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The mating system and genetic significance of polycarpy in the neem tree (*Azadirachta indica*)

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Abstract The objectives of this study were to estimate the outcrossing rate and to explain genetic consequences of the development of seed in the endocarp in a natural population of neem in Bangladesh. Cotyledons of germinated open-pollinated seeds of individual trees were analyzed by starch-gel electrophoresis to examine allozymes. Three loci with clear Mendelian segregation were used to estimate outcrossing rate. A multilocus mixed mating model was used to evaluate the mating system. The population exhibited high outcrossing rates both for multilocus (t_m=0.90±0.024) and mean single-locus (t_s=0.92±0.020) estimates. The difference between these two parameters (t_m–t_s=0±SE 0.038) was insignificant, indicating that there was no 'biparental inbreeding' in the population. The degree of variance of the estimates of multilocus outcrossing rates decreased when two or more loci were included. In order to elucidate the significance of polycarpy a total of 471 seeds were counted out of 440 endocarps. This mechanism appears to be a possible way of avoiding inbreeding. The results indicated that the studied neem population was predominantly allogamous.

Key words Allozyme · Bangladesh · Endocarp · Neem · Outcrossing rate · Protandry

Introduction

The pattern of genetic variation in a plant species is determined by its mating system, which affects the genetic structure and dynamics of populations within the species (Allard 1975; Tigerstedt 1984; Muona 1990). Mating systems control the mode of transmission of genes from one generation to the next, and plant species exhibit a great variety of such systems. It is important to have an account of mating processes for a tree species in order effectively to conserve its genetic resources and to optimize genetic improvement.

The neem tree *(Azadirachta indica* A. Juss.) (Meliaceae) is a remarkable multipurpose species native to the Indian subcontinent and Myanmar. This tree species was introduced to Africa early this century, and nowadays its cultivation has spread to most of the warmer parts of the world. The recent surge of interest in this tree is due to the spectacular biological activity of ingredients found in the bark, leaves and seeds against a wide range of pests and pathogens (Schmutterer 1995; Govindachari 1998; Immaraju 1998).

The neem is an attractive broad-leaved evergreen tree (deciduous in arid and semi-arid conditions) that can grow up to 30 m tall and 2.5 m in girth. Natural stands of neem are rare. In its natural environment, neem forms a component of mixed deciduous forests. It thrives in nutrient-poor, dry soils and is tolerant of high temperatures. The native habitat of neem lies between altitudes of 50–100 m, and an annual rainfall of only 130 mm is sufficient for its normal growth. The tree can withstand long periods of drought.

Neem normally starts producing seed after 5 years. The timing of flowering and fruiting varies from place to place. In India, neem flowers from January to April, and the fruits mature from June to August. Flowering starts in different parts of the tree at different times. Bisexual and male flowers occur on the same individual, i.e., the species is 'andromonoecious'. The pollen matures before the stigma becomes receptive (protandrous). The anthers start to dehisce around 8 a.m. in the closed flower (Gupta et al. 1996). Pollination is performed by insects. The ovary is trilocular, having two ovules in each chamber; thus each ovary contains six ovules. The endocarp encloses one, sometimes two and rarely three seeds (Schmutterer 1995; Singh et al. 1995). This phenomenon may be termed as 'polycarpy'. The seeds are recalcitrant or of the orthodox type. In nature, seeds are mainly dispersed

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by birds and animals. Although the flowers are protandrous, selfing has been reported (Gupta et al. 1996). Selfing may occur when insects visit close neighbors or different branches of the same tree (Mathew and Das 1987).

The International Neem Network has undertaken a provenance trial aimed at genetic improvement in 16 countries (Anon 1997). Studies have shown significant variability between provenances in survival, growth, morphology and physiology (Rajawat et al. 1994; Kundu and Tigerstedt 1997; 1999; Kundu et al. 1998). Isozyme and seed morphometric data (Kundu 1999) suggest that there is a high degree of genetic variation among neem populations.

The objectives of the study reported here were to estimate the outcrossing rate and to explain the genetic significance of polycarpy in a natural population of neem.

Materials and Methods

Seed materials

Open-pollinated individual tree seeds from 15 trees (Nawabganj population, latitude 23°25´N; longitude 89°15´E; altitude 15 m; mean annual rainfall 1540 mm) were received from the Bangladesh Forest Research Institute, Chittagong.

Electrophoretic analyses

From each tree, 8 seeds were analyzed. The anterior part of a germinated seed (0.3–0.5 cm in length) with radicle was excised with a scalpel and used for extraction of enzymes. The excised materials were crushed and homogenized in 4–5 drops of seed extraction buffer (Cheliak and Pitel 1985). The homogenate was absorbed onto Whatman No. 3 filter-paper wicks (3 mm×1.0 cm) and loaded onto 11% starch gels prepared with a histidine-citrate buffer at pH 6.5 (Stuber et al. 1988). The gel buffer consisted of 0.016 *M* L-histidine and 0.002 *M* citric acid, and the tray buffer of 0.065 *M* L-histidine and 0.007 *M* citric acid. Gels were cooled to 4°C before loading the samples. Electrophoresis was conducted in a cold room at 4°C±2°C with a RM6 LAUDA cooling system. Sample wicks were removed (with power off) after running for 1 h at 50 mA. The amperage was then set at 55 mA for two gels, and total running time was kept at 5.5 h.

The enzyme systems investigated malate dehydrogenase (MDH; E.C.1.1.1.37) and 6-phosphogluconate dehydrogenase (6PGD; E.C.1.1.1.44). The staining methods of Cheliak and Pitel (1985) were followed. Allelic bands were interpreted in accordance with Mendelian segregation of progeny arrays from open-pollinated families. The allelic designation followed was according to House and Bell (1994). The alleles of the loci were inferred from the single tree progeny arrays from four different populations (Kundu 1999).

Mating systems and model assumptions

Brown (1990) grouped plant mating systems into five categories: (1) predominantly self fertilizing; (2) predominantly outcrossing; (3) mixed selfing and outcrossing; (4) partially apomictic and (5) partial selfing of gametophytes. Different procedures have been developed to investigate the related issues of mode of mating systems. In a mixed mating model each zygote is assumed to result from either a self-fertilization of probability ‘s’, or outcrossed with a pollen grain chosen at random from the whole population with probability t=1–s. (A population is considered as predominantly selfing when the outcrossing rate t<0.01, but a predominately outcrossing population maintains a selfing rate of s<0.05). The mixed mating model is based upon the following assumptions: independent segregation of alleles at the different marker loci; no selection or mutation between fertilization and progeny assay; homogeneity of both the outcrossing rate and the pollen pool composition over maternal genotypes (i.e. random mating for outcrosses), and over maternal trees themselves if mother genotypes are inferred from their progeny genotypes, which is the case in the present study.

Estimation of mating system parameters

The mating system was analyzed using the multilocus mixed mating program of Ritland (1990; module MLTR, 1998 version 1.0). From progeny array data, the program simultaneously estimated: (1) the multilocus population outcrossing rate (t_m); (2) the average single-locus outcrossing rate (t_s); (3) the average single-locus inbreeding coefficient (F) and (4) the pollen and ovule allele frequencies (p’s) by the expectation maximization method. To find the confidence limit, an option of 500 bootstraps was used in the analysis. To test for homogeneity of maternal genotypes a Chi-squared test was performed.

Observation on seed development

A total of 471 seeds were counted out of 440 endocarps from the above material of 15 trees. The endocarps were grouped according to 0 (absence of seed), 1 (presence of a single seed) and 2 (presence of two seeds). Theoretically, up to 6 seeds could develop in one endocarp. The percentage table of these groups was analyzed with a chi-squared test by comparing observed frequencies in rows and columns by using the PORC FREQ procedure of the SAS statistical software package (SAS Institute, Cary, N.C.).

Results

The multilocus and mean single-locus outcrossing estimates for the population were t_m=0.904 (SE±0.034) and t_s=0.915 (SE±0.024), respectively (Table 1). The difference t_m–t_s was effectively zero (±SE 0.038), indicating there was no ‘biparental inbreeding’ in the population. The allele frequency estimates and their standard errors attributed to pollen and ovule in the progeny are presented in Table 2. Chi-squared tests of homogeneity of pollen allelic frequencies among maternal genotypes showed a highly significant difference for MDH-2 (χ^2=57.69; *df*=3; *P*<0.001); the two others MDH-3 and 6PGD-1 being non-significant (Table 3). Figure 1 demonstrates that the variance of the estimates of outcrossing rates decreases when more loci are included in the analyses. The chi-squared test on the number of seeds per

Table 1 Single and multilocus estimates of outcrossing rate in a population of *A. indica*

Locus	Outcrossing rate	SE
MDH-2	0.634	(±0.037)
MDH-3	0.625	(±0.051)
6PGD-1	1.999	(±0.000)
Mean single locus (t_s)	0.915	(±0.020)
Multilocus (t_m)	0.904	(±0.024)

Table 2 Allele frequency estimates attributed to pollen and ovules in the progeny of 15 neem trees

Locus	*N*	Allele	Pollen pool (SE)	Ovule pool (SE)
MDH-2	120	1	0.000 (0.000)	0.200 (0.068)
		2	0.806 (0.103)	0.600 (0.056)
		3	0.194 (0.103)	0.200 (0.069)
MDH-3	120	1	0.000 (0.000)	0.167 (0.071)
		2	0.875 (0.086)	0.800 (0.074)
		3	0.125 (0.086)	0.033 (0.034)
6PGD-1	120	1	0.000 (0.000)	0.333 (0.071)
		2	1.000 (0.000)	0.667 (0.071)

Table 3 Chi-square tests of homogeneity of pollen allelic frequencies among maternal genotypes (exact probabilities) (*NS*, not significant)

Locus	Maternal genotype	Chi-square	*d.f.*
MDH-2	11	57.69***	3
	12		
	13		
	23		
MDH-3	12	7.27^{NS}	3
	22		
	13		
	33		
6PGD-1	33	0.25^{NS}	1
	34		

*** $P \leq 0.001$

Table 4 Percentage of trilocular endocarps containing 0–2 seeds of *A. indica*

Tree	Number of seeds per endocarp			Number of fully grown seed	Number of endocarps
	0 (%)	1 (%)	2 (%)		
1	–	85.71	14.29	30	28
2	–	82.14	17.86	33	28
3	4.35	95.65	–	22	23
4	–	91.89	8.11	40	37
5	–	100.00	–	33	33
6	2.94	88.24	8.82	35	34
7	–	100.00	–	30	30
8	–	80.65	19.35	37	31
9	–	100.00	–	35	36
10	–	89.74	10.26	43	39
11	–	85.19	14.81	31	27
12	–	100.00	–	18	18
13	–	83.33	16.67	28	24
14	–	85.71	14.29	24	21
15	–	100.00	–	31	31
Mean	0.45	91.37	8.18	31.14	29.33

χ^2=44.39*; *N*=440; *df*=28; *P*<0.025

endocarp (Table 4) showed significant heterogeneity (χ^2=44.39; *df*=28; *P*<0.025), indicating statistically significant differences among the trees for the occurrence of seeds per endocarp. Of the 15 mother trees, two had three classes (0–2), eight possessed two classes (1–2) and the remaining 5 contained only a single seed (1) in the endocarp.

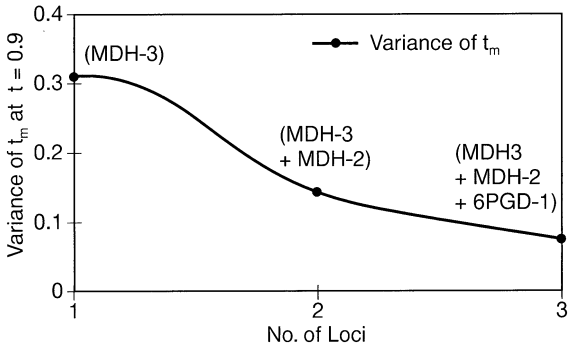

Fig. 1 Results demonstrating the decrease in variance of the estimate of outcrossing rate (t_m)

Discussion

Both multilocus (t_m=0.92) and mean single-locus (t_s=0.90) outcrossing rates were relatively high, indicating that neem is predominantly an allogamous species. The 'protandrous' phenomenon (Gupta et al. 1996) could reasonably account for the high degree of outcrossing in this species. The high level of heterozygosity detected in this species (Kundu 1999) is in accordance with the high outcrossing rate. The outcrossing rate found here is comparable to most of the studies listed by Doligez and Joly (1997) for tropical forest trees. More specifically, the present 't_m' value closely concurs with the results of Murawski and Hamrick (1991) for *Beilschmedia pendula* (Lauraceae) and *Platypodium elegans* (Fabaceae), Hall et al. (1994) for *Carapa guianensis* (Meliaceae), House and Bell (1994) for *Eucalyptus urophylla* and Murawski et al. (1994) for *Shorea congestiflora* (Diptocarpaceae). This is the first published report on the outcrossing rate in neem (*A. indica*) and could be important in formulating breeding strategies and designing seed orchards.

Single-locus outcrossing estimates varied widely between the three loci MDH-2, MDH-3 and 6PGD-1. Such variability is common for estimates of 't' determined for many predominantly outcrossing plants (Brown and Allard 1970; Brown et al. 1975). Shaw and Allard (1979) suggested including several loci in a study to obtain a reliable estimate. The outcrossing rate detected for 6PGD-1 is more than unity (t<1.999). This result is conceivably due to the unequal sampling of the pollen pool (nonrandom mating) that leads to a higher number of observed heterozygote individuals in the progeny. A large sample size could provide more reliable estimates for this locus.

Biparental inbreeding (mating of relatives within distinct populations) can be adaptive, constituting a mode of evolving reproductive isolation (Antonovics 1968). In the present study, the lack of any difference between the multilocus (t_m) and single-locus (t_s) estimates indicates that there is no 'biparental inbreeding' in the population. This result is in agreement with the study of Pascarella (1997) for *Ardisia escallonioides* (Myrsinaceae). Biparental inbreeding in tropical tree species has been reported by Doligez and Joly (1997) for *Carapa procera* (Me-

liaceae). The absence of 'biparental inbreeding' in this study strengthens the somewhat tentative conclusion that the studied population was predominantly outcrossing.

A chi-squared test indicated that observed progeny genotype frequencies did not conform to those expected under mixed mating for the locus MDH-2. Such deviation may be due to selection against homozygote genotypes, genotype-dependent outcrossing and unbalanced frequencies of pollen in the population (Ritland 1983). Thus, the present results support the hypothesis that selection against homozygotes could have occurred in the early stages of seed or seedling development. The "trilocular ovary" structure with its six ovules may be a special mechanism in neem to avoid production of inbred seed. This is closely cognate to the observations of polyembryony in conifer seeds (Koski 1971), which has been shown to be an effective mechanism against inbreeding. In this respect, it is suggested that neem suffers from inbreeding depression, contrary to what Gupta et al. (1996) observed on selfed seedlings. In the present study, a total of 471 seeds obtained from 440 endocarps indicated that, on average, an endocarp produced only 0–2 seeds- so that four to six ovules must have been eliminated during seed development. This finding could be tentatively interpreted as indicating that inbreds are selected against. The analysis on seeds per endocarp (Table 4) also indicated significant heterogeneity, a result which could point to genotypic differences in tolerance to inbreeding, a typical phenomenon of many plant species.

The degree of variance of the estimates of outcrossing rates decreased sharply when two or three loci were included in the analyses. The variance of the estimates decreased twofold when two loci, and four times when three loci were included in the analyses. Ritland and Jain (1981) in their original paper describing the mixed-mating model predicted this phenomenon of decreasing variance of the estimates of outcrossing rates through simulation studies. They explained that three or four loci with intermediate gene frequencies would provide a good estimate of the outcrossing rate. Similar results using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers were reported by Gaiotto et al. (1997) for *Eucalyptus urophylla*.

It is important to know not only how much outcrossing is taking place in a breeding population but also how much selfing occurs. Selfing in neem (*A. indica*) has been reported by Gupta et al. (1996), and inbreeding depression was not apparent. On the contrary, viable seedlings do not result from selfing of cross-pollinating teak (*Tectona grandis*) (Headegart 1973). Insects assure pollination, but by staying on the same tree for a long time they tend to favor selfing (Mathew et al. 1987). In neem the protandrous bisexual flowers and the polycarpic endocarp structure may form natural barriers against widespread selfing.

In conclusion, the reasonably high value of the present estimate of 't_m', the probable absence of 'biparental inbreeding', and variation of 'seeds' in the endocarp (polycarpy) all indicate that neem is most likely a predominantly outcrossing species. The "trilocular ovary" may constitute an efficient mechanism for avoiding inbreeding. High heterozygosity is probably maintained by protandry, early exclusion of selfed zygotes and by progressive selection against homozygous genotypes during the life of a stand.

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