Positioning of Sex-Correlated Markers for *Populus* in a AFLP- and SSR-Marker Based Genetic Map of *Populus tremula* x *tremuloides*

By T. MARKUSSEN[1)], B. PAKULL and M. FLADUNG[1)]

Federal Research Centre for Forestry and Forest Products (BFH)
Institute for Forest Genetics and Forest Tree Breeding, Sieker Landstrasse 2, D-22927 Grosshansdorf, Germany

(Received 2nd June 2006)

Abstract

A preliminary consensus map of *Populus tremula* x *tremuloides* has been constructed from an interspecific hybrid population of 66 seedlings of the cross Brauna 11 (*P. tremula*) x Turresson 141 (*P. tremuloides*). The map was constructed based on 205 AFLP- and 29 SSR-markers covering 1875cM on 19 linkage groups. A single locus correlating to sex in *Populus* was mapped close to two AFLP-markers. The map will be used as a starting point for the identification of sex-related genes or molecular markers and their fine mapping based on a BAC-library screening.

Key words: Populus, sex-related marker, Linkage map, AFLP, SCAR

Introduction

In the last 20 years a high number of genetically modified (transgenic) trees have been produced in line with the development of gene transfer methods in the different tree species. The genes transferred are coding for important features like herbicide and insect resistance and growth parameters, and in particular for wood characteristics, e.g. alteration of lignin and cellulose biosynthesis (PENA and SEGUIN, 2001). The commercialisation of e.g. transgenic poplar is on the way in many countries. However, most *Populus* species are dioecious and thus obligatory out-crossers. Major concerns have been raised in relation to gene flow and gene introgression into natural populations (STRAUSS et al., 1995). In addition to being wind-pollinated, seeds can be dispersed over long distances, resulting in high rates of migration (SCHREINER, 1974). These factors will be especially important, if transgenic modified poplar will be planted out into fields.

Gene flow in poplar plantations and its implications for transgenic risk assessment have been studied extensively by DIFAZIO (2002). An option to reduce or even avoid an undesired gene flow of transgenes into non-transgenic trees may be the incorporation of sterility genes like barnase into transgenic lines (STRAUSS et al., 1995). But, beside that, bacterial RNAse genes may be undesired in transgenic plants, because of possible pleiotropic effects. Additionally, transgenes can be less stable than originally been thought or influenced by gene silencing effects (HOENICKA and FLADUNG, 2006). An alternative and even more attractive option will be the identification of sex-related genes or molecular markers correlated to sex.

By the availability of sex-related genes or markers, respectively, transgenic trees can be screened on gender before planting out into the field. This option can be used to select only female sex for e.g. short rotation planting (up to ten years) thus will be efficient to avoid uncontrolled pollen flow between transgenic and non-transgenic trees. In case the female flowers are unexpectedly formed early during plantation either these can be easily removed before seed set and seed flow, or flowering trees can be selectively felled.

The intention of this study was the identification and development of sex-correlated molecular markers by a bulked segregant analysis and their positioning in a preliminary linkage map for an interspecific cross of *P. tremula* x *tremuloides*. While several linkage maps have already been published for favored *Populus* species like *P. deltoides*, *P. trichocarpa*, *P. alba*, *P. nigra* (BRADSHAW et al., 1994; CERVERA et al., 2001; YIN et al., 2004; JORGE et al., 2005), so far only one linkage map was published for trembling aspen *P. tremuloides* by LIU und FURNIER (1993) based on a total of 75 RFLP-marker.

Here we describe the localization of sex-correlated markers for *Populus* in a linkage map for hybrid aspen of a *P. tremuloides* x *tremula* cross for the first time. For the future, the sex-correlated markers presented here will be used to screen a BAC library from the male parent Turresson 141 (FLADUNG et al., manuscript in preparation). The aim is to construct a BAC-contig containing the sex-locus. This BAC-contig will be further characterized by fine-mapping approaches.

Material and Methods

Plant Material

The F1 population from a cross between the female parent Brauna 11 (*P. tremula*) and the male parent Turresson 141 (*P. tremuloides*) was used for the identification of sex-correlated markers and map construction. The cross was performed in 1951 and a total of 450 resulting progeny trees were established in the field in 1953 on different sites in Northern Germany. Out of the 450 progenies a residual of 146 progeny genotypes survived to date. For these 146 trees, the gender could be determined only for 117 trees in 2002/03, because it was not appraisable for all progenies within these years. For

[1)] Authors for further correspondence:
T. MARKUSSEN: E-mail: t.markussen@holz.uni-hamburg.de,
Phone: +49 (0)4102696157, Fax: +49 (0)4102696200.
M. FLADUNG: E-mail: mfladung@uni-hamburg.de,
Phone: +49 (0)4102696156.

DOI:10.1515/sg-2007-0027
edited by Thünen Institute of Forest Genetics

construction of a preliminary linkage map, a total of 66 progeny (46 male, 20 female) genotypes were used.

AFLP and SSR analyses

DNA was extracted according to the protocol of DOYLE and DOYLE (1990). AFLP fingerprints were generated based on the modified protocol described by VOS et al. (1995). A total of 250 ng genomic DNA was restricted with either *Eco*RI and *Mse*I (4–5 U of each) for 2–2.5 h at 37 °C in 1 x restriction buffer OPA (One-Phor-all; Amersham-Pharmacia, Freiburg, Germany) in a final volume of 50 µl. After controlling for complete digestion, 10 µl of a ligation mix [50 pmol *Mse*I adaptor, 5 pmol *Eco*RI adaptor, 10 mM ATP, 1 x OPA buffer and 2.5 U T4 DNA ligase] was added to the samples and incubated for 3 h at 37 °C. A pre-amplification reaction was performed in a 50 µl reaction containing: 5 µl of template DNA, 150 ng of *Eco*RI and *Mse*I primer with one nucleotide extension, 0.25 mM dNTPs, 1 x PCR buffer (Eurogentec, Seraing, Belgium), 1.2 mM $MgCl_2$ and 1 U *Taq* Polymerase (Eurogentec, Seraing, Belgium). Samples were run on a TGradient cycler (Biometra, Göttingen, Germany) using the following parameter: 94 °C (1 min), 19 cycles of 94 °C (30s), 60 °C (30 s), 72 °C (1 min) and a final extension 72 °C (5 min). Pre-amplified DNA was diluted (1:5). Equal aliquots for each individual selected for the different bulks were mixed and used for selective amplification. Selective amplification was performed in a 20 µl reaction containing: 5 µl of the diluted template DNA, 10 ng *Eco*RI primer (Cy5 labelled, MWG-Biotech, Ebersberg, Germany) and 50 ng *Mse*I primer each having three selective nucleotides, 1 x PCR buffer (Eurogentec, Seraing, Belgium), 1.2 mM $MgCl_2$ and 1 U *Taq* Polymerase (Eurogentec, Seraing, Belgium). Samples were run on a TGradient cycler (Biometra, Göttingen, Germany) using the following cycling parameter: initial denaturation step 94 °C (5 min); 1 x 94 °C (30 s), 65 °C (30 s), 72 °C (1 min); 2 x 94 °C (30 s), 64 °C (30 s), 72 °C (1 min); 2 x 94 °C (30 s), 62 °C (30 s), 72 °C (1 min); 2 x 94 °C (30 s), 58 °C (30 s), 72 °C (1 min); 23 x 94 °C (30 s), 56 °C (30 s), 72 °C (1 min) and a final extension of 72 °C (5 min). 8.5 µl reaction products were resolved on high-resolution polyacrylamide gels on the automatic sequencer ALFexpress II (Amersham-Pharamcia, Freiburg, Germany) and analysed by using the Fragment Analyser software (Vers. 1.03; Amersham-Pharamcia, Freiburg, Germany). For each primer enzyme combination (PEC), the bulks were compared on polymorphic fragments, reflecting putative markers linked to gender. By detection of putatively linked markers, each progeny was successional analysed separately on marker presence. The significance level for marker correlation to gender was estimated by a two-sided Fisher exact test.

SSR-primer used for map construction were selected based on the publications of DAYANANDAN et al. (1998) and RAHMAN et al. (2000) as well as the SSR-library presented by the Oak Ridge National Laboratory http://www.ornl.gov/sci/ipgc/ssr_resource.htm. SSR analysis was performed according to the authors protocols.

Conversion of AFLP into SCAR marker and data base comparison

Fragments were isolated from a high-resolution polyacrylamide gel (Amersham-Pharmacia, Freiburg, Germany), purified by using the QIAEX II-Kit (QIAGEN, Hilden, Germany) according to manufacturer instructions and reamplified in an final volume of 50 µl by using unlabeled primers set corresponding to the AFLP primers used for selective amplification. A 10 µl sample of each PCR product was electrophoresed on a 2% agarose gel for size comparison. The concentration of the PCR products was determined and an aliquot cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, Calif. USA) according to manufacturer instructions. Forty colonies were chosen for each transformation event, transferred to a microtiter plate containing 100 µl LB-media with 50 µg/ml of ampicillin and cultured overnight. An one µl aliquot of each culture was used for PCR amplification by using the M13 forward- and reverse-primer. PCR products were screened on size homologies corresponding to the original AFLP fragments on a 1.4% agarose gel. Five clones per transformation event were selected and sequenced by using the BIG-Dye Terminator Sequencing-Kit (PE Applied Biosystems, Foster City, USA) according to manufacturer instructions. Generated sequences were compared to the MAFF DNA bank (http://www.dna.affrc.go.jp/) and the Populus specific gene bank (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) by BLAST N- and BLAST X-search.

Map construction

Polymorphic fragments were scored for presence and absence in parents and F1 progenies. Linkage analysis

Table 1. – Results of the two-sided fisher-exact-test performed for the estimation of marker linkage to gender. Markers significant correlated to gender are marked as bold. a: Primer-enzyme-combination plus selective nucleotides with 3 or 4 nt extension, respectively; E = *Eco*RI-adaptor, M = *Mse*I-adaptor. b: Size of AFLP-generated fragments in base pairs. c: Marker correlation to gender. d, e: number of male and female individuals analysed for marker presence (20 females and 46 males). f: number of deviations from expectation on presence or absence of fragments out of 66 individuals. 5% significance level.

PEC [a]	Size [b]	Character [c]	Female [d]	Male [e]	Deviation events % [f]	p-value
EAAC/MGCC	496	♀	13(20)	17(46)	24(66) / 36.4	0.0598
EAAC/MCCC	**153**	♂	**2(20)**	**34(46)**	**14(66) / 21.2**	**‹0.001**
EAAC/MTCC	306	♂	5(20)	25(46)	26(66) / 39.4	0.0340
EACA/MCAA	245	♂	11(20)	39(46)	18(66) / 27.3	0.0268
EACGC/MATA	**221**	♂	**0(20)**	**39(46)**	**7(66) / 10.6**	**‹0.001**
EAAG/MCTAT	**72**	♂	**1(20)**	**36(46)**	**11(66) / 16.6**	**‹0.001**

DOI:10.1515/sg-2007-0027
edited by Thünen Institute of Forest Genetics

was performed according to RITTER et al. (1990, 1996). The MAPRF program (RITTER et al., 1996) was applied for the computational methods. Linkage maps were constructed based on fragments specific to either parent. Linked fragments were arranged into linkage groups using a minimum LOD threshold of 3.0 between consecutive markers. Subsequently, fragments common for both parents were integrated into linkage groups as anchor points as described in RITTER et al. (1990). Only common markers linked with more than LOD 6.0 to at least one parent and with LOD 3.0 to the other were considered for this purpose.

Results and Discussion

Conversion of AFLP and data base comparison

For the identification of sex-correlated markers by a bulked segregant analysis (BSA) approach, two pools were collected each for male and female gender containing ten progenies of the mapping population per pool. A total of 201 AFLP Primer-enzyme-combinations (PECs) were analyzed for markers co-segregating with gender. Six AFLP-markers were putatively associated with gender by the Fisher-exact test (*Table 1*), in particular, of which three markers were significantly correlated to gender (at $\alpha = 0.001$).

Based on the results of the fisher-exact-test, three markers showing the highest significance level were selected for conversion into SCAR markers (*Table 1*, marked as bold). By sequencing of these fragments, the following sequences were obtained for the different markers as presented in *Table 2*.

Sequence similarity comparisons in public libraries revealed the results presented in *Table 3*. While non-significant correlations were found for markers 153, 221-1 and 221-2, a significant similarity to a flower specific

Table 2. – Results of the sequencing approach for selected fragments. a: Primer-enzyme-combination plus selective nucleotides with 3 or 4 nt extension, respectively; E = *Eco*RI-adaptor, M = *Mse*I-adaptor. b: Size of the selected fragment according to AFLP-analysis. c: Fragment size excluding *Eco*/*Mse* adaptors (16bp each). Selective nucleotides are marked as bold.

PEC [a]	Fragment [b]	Length (bp) [c]	Sequence
EAAG/MCTAT	72	45	**CTAT**CTTGCTCTTATTCCTGCCAGGTAACGAACTTTTCAA TT**CTT**
EAAC/MCCC	153	129	**AAC**AAGTGAAGCTACCAACTGGTTATTTAYGTGCAACAG ATAACAGATACACTAMCATGGACTGAATGGTAGAAGGA TAASTAAAACRTAGAACTTGATTGGTTGGGATCGRACCT GSRRTTWGAT**GGG**
EACGC/MATA	221-1	204	**ACGC**ATGCTTGCCGACTGTGGCTATTTTGGTCACTGGGC TGGGCCAGTGACAAGCTGGTTAGGCTGCATCAATCCCAG CTTTATTGGCTGGCTAGATCCAGTCCATAAAAAAAAAGT TGAGACGGGCTGGGCCTGAGCCTGAGGCCCATCCAACCT ATTTTTTGGGCTTTATACCCTTTCCCTTTTTTTATTCTTTTT TTT**TAT**
EACGC/MATA	221-2	186	**ACGC**TGGGATGGAAGTGGGTGATTATGAAGAATGTTTG AGTTTTAGATTGGTGTGATGATATACAAGTAGTGGATCA AGAGCTGGGTCAGGTTATATTGTGATGTATTCATTAGTTT TTCTGGTTTTTTCTTGCTCTCTCGTTTCATTTATCTTATGT ATATGGTGGCTATTGTTTGTCTTTG**TAT**

Table 3. – Results of database comparison with sequence information of converted fragments. A: Data according to BLAST-N search. B: Data according to the *Populus* specific database.

A

PEC/ Fragment size in bp	Highest similarity to	e-Value
EAAG/MCTAT-72	UM57TB02 *Populus* flower cDNA library *Populus balsamifera* subsp. *trichocarpa*, cDNA 5 prime, mRNA sequence (*gender not specified)*	8e-05
EAAC/MCCC-153	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 2, BAC	0,088
EACGC/MATA-221-1	*Solanum demissum* chromosome 5 BAC clone PGEC287I17, complete sequence	0,009
EACGC/MATA-221-2	BOGZS82TR BOGZ *Brassica oleracea* genomic clone BOGZS82, DNA sequence	0,130

B

PEC/Fragment size in bp	Highest similarity to	e-value
EAAG/MCTAT-72	XXI887102.b1 CHROMAT_FILE: XXI887102.b1 PHD_FILE	3e-14
EAAC/MCCC-153	VAO91251.b1 CHROMAT_FILE: VAO91251.b1 PHD_FILE	1e-48
EACGC/MATA-221-1	XXI515714.b1 CHROMAT_FILE: XXI515714.b1 PHD_FILE	0,003
EACGC/MATA-221-2	TRE169804.y1 CHROMAT_FILE: TRE169804.y1 PHD_FILE	6e-21

DOI:10.1515/sg-2007-0027
edited by Thünen Institute of Forest Genetics

cDNA-library of *P. balsamifera* (Acc. No. BU880966) revealed for the 72-bp fragment.

Construction of a preliminary linkage map and location of sex-related markers

For map construction, a total of 36 different AFLP-primer enzyme combinations (PECs) and 108 SSR markers (DAYANANDAN et al., 1998; RAHMAN et al., 2000) analyzed in the 66 progeny genotypes yielded 261 segregating fragments (27 distorted fragments were excluded from analyses). A low degree of polymorphisms between parental alleles together with a large degree of homozygosity (non segregating fragments) was observed for AFLP as for SSR fragments.

Initially, individual linkage maps of 19 linkage groups each were obtained for the two parents of the mapping population. Linkage groups of the female parent (*Brauna 11*) contained 2 to 32 individual and common markers and were between 3 and 230 cM in length. The total map length for the female parent was 1875 cM. The male parent map (*Turresson 141*) was 1730 cM in length and up of linkage groups with 2 to 23 markers each. The size of the linkage groups varied between 3 and 112 cM. Based on the integration of markers common to both parents and codominant markers (SSRs) into linkage groups for both parents, 19 homologous chromosomes were assigned for both parents and an integrated consensus map was obtained with a total of 234 markers (3-286cM).

For map construction, the G-Locus was considered to be equal to a marker which is correlated to male gender. The sex related markers identified by bulked segregant analysis and converted into SCAR marker were mapped on the linkage group 5 (LG5, *Fig. 1*). The Marker EACGCMATA-221 mapped 7.4 cM, EAAGMCTAT-72 mapped 16.9 cM and the marker EAACMCCC-153 mapped 20.3 cM distant to the sex-related locus G (*Fig. 1*)

The maps which have already been published for the different poplar species showing a total length of 664 cM distributed on 14 linkage groups to more than 3000 cM distributed over 30 linkage groups (overview CERVERA et al., 2004). The number of mapped markers (AFLP, ISSR, RFLP, RAPD) of these published linkage maps varied between 57 and 350.

While the linkage map presented here for hybrid aspen corresponds to the number of *Populus* chromosomes (N = 19) and expected map length (see CERVERA et al., 2004), the number of markers are inhomogeneous dispersed over the linkage groups.

Therefore, the population will be enlarged by nearly 40 individuals for consolidation of marker positions by screening these individuals for all markers used for map construction in the near future. Next this, the map will be filled up by additional markers for the creation of a saturated map and flower-related candidate genes (e.g. *Leafy*, *Agamous*, *FT*) will be analyzed on their segregation for map integration. The marker specific primers presented here will be also used for screening their correlation to gender within other populations in the near future.

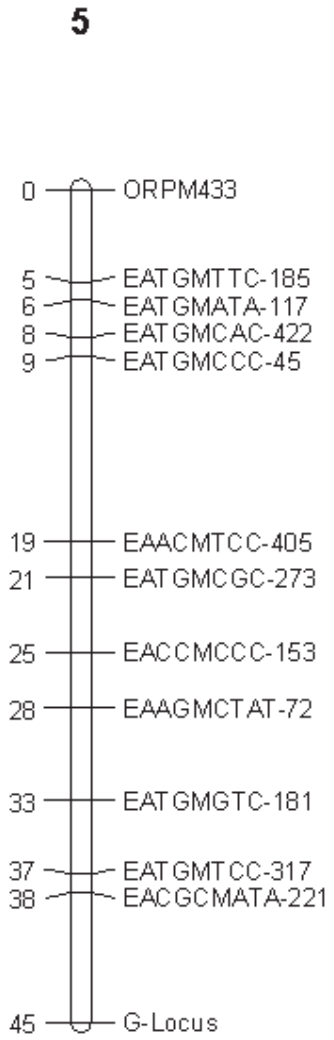

Figure 1. – Extract of the whole linkage map constructed for *P. tremula* x *P. tremuloides.* Linkage group 5 (LG5) contains the sex-correlated locus (G-Locus) and the sex-correlated markers identified by bulked segregant analysis.

Furthermore, a consensus map will be constructed for the map presented here and a second independent map of a *P. tremula* x *P. tremula* cross and QTLs localized for 13C-isotope signatures (MEYER et al., manuscript in preparation). Additionally, the map will be used as a starting point for the identification of sex-related genes or additional molecular markers and their fine mapping based on a BAC-library with a total of 60.000 clones.

Acknowledgements

This research was funded by the German Research Foundation (DFG-FL263/2-1, FL263/2-2).

Thanks to G. VON WÜHLISCH and C. DÜHRING for providing the plant material.

References

BRADSHAW, H. D., M. VILLAR, B. D. WATSON, K. G. OTTO, S. STEWART and R. F. STETTLER (1994): Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS and RAPD markers. Theor Appl Genet **89**: 167–178.

DOI:10.1515/sg-2007-0027
edited by Thünen Institute of Forest Genetics

CERVERA, M. T., V. STORME, A. SOTO, B. IVENS, M. VAN MONTAGU, O. P. RAJORA and W. BOERJAN (2005): Intraspecific and interspecific genetic and phylogenetic relationships in the genus *Populus* based on AFLP markers. Theor Appl Genet **111**: 1440–1456.

DAYANANDAN, S., O. P. RAJORA and K. S. BAWA (1998): Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*) Theor Appl Genet **96**: 950–956.

DIFAZIO, S. P. (2002): Measuring and modelling gene flow from hybrid poplar plantations: Implications for transgenic risk assessment. PhD Dissertation, Oregon State University.

DOYLE, J. J. and J. L. DOYLE (1990): Isolation of Plant DNA from fresh tissue. Focus **12**: 13–15.

HOENICKA, H. and M. FLADUNG (2006): Biosafety in *Populus* ssp. and other forest trees: from non-native species to taxa derived from traditional breeding and genetic engineering. Trees **20**: 131–144.

JORGE, V., A. DOWKIW, P. FAIVRE-RAMPANT and C. BASTIEN (2005): Genetic architecture of qualitative and quantitative *Melampsora larici-populina* leaf rust resitance in hybrid poplar: genetic mapping and QTL detection. New Physiologist **167**: 113–127.

LIU, Z. and G. R. FURNIER (1993): Inheritance and linkage of allozymes and restriction fragment length polymorphisms in trembling aspen. J. Heredity **84**: 419–424.

MARKUSSEN, T., A. TUSCH, B. R. STEPHAN and M. FLADUNG (2004): Identification of molecular markers for selected wood properties of Norway Spruce (*Picea abies* L.[Karst.]) I. Wood density. Silvae Genetica **53**: 45–50.

PENA, L. and A. SEGUIN (2001): Recent advances in the genetic transformation of trees. Trends in Biotechnology **19**: 500–506.

RAHMAN, M. H., S. DAYANANDAN and O. P. RAJORA (2000): Microsatellite DNA markers in *Populus tremuloides*. Genome **42**: 293–297.

RITTER, E., C. GEBHARDT and F. SALAMINI (1990): Estimation of recombination frequencies and construction of RFLP linkage maps in plants from crosses between heterozygous parents. Genetics **224**: 645–654.

RITTER, E. and F. SALAMINI (1996): The calculation of recombination frequencies in crosses of allogamous plant species with application to linkage mapping. Genet. Res. **67**: 55–65.

SCHREINER, E. J. (1974): *Populus* L. Poplar. *In:* SCHOPMEYER, C. S. (ed). Agriculture Handbook No 450, Forest Service, Seeds of woody plants in the United States. USDA, Washington, DC, pp 645–655.

STRAUSS, S. H., W. H. ROTTMANN, A. M. BRUNNER and L. A. SHEPPARD (1995): Genetic engineering of reproductive sterility in forest trees. Mol Breed **1**: 5–26.

TUSKAN, G. A., L. E. GUNTER, Z. K. YANG, T. M. YIN, M. M. SEWELL and S. P. DIFAZIO (2004): Characterization of microsatellites revealed by genomic sequencing of *Populus trichocarpa*. Canadian Journal of Forest Research. **34**: 85–93.

YIN, T. M., S. P. DIFAZIO, L. E. GUNTER, D. RIEMENSCHNEIDER and G. A. TUSKAN (2004): Large-scale heterospecific segregation distortion in *Populus* revealed by a dense genetic map. Theor Appl Genet **109**: 451–463.

Studies on Cytogenetical Variation in *Prosopis cineraria* (Linn.) Druce – A Key Stone Tree Species of Indian Desert

By D. RAWAT, A. KUMAR and S. R. RAO[1)]

Cytogenetics and Molecular Biology Laboratory, Department of Botany, J. N. Vyas University, Jodhpur- 342005, Rajasthan, India

(Received 3rd June 2006)

Abstract

Prosopis cineraria (Mimosaceae) is an important tree of agro-forestry and ethano- botanical importance with multipurpose utility as wood yielding, fodder, food and medicinal uses. Some remarkable features are observed in the form of phenotypic variation in various populations inhabiting different regions of the Indian desert. To asses these variations male meiotic studies were conducted in ten different accessions collected from four proviences of Rajasthan, India. Analysis of data on chromosome associations, chiasma frequency and their distributions pattern concluded that the somatic chromosome number of *P. cineraria* is 2n = 2x = 28. The complete absence of accessory chromosomes (B) and percentage of pollen stainability indicates an over all genomic stability in *P. cineraria.* Numerical changes like aneuploidy might have played an important role in origin and adoptation of *P. cineraria* against all the odds of the climatic condition of the Indian desert.

Key words: Aneuploidy, Chromosome associations, Chiasma frequency, Genetic variation, Meiosis, Pollen stainability, *Prosopis cineraria.*

Introduction

Prosopis cineraria is one of the most common tree of the Indian desert belonging to family Mimosaceae and

[1)] Corresponding Author: Telephone #: +91 - 291-2711227 (R), 91 9414919943 (M). E-mail: srrao22@yahoo.com

DOI:10.1515/sg-2007-0028
edited by Thünen Institute of Forest Genetics