that deviated from the theoretical expected ratios were considered as distorted with significance level ($P < 0.01$, $P < 0.001$, and $P < 0.0001$) and used for analysis. Linkage analysis was performed using JoinMap 4.0 software (Van Ooijen 2011) with cross pollinator (CP) option. Linkage phase determination and grouping was done independently for the parental data sets. Linkage group (LG) was estimated by applying independence logarithm of odds (LOD) ranges from 1 to 10. The SSR markers were assigned to the LG based on the information in published literature (Grattapaglia *et al.* 2015). Marker ordering was performed with regression mapping using the standard parameters and markers mapped in first and second round were included and maps were generated with Mapchart 2.1 software (Voorrips 2002). The parent specific maps were integrated using the "Combine groups for map integration" function of JoinMap to produce consensus map using common SSR markers.

Stomatal morphology measurements and data analysis

Leaves of coppice shoots from the parents and hybrid individuals were selected for stomatal morphology studies. Stomatal observations were carried out in the 3rd and 4th fully expanded leaves on 30th day after coppicing the plants following the methodology described by James and Bell (2000). From each plant, two leaves were smeared with a thin film of nail varnish at the center of the leaf blade on the abaxial and adaxial surface to make an imprint. Stomatal density per unit area (number of stomata per mm^2) was determined at 400X magnification from 10 field views per slide using photo microscope (Leitz, Japan). Images of the stomata were taken using an ultrascope fitted with microscope and stomatal length (SL), width (SW) and pore length (PL) were measured using Scope Image 9.0. SL, SW and PL values were measured in micrometer (μm) from abaxial and adaxial leaf surfaces.

Stomatal area (μm^2) was calculated using the following formula (Wang *et al.* 2012)

$$\frac{SL \times SW \times \pi}{4}$$

Descriptive statistics and one way analysis of variance (ANOVA) was used to analyse the variation in stomatal traits between the parents and F1 individuals using the SPSS v 22.0 software (IBM Corporation, Armonk, NY).

Quantitative trait loci (QTL) analysis

QTL analysis was carried out using composite interval mapping (CIM) procedure in WinQTL Cartographer v 2.5 (Wang *et al.* 2007) to identify QTLs for stomatal density, stomatal area and pore length in adaxial and abaxial leaf surfaces adopting backcross model. The LOD threshold was determined by permutation analysis with 1000 repetitions. The size of the analysis window was maintained at 10 cM with a walk speed (mapping resolution) of 1 cM and control marker number five. Stepwise regression (forward method) under model 6 and the LOD score corresponding to P= 0.05 was used to identify significant QTL.

Results

Marker production and linkage map generation

A total of 114, 115 and 129 SSR, ISSR and SRAP markers were generated respectively and the details of SSR segregation pattern, ISSR and SRAP polymorphism status are given in the supplementary table 1, 2 and 3. These markers were used for framework map construction in female and male parents separately at LOD 3.0, where 11 groups, equivalent to the haploid

chromosome number were formed. The details on marker types, segregation pattern, parent informativeness and chi square significance are presented in supplementary table 4. Details on number of markers mapped, segregation deviation (SD) markers for SSR, ISSR and SRAP of parental maps are given in the supplementary table 5 and 6. The female map had 204 markers covering 1023.56 cM (supplementary table 5; supplementary figure 1). The male map had 172 markers with 1046.64cM length (supplementary table 6; supplementary figure 2). The consensus map with total length of 1049.4cM had an average of 28.55 markers per LG, with an average spacing of 3.9 cM (Table 1; Figure 1).

Phenotypic variations in stomatal traits

The univariate statistics for stomatal density, stomatal area and pore length of the Et86×Eg9 mapping population is presented in Table 2. Analysis of variance (F-test) indicated significant differences among the parents and F1 hybrids for the traits such as stomatal density and area on the adaxial and abaxial surface of the leaf. However, pore length did not show significant difference for the abaxial surface. The absolute values of skewness and kurtosis were less than one for all traits, indicating that the phenotypic values of these traits were normally distributed and suitable for QTL analysis.

QTL analysis

Phenotypic variance (PV; R^2) controlled by these QTL ranged from 11.36 to 27.3% (Table 3). No QTL were identified for the stomatal density on abaxial leaf surface. One QTL of large effect (explaining 27% of phenotypic variance) in LG 8 associated with marker locus M14E12-350 for stomatal pore length at abaxial surface was identified. Two SSR loci (Embra204 in LG9 and Embra2 in LG11) were associated with stomatal area at abaxial surface and the PV explained were 12 and 14% respectively. Two SRAP markers and three ISSR markers were associated with adaxial stomatal area, abaxial and adaxial stomatal pore length and stomatal density in adaxial surface of the leaves.

Discussion

Genetic linkage map

A number of linkage maps are reported in eucalypts including high density maps in interspecific crosses of *E. grandis* and *E. urophylla*, the hybrid combinations predominantly grown in Brazil, Australia and South Africa (Freeman 2014). Only very few linkage maps are available for the hybrid combinations with *E. tereticornis* as parent species. The individual

parent specific map generated in this study for *E. tereticornis* (Et86) was 1023.56cM. Yu *et al* (2012) reported a map of size 1,488cM with 21 LGs using expressed sequence tag-based cleaved amplified polymorphic sequence markers (EST-CAPS) and random amplified polymorphic DNA (RAPD) markers in *E. tereticornis*. Recently, for the same species Li *et al* (2015) generated linkage map of 1241cM length with 585 loci distributed in 11 LGs. In *E. grandis*, several genetic maps were developed using different types of DNA markers and linkage map length ranged from 925cM (Kullan *et al*. 2012) to 1216cM (Gion *et al*. 2011). The present study also observed the map length of 1046.64cM with 172 markers belonging to SSR, ISSR and SRAP. Most of the reports on *Eucalyptus* maps constructed till date reported the haploid number of chromosomes, despite of various types of DNA markers, pedigree structure and mapping software packages (Freeman 2014) and the present study also showed marker distribution on 11 LGs.

QTL mapping for stomatal traits

Size, density and pattern of leaf stomata play an important role in plant growth and yield. Genetic relationships between stomatal density and size with yield were documented in cereals (Shahinnia *et al.* 2016). In *Quercus*, based on the QTL for stomatal density, their role in adaption towards climate change was discussed (Gailing *et al.* 2008).The aim of this study was to identify the QTL regions controlling stomatal density, stomatal area and pore length on adaxial and abaxial surface of the Et86 × Eg9 mapping population. The marker loci M14E12-350 in LG8 showed co-segregation for stomatal pore length at abaxial surface with PV of 27.3%. The higher PV (>25%) recorded in this study could be attributed towards the use of smaller populations, lack of multi-environment measurements and wide physical linkage between adjacent causal loci within single crosses (Hall *et al.* 2016).

This result is a step forward in understanding the function of these loci and to correlate their role in yield and biotic and abiotic stress tolerance. The accuracy of QTL mapping depends on several factors such as density of genetic map, genetic architecture of the trait, phenotyping efforts, statistical methods and population size (Semagn *et al.* 2010). In the present study the mapping population was small sized and established only in a single environment. Nevertheless, practical applications for such QTL linked markers will require validating them in different genetic backgrounds and environments.

Stomatal parameters like density, area and pore length gains significance in eucalypts because of the variation expressed towards abiotic stress tolerance, photosynthetic and transpiration efficiency. These characteristics affect the growth and productivity of eucalypts, wherein stomata play a major role in the control of water evaporation, gas exchange and pathogen entry (Tong *et al*. 2016). Stomata size and density are under a complex genetic control and thus provide multiple levels of regulation for stomatal functions (Chaves *et al.* 2016). Therefore, a better understanding of the genetic control of stomatal parameters is an important aspect in breeding of eucalypts for productivity, abiotic stress tolerance and disease resistance (Héroult *et al.* 2013; Tong *et al*. 2016). Wider variations for stomatal density, area and pore length in Et86 × Eg9 provide opportunity to assess their influence on various physiologically adaptive characteristics of hybrid individuals. The individuals which compromise between high water use efficiency and leaf cooling capacity would be the best adapted genotypes under semiarid conditions (Chaves *et al.* 2016). Thus, correlation of stomatal traits with yield and adaptability would help to improve productivity of *Eucalyptus* plantations in arid conditions.

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Table 1: Mapping statistics for consensus map of *E. tereticornis* (Et86) × *E. grandis* (Eg9)

Linkage group	Total number of loci mapped		Common SSR markers	Total number of loci mapped in Et86 × Eg9	Size in cM	Mean distance between markers
	Et86	Eg9				
1	25	16	7	33	91.7	2.8
2	21	12	6	27	99.8	4
3	8	18	3	22	93.9	6.8
4	13	28	7	33	83.1	2.5
5	22	21	6	37	86.3	2.3
6	15	9	2	21	97.5	4.6
7	20	9	6	23	92.9	4
8	31	9	7	33	92.3	2.8
9	15	8	6	16	107.9	6.7
10	22	28	3	47	114.7	2.4
11	12	14	4	22	89.3	4.1
Total	**204**	**172**	**57**	**314**	**1049.4**	**-**
AVG	**18.55**	**15.64**	**5.18**	**28.55**	**95.40**	**3.9**

Table 2: Descriptive statistics and ANOVA for stomatal density, stomatal area and pore length of *E. tereticornis* (Et86), *E. grandis* (Eg9) and F1 individuals.

Genotype	Density (no. mm^{-2})		Stomatal area (μm^2)		Pore length (µm)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Et 86	132.00±22.9	213.00±50.8	135.19±26.6	92.26±33.3	7.53±1.2	5.22±1.0
Eg 9	92.00±14.7	168.00±20.4	204.22±34.0	133.60±20.2	7.69±1.6	6.17±1.0
F1 individuals (n=98)						
Mean	112.85	246.91	153.97	143.25	5.12	5.13
Max	169	351	244.11	238	9.17	8.85
Min	57	123	54.85	73.29	2.09	2.68
Skewness	0.19	-0.21	0.32	0.64	0.4	0.21
Kurtosis	-0.17	-0.45	0.74	0.33	0.6	-0.51
SE (±)	22.43	49.37	32.81	32.27	1.32	1.28
F-test	*	**	**	*	**	ns

**, * and ns are $p < 0.01$, $p < 0.05$ and not-significant, respectively.

Table 3: QTLs for stomatal traits mapped in Et86×Eg9 using WinQTL Cartographer v2.5 (LG-linkage group; PV(%) – phenotypic variation (R^2))

Traits	LG	Locus name	LOD	Left Marker	Right Marker	PV (%)	Additive
Stomatal density adaxial	6	ISSR3-550	4.15	ISSR22-425	M12E10-146	14.9	21.07
Stomatal area abaxial	9	EMBRA204	4.37	CD519	EMBRA1811	12.4	-23.21
Stomatal area abaxial	11	EMBRA2	3.93	M11E11-297	ISSR3-850	14.4	-24.81
Stomatal area adaxial	1	M2E9-292	3.42	EMBRA303	M1E9-388	11.4	22.85
Pore length abaxial	8	M14E12-350	11.34	ISSR27-1000	EMBRA1468	27.3	-1.69
Pore length abaxial	11	ISSR22-475	6.8	ISSR3-850	M12E13-194	14.8	1.39
Pore length adaxial	10	ISSR27-675	7.37	M11E11-200	ISSR11-900	24	-1.51

Figure 1: Consensus linkage map of the cross *Eucalyptus tereticornis* (Et86) and *E. grandis* (Eg9) showing QTL regions for stomatal traits. Distances among markers are indicated in cM to the left of the linkage groups; Markers in red, blue and black colors are SSR, ISSR and SRAP markers respectively and markers with underline are common between maternal and paternal maps. QTL for the traits analysed are depicted as colored vertical bars to the right of the linkage groups 1 (stomatal area adaxial), 6 (stomatal density adaxial), 8 (pore length abaxial), 9 (stomatal area abaxial), 10 (pore length adaxial) and 11 (pore length abaxial).