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MATING SYSTEM AND GENE FLOW ANALYSIS WITHIN A CLONAL SEED ORCHARD OF *TECTONA GRANDIS* L.F. USING MICROSATELLITE MARKERS

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ABSTRACT

Microsatellite markers were used to analyze pollen dispersal and mating system within a clonal seed orchard of *Tectona grandis*. A total of 105 offsprings, five known seed bearers and 307 putative pollen donors were genotyped at four microsatellite loci. This dataset was analyzed using maximum likelihood approach to determine paternity of 105 offsprings. Spatial analysis of known seed bearers and identified pollen donors was used to trace pollen dispersal in this insect pollinated species. The analysis revealed the mating system to be dominated by cross fertilization (93.4%), with very low rate of selfing (6.6%). The average pollen dispersal distance varied from 84.0±18.8 m (SEM) to 153.4±14.2 m (SEM). About 80.7% of the pollen donors were beyond 50 m of the seed bearers. Of the thirty clones planted in the orchard, 23 participated in the breeding process. The number of pollen donors per seed tree varied from 10 to 14. Management implications of these findings are discussed in the paper.

Key words: Tectona grandis, Microsatellite marker, Mating system, Pollen dispersal, Gene flow.

Introduction

Traditionally, observational methods have been used to quantify gene flow between and within populations. These methods take the form of directly marking gametes and/or zygotes and either recapturing a sample to estimate mobility or to follow dispersal directly from a source (Campbell, 1985). These observational methods do not take into account post-dispersal factors, such as viability, sexual compatibility, gamete competition or selective embryo abortion. Therefore, these methods only measure dispersal, not successful gene flow (Cain *et al*., 2000). Recently, molecular markers have been successfully introduced in tree improvement programmes to study mating systems and gene flow in natural populations and orchards. Molecular markers provide a direct method to assess contemporary gene flow by way of identifying parents of progeny arrays. Microsatellite markers, because of their hyper-variability, co-dominance and mendelian segregation, have proved to be the marker of choice for studying contemporary gene flow and mating system in natural forests (Prabha *et al.*, 2011; Dow and Ashley, 1996) and breeding pattern in seed orchards (Grattapaglia *et al*., 2004).

Tectona grandis, belonging to family Verbenaceae, is a large deciduous tree distributed in South and South-east Asia. It is mainly valued for its decorative and very durable timber. Teak trees produce several large inflorescences, each consisting of hundreds of small flowers. The hermaphroditic flowers are arranged in panicles and it is primarily an outcrossed, insect pollinated species with adaptations like protandry. Several bee species viz., *Heriades parvula*, *Ceratina hieroglyphica*, ants and other insects have been recorded as Teak pollinators (Hedegart, 1976).

Under the planting stock improvement programme of Teak, clonal seed orchards have been established at several locations in Telangana and Andhra Pradesh with an objective to produce genetically superior seeds. This objective can be met only when there is random mating among clones planted in the clonal seed orchards, thereby, capturing all possible gene combinations of the parent clones into their progenies. However, biotic factors (asynchronous flowering, pollinator behaviour, plant density, etc.) and environmental factors (rainfall, temperature, humidity, altitude, etc.) have the potential to effect non-random mating among some clones.It may cause depletion of genetic diversity in plantations raised from seeds, sourced from such clonal seed orchards. Selfing and pollen contamination from trees outside the orchard are other potential factors that defeat the purpose of producing genetically superior seeds. With this knowledge in background, a study was undertaken at the Institute of Forest Biodiversity, Hyderabad, to assess mating system and gene flow within a Teak CSO, located at Achuthapuram, Telangana, using microsatellite markers.

Paternity analysis of progeny arrays of *Tectona grandis* with microsatellite markers revealed a mating system dominated by cross fertilization and high levels of long distance (> 50 m) pollination.

Material and Methods

Study site

The study site is located at the Forest Research Station, Achuthapuram (17^0 14' 59.7516'' N and 81^0 2' 51.1044'' E), under the Forest Geneticist, Telangana Forest Department, Warangal. The Teak clonal seed orchard was established during the year 1996, in permuted neighbourhood design, with double ring isolation and contained a total of 78 clones in three blocks. Block I, where the study was undertaken, contained 30 clones, replicated ten times with four ramets per clone. Thus, a total of 1200 ramets were planted at the time of CSO establishment during 1996. The total count of plants was 307 during the year 2011, when this study was initiated.

Leaf sample collection from clones and progenies

Genetic material in the form of fresh terminal leaf (about 200 mg) was collected from all retained ramets of the thirty clones. The leaves were swabbed with 70% ethanol to physically remove dirt, other contaminating particles and micro-organisms. Then, the leaves were dried following the protocol of Chase and Hills (1991), by putting them inside zip lock bags containing silica gel. The bags were labelled and then transported to laboratory for extraction of DNA. About one hundred open-pollinated fruits were collected from five seed bearers, located at different parts of the orchard, representing five clones. The fruits were bagged and tagged to maintain clonal and ramet identity. Fruits were germinated at the Institute of Forest Biodiversity nursery using alternate wetting and drying method. Following germination, which was about 20%, leaf samples were collected from half-sib progeny arrays and dried with silica gel using the above mentioned protocol. Progeny samples were serially numbered along with the mother plant identity.

DNA extraction

Genomic DNA of each sample of *Tectona grandis* was extracted using the cetyl trimethyl ammonium bromide (CTAB) protocol of Doyle and Doyle (1990). The protocol was slightly modified by adding a higher concentration (2%) of β-mercapto ethanol. About 75 mg sample tissue was ground manually, in a mortar and pestle, in presence of liquid nitrogen. The finely ground tissue was incubated in 2% CTAB buffer for one hour at 65^{0}C. Following incubation, DNA was extracted using a mixture of chloroform and isoamyl alcohol, and precipitated using isopropanol. The precipitated DNA was washed in ethanol and suspended in Tris buffer. The suspended DNA was treated with enzyme RNaseA (10µg/ml) to degrade any contaminating RNA.

Microsatellite genotyping

Fifteen microsatellite markers developed by Verhaegen *et al.* (2005) were screened for polymorphisms to identify markers with higher information content for the current study. Microsatellite marker amplifications were performed for a total volume of 10.0 µl [Master mix (2x)-5.0 µl; Forward primer (0.2µm) - 0.2µl; Reverse primer (0.2µm) - 0.2 µl; Template – 0.5µl (25ng/µl); Nuclease free water- 4.1 µl]. The polymerase chain reaction was performed in 35 cycles and consisted of (a) Initial denaturation at 94^{0}C for 4min., (b) Denaturation at 94^{0}C for 30 sec., (c) Primer Annealing at 48^{0}C to 53^{0}C for 30 sec., (d) Primer extension at 68^{0}C for 30 sec., (e) Final extension at 68^{0}C for 10 min., (e) Reaction termination at 5^{0}C for 5min.

Four primer pairs showing clear amplicons and heterozygous banding pattern were short listed (Table 1) for genotyping of entire samples consisting of progenies (105 Nos.), known mothers (5 Nos.) and candidate fathers (307 Nos.) (Table 1).

Following final PCR amplification of target microsatellites, the amplicons were separated on non-denaturing polyacrylamide gel (5 or 7%), run at constant current (30mA) for 2hour 45 minutes, alongside 100bp DNA ladder. The gels were post-stained with GelStar Nucleic Acid Gel Stain following manufacturer instructions. Gel images were captured in a Syngene GelDoc system. Then, alleles were scored at each locus taking into account the allelic range at that loci (Verhaegen *et al.,* 2005).

Table 1: Microsatellite primers used in the present study for genotyping of T*ectona grandis*.

Sl. No.	Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Repeat motif	Annealing temperature (°C)	Reference
1	CIRAD3TeakB02	ATG AAG ACA AGC CTG GTA GCC	GGA AGA CTG GGG AAT AAC ACG	$(TC)_{11}GC(TC)_4$ $(N)_{62}(AC)_7$	53.0	Verhaegen *et al.,* 2005
2	CIRAD3TeakF01	GCT CTC CAC CAA CCT AAA CAA	AAA ACG TCT CAC CTT CTC ACT	$(TC)_{16}$	51.0	
3	CIRAD4TeakDA12	CGC ACA CCA GTA GCA GTA GCC	GCC GGA AAA AGA AAA ACC AAA	$(GA)_4(N)_5(GA)_{11}A(GA)_4$	49.0	
4	CIRAD4TeakF02	CCG GTA AAA AGG TGT GTC A	GAG TGG AAG TGC TAA TGG A	$(TC)_4(AC)_3(N)_{16}(TC)_{11}$	48.0	

Paternity assignment

Maximum likelihood approach was adopted to find the single most likely pollen parent (father) for each of the focal offspring with known seed parent (mother). The maximum likelihood approach was implemented through the software CERVUS 3.0.7 (Marshall, 1998-2014). In this study, the simulation parameters for generating Delta (?) were set as follows: Offspring – 100,000; Candidate parents – 220 (only those flowered out of total 307); Proportion of loci typed – 0.9806; Proportion of loci mistyped – 0.01; Confidence levels – 95% and 80%. CERVUS 3.0.7 was also used to determine the number of alleles (K), observed heterozygosity (Ho), expected heterozygosity (He) and non-exclusion probability.

Spatial analysis

In the present study, the row-row and plant-plant distance in the clonal seed orchard was known (5x5 m). A grid was prepared and each plant was identified with X-axis and Y-axis co-ordinate (X_1, Y_1; X_2, Y_2,.....). The straight line distance between the known seed parent and the identified pollen parent was calculated using the Pythagorean theorem:

$$d= \sqrt{(x_2-x_1)^2 + (y_2-y_1)^2}$$

The pollen dispersal distance was calculated by taking average of only those pollen parents identified with 95% confidence level.

Results and Discussion

The four microsatellite loci used in the present study produced distinct banding patterns. The number of alleles observed per locus varied from 12 to 18 with an average of 15.5. The mean observed heterozygosity (0.766) was lower than expected heterozygosity (0.896). Paternity analysis with maximum likelihood approach identified the most likely pollen donors for 61 progenies (58.1%) with 95% confidence level. A higher assignment rate (83 progenies: 79.1%) could be achieved at relaxed confidence level of 80% (Table 2). Out of the 61 progenies for which paternity could be established with 95% confidence, only four progenies were selfed (6.6%) and rest was the result of cross-fertilization (93.4%) (Table 3). This high outcrossing rate in Teak is in agreement with Prabha *et al*. (2011), who had reported 96.1% cross-fertilization in a natural Teak forest in the Kerala state of India. Kjaer and Suangtho (1995) had reported outcrossing rate of 89-95% in Teak using isozyme markers.

Spatial analysis of known seed bearers and identified pollen donors (identified at 95% confidence level) revealed pollen dispersal in all directions of the orchard (Fig. 1 and 2). The average pollen dispersal distance to five focal seed bearers varied from 84.0± 18.8 (SEM) m to 153.4± 14.2 m (SEM) (Table 3). Further spatial analysis (Table 4) revealed that 80.7 % of the pollen donors were beyond 50.0 m of the seed bearers, whereas, only 19.3 % pollen donors were within 50.0 m from the seed bearers. Pollination success was maximum (35.1 %) when pollen donors were in between 101 to 150 m from the seed trees.

Out of the thirty clones planted in the CSO, only 23 clones participated in the breeding process. The contribution of other 7 clones could not be detected. The number of pollen donors varied from 10 to 14, with an average of 11.4 pollen donors per seed tree. Further analysis revealed that these pollen donors belonged to 8 - 10 clones, with a maximum of 10 clones fertilizing seed bearer 7B. This indicates through mixing of alleles in the orchard, thus, helping in the production of genetically diverse and high quality seeds.

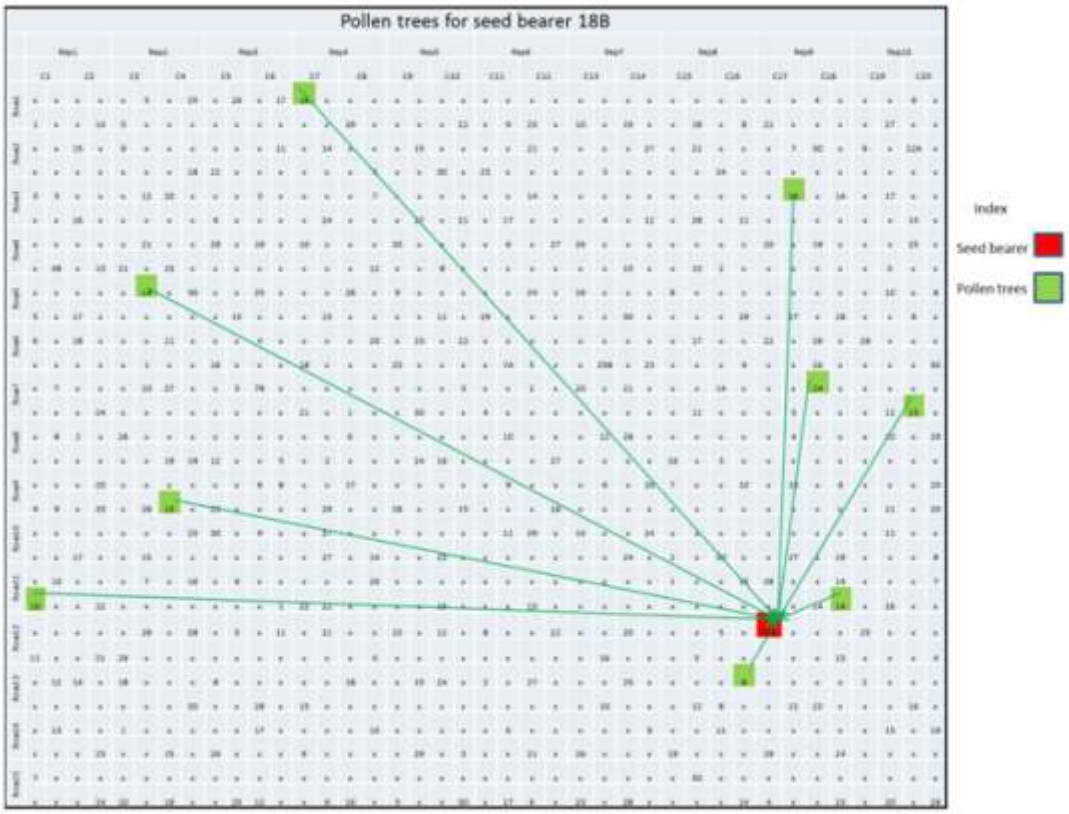
In most studies on tropical plant species, gene flow through pollen acts over longer distances than through seed dispersal (Marico *et al*., 2009). There are two main factors responsible for mating distance in tropical trees *viz*., performance of pollinators and flowering tree density (Konuma *et al*., 2000). The impact of population density, species composition and pollinator abundance on

Table 2: Paternity analysis for 105 *Tectona grandis* progenies.

Sl. No.	Seed bearer (Mother tree)	No. of progenies analyzed for paternity	No. of progenies for which most likely pollen donors (Father tree) identified		No. of progenies for which pollen donors not identified
			Confidence level		
			95%	80%	
1	4B (Clone 4)	22	12	17	5
2	25B (Clone 25)	20	12	15	5
3	7B (Clone 7)	22	11	17	5
4	18B (Clone 18)	22	12	19	3
5	22A (Clone 22)	19	14	15	4
	Total	105	61	83	22
	Percentage of progeny analyzed		58.1	79.1	20.9

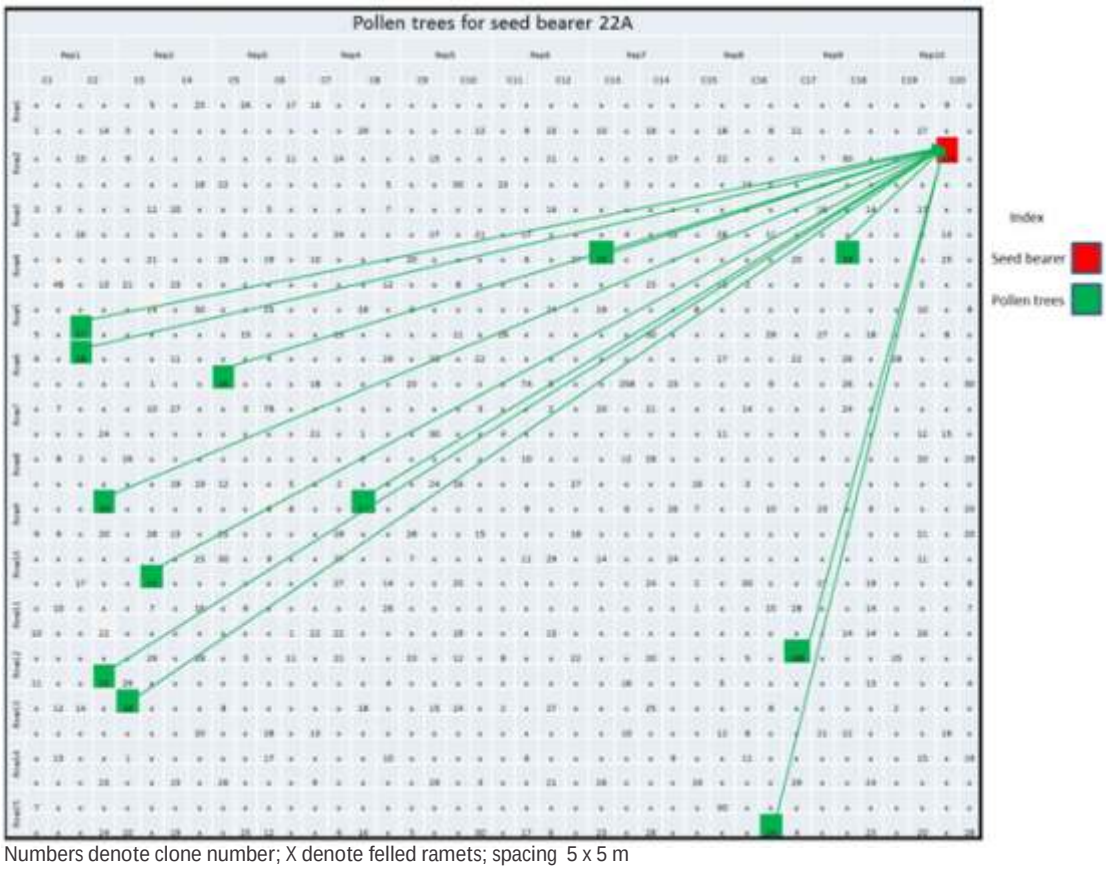
Table 3: Pollen dispersal distance for sixty-one *Tectona grandis* progenies.

Progeny No.	Seed tree	Seed tree position in orchard	Most likely pollen tree	Pollen tree position in orchard	Pollen dispersal distance (m)	Average pollen dispersal distance ±SEM (m)	Remarks
1	4B	R4C1	C8-8	R9C6(2)	67.3	87.160 ± 18.034	• Outcrossing rate - 83.3%
2			C17-7	R10C2	60.2		• Selfing rate - 16.7%
3			C4-2	R3C13	120.4		
4			C20-2	R4C9	75.2		
5			C6-1	R2C19	176.8		
6			C4-3	R4C1	0 (Selfing)		
7			C8-5	R5C20(2)	185.3		
8			C12-1	R3C3	25.0		
9			C21-3	R3C10	90.6		
10			C4-3	R4C1	0 (Selfing)		
11			C27-6	R7C4	35.4		
12			C27-6	R7C4	35.4		
13	25B	R6C13	C6-1	R2C19	71.1	91.367 ± 10.072	• Outcrossing rate - 100%
14			C16-4	R6C5	85.0		
15			C8-6	R8C1	120.9		
16			C8-6	R8C1	120.9		
17			C10-8	R11C1(2)	134.6		
18			C4-9	R15C17	96.6		
19			C3-7	R7C10	30.4		
20			C8-6	R8C1	120.9		
21			C27-4	R4C12	26.9		
22			C16-4	R6C5	85.0		
23			C20-11	R13C4	114.0		
24			C10-10	R14C8	90.1		
25	7B	R7C6	C29-4	R5C16	106.1	93.473 ± 10.382	• Outcrossing rate - 100%
26			C21-8	R9C19	137.3		
27			C11-6	R10C11	62.7		
28			C19-7	R10C18	129.8		
29			C7-3	R6C11	55.2		
30			C7-3	R6C11	55.2		
31			C8-2	R4C10	47.2		
32			C18-7	R12C17	120.8		
33			C4-2	R3C13	82.8		
34			C14-10	R11C18(3)	132.9		
35			C13-3	R4C15	98.2		
36	18B	R12C17	C14-10	R11C18(3)	15.8	84.010 ± 18.799	• Outcrossing rate - 83.3%
37			C15-8	R11C16	11.2		• Selfing rate - 16.7%
38			C18-7	R12C17	0 (Selfing)		
39			C19-6	R8C4(2)	129.8		
40			C8-12	R13C16(1)	11.2		
41			C18-7	R12C17	0 (Selfing)		
42			C23-3	R4C4	150.1		
43			C16-4	R6C5	132.0		
44			C19-10	R14C15	32.0		
45			C22-3	R2C20	104.4		
46			C22-3	R2C20	104.4		
47			C24-11	R15C2	149.2		
48	22A	R2C20	C17-6	R9C8	138.9	153.436 ± 14.160	• Outcrossing rate - 100%
49			C18-7	R12C17	104.4		
50			C17-4	R5C2	183.4		
51			C14-12	R15C16	139.5		
52			C15-7	R10C3	185.6		
53			C20-6	R9C2(1)	188.5		
54			C26-2	R4C13	72.8		
55			C18-8	R13C3	202.5		
56			C19-3	R4C18	28.3		
57			C18-8	R13C3	202.5		
58			C18-4	R6C2	184.4		
59			C16-4	R6C5	156.6		
60			C21-10	R12C2	204.1		
61			C16-4	R6C5	156.6		



Numbers denote clone number; X denote felled ramets; spacing 5x5 m

Fig. 1: Layout map of *Tectona grandis* CSO depicting pollen dispersal from the most likelypollen donors to seed bearer 18B.



Numbers denote clone number; X denote felled ramets; spacing 5 x 5 m

Fig. 2: Layout map of *Tectona grandis* CSO depicting pollen dispersal from the most likely pollen donors to seed bearer 22A.

Table 4: Spatial analysis of pollen donors for fifty seven progenies of *Tectona grandis*.

Sl.No.	Distance of pollen dispersal (m)	No. of progenies having their pollen doors falling within the range*	Percentage of progenies (%)
1	0-50	11	19.298
2	51-100	15	26.316
3	101-150	20	35.088
4	151-200	11	19.298

*Excluding four selfed progenies.

outcrossing rate at a landscape level has been discussed by many researchers (Dick *et al*., 2003; Degen *et al*., 2004). At an intermediate scale, pollinators are responsible for substantial pollen flow (Martins *et al*., 2011).

Though cross pollination did not seem to be a problem in the present study, however, the low amount of viable seed production per seed tree indicated that such cross pollination events are very less in number. Insufficient pollination and high incidence of selfing events, probably due to the inefficient pollinators, might be two of the reasons for low fruit productivity. Teak has early-acting self-incompatibility during pollen tube entry into the ovule through the micropyle (Mohandas *et al*., 2002), as a result of which most of the selfed embryos are aborted. Other factors observed in the present study that may be contributing to the low seed production are the less number of inflorescence per tree, temporal variation in flowering and seed infestation. These problems need to be addressed in order to increase production of good quality seeds from the clonal seed orchard. As a general measure to enhance cross pollination events, a number of bee cages may be introduced in the orchard to attract and keep the bees from flying longer distances to harvest pollen and nectar. Provision of sugar syrup and water may be made available during lean flowering periods. Intensive fertilization regimes along with application of Paclobutrazol may be considered to stimulate abundant flowering.

Conclusion

The present study used paternity analysis of progeny arrays with microsatellite markers to gain insight into the mating system and gene flow within a clonal seed orchard of *Tectona grandis*. The mating system was dominated by cross fertilization. The early-acting self-incompatibility in Teak is very effective in aborting selfed seeds, thus, keeping the effective selfing rate low. The average pollen dispersal distance varied from 84.0±18.8 m (SEM) to 153.4±14.2 m (SEM). The average number of pollen donors per seed tree was 11.4, which indicated multi-parental mating leading to approximate mixing of alleles in the orchard.

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माइक्रोसैटेलाइट मार्कर्रों का उपयोग करके *टैक्टोना ग्रैन्डिस* एल.एफ. के क्लोनीय बीजोद्यान के भीतर संगम प्रणाली और जीन प्रवाह

एस. पटनायक और के. शिवा

सारांश

टैक्टोना ग्रैन्डिस के क्लोनीय बीजोद्यान के भीतर पराग छितराव तथा संगम प्रणाली का विश्लेषण करने के लिए माइक्रोसैटेलाइट मार्कर्रों का उपयोग किया गया। कुल 105 सन्ततियों, पांच ज्ञात बीज धारकों और 307 अनुमानित पराग दाताओं को चार माइक्रोसैटेलाइट लोसी पर जीन प्ररूपित किया गया। 105 सन्ततियों के पैतृत्व का निर्धारण करने के लिए अधिकतम संभावित एप्रोच का उपयोग करने इस डाटासैट को विश्लेषित किया गया। इस कीट परागित प्रजाति में पराग छितराव का पता लगाने के लिए ज्ञात बीज धारकों एवं पहचान किए गए पराग दाताओं के स्थानिक विश्लेषण का उपयोग किया गया। विश्लेषण ने संगम प्रणाली के उद्घाटित किया, जिसमें स्व-निषेचन (6.6%) की बहुत निम्न दर के साथ पर-संसेचन (93.4%) की प्रधानता है। औसत पराग छितराव दूरी 84.0 ± 18.8 m (SEM) से 153.4 ± 14.2 m (SEM) तक है। करीब 80.7% पराग दाता बीज धारकों के 50 मी. के पार थे। उद्यान में रोपित तीस क्लोनों में से 23 ने प्रजनन प्रक्रिया में भाग लिया। प्रति बीज वृक्ष पराग दाताओं की संख्या 10 से 14 थी। इस शोधपत्र में इन निष्कर्षों की प्रबंधन जटिलताओं पर विचार-विमर्श किया गया है।

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