causative agent of European stone fruit yellows using SYBR Green-based real-time PCR assay was developed by designing primers from the highly conserved 16S rDNA within the 16SrX phytoplasma group[13].

Phytoplasmas are difficult to detect due to their low concentration, especially in woody hosts and their erratic distribution in the infected plants[14]. Real-time PCR is a valuable alternative to the classical PCR procedure for routine diagnosis, because it is more sensitive and specific and avoids time- and resource-consuming steps like nested-PCR and agarose gel electrophoresis that can further increase the risk of sample cross-contamination.

Thus SYBR Green-based real-time PCR assay can be used in future for quick detection of phytoplasma in coconut and for identification of disease-free planting material, which is the most important strategy for management of coconut root (wilt) disease.

1. Koshy, P. K., Root (wilt) disease of coconut. *Indian Phytopathol*., 1999, **52**, 335–353.
2. Butler, E. J., Report on coconut palm disease in Travancore. Agriculture Research Institute, PUSA, Bulletin No 9, 1908, p. 23.
3. Manimekalai, R. *et al.*, Molecular detection of 16SrXI group phytoplasma associated with root (wilt) disease of coconut (*Cocos nucifera*) in India. *Plant Dis.*, 2010, **94**, 636.
4. Gasparich, G. E., Spiroplasmas and phytoplasmas: Microbes associated with plant hosts. *Biologicals*, 2010, **38**, 193–203.
5. Nejat, N. and Vadamalai, G., Phytoplasma detection in coconut palms and other tropical crops. *Plant Pathol. J.*, 2010, **9**, 101–110.
6. Solomon, J. J., Nair, C. P. R., Srinivasan, N., Gunasekaran, M. and Sasikala, M., Coconut root (wilt) – the malady and remedy. *J. Plantation Crops*, 1999, **27**, 71–92.
7. Sasikala, M., Prakash, V. R., Sapna, V. P., Mayilvaganan, M. and Leena, S. N., Refinement of ELISA and its use in early detection of coconut root (wilt) disease. *Cord*, 2005, **212**, 37–44.
8. Lee, I. M., Davis, R. E. and Gundersen-Rindal, D. E., Phytoplasma: phytopathogenic mollicutes. *Annu. Rev. Microbiol.*, 2000, **54**, 221–255.
9. Torres, E., Bertolini, E., Cambra, M., Monton, C. and Martin, M. P., Real-time PCR for simultaneous and quantitave detection of quarantine phytoplasmas from apple proliferation (16SrX) group. *Mol. Cell. Probes*, 2005, **19**, 334–340.
10. Martini, M., Loi, N., Ermacora, P., Carraro, L. and Pastore, M., A real-time PCR method for detection and quantification of '*Candidatus* Phytoplasma prunorum' in its natural hosts. *Bull. Insectol*., 2007, **60**, 251–252.
11. Marzachi, C. and Bosco, D., Relative quantification of chrysanthemum yellows (16SrI) phytoplasma in its plant and insect host using real-time polymerase chain reaction. *Mol. Biotechnol*., 2005, **30**, 117–127.
12. Ririe, K. M., Rasmussen, R. P. and Wittwer, C. T., Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem*., 1997, **245**, 154–160.
13. Yvon, M., Thebaud, G., Alary, R. and Labonne, G., Specific detection and quantification of the phytopathogenic agent '*Candidatus* Phytoplasma prunorum'. *Mol. Cell. Probes*, 2009, **23**, 227–234.
14. Marzachì, C., Molecular diagnosis of phytoplasmas. *Arabian J. Plant Prot*., 2006, **24**, 139–142.

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Contemporary gene flow and mating system analysis in natural teak forest using microsatellite markers

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Mating system as well as contemporary gene flow through pollen and seed dispersal were analysed in a disturbed natural teak population in the Peechi–Vazhani Wildlife Sanctuary, Kerala, India, using microsatellite markers. DNA analysis of 174 adult teak trees, 180 seed/fruit progenies and 100 seedlings on the forest floor revealed that this teak stand has a gene diversity of 0.563 harbouring 7% inbreeding. On comparing the genotypic fingerprints of each of the progenies and the known maternal parents as well as all the adult trees in the population, the unknown parents could be identified using the maximum likelihood method. The results showed that the gene flow through pollen acts over longer distances than through seed dispersal, since the main range of pollen dispersal distance was 151–200 m and that of seed dispersal was 50–100 m. Estimation of the multilocus outcrossing rate in this population showed that *Tectona grandis* is predominantly an outcrossing (96.11%) species. The results also showed that teak prefers multi-parental mating even up to the extent of having genetically non-identical seeds even within individual fruits. The data generated through the present study on pollen and seed migration rates and their relative contribution to total gene flow at different spatial scales are essential for developing strategies for *in situ* conservation. The information gathered is also vital for effective management of seed orchards and for formulating genetic conservation measures, as the pattern of gene flow strongly influences the genetic structure within populations.

Keywords: Contemporary gene flow, mating system, pollen and seed dispersal, *Tectona grandis*.

THE mating system of a plant species determines how the genetic information is transferred from one generation to the next[1], and it has fundamental importance for genetic conservation and breeding programmes. The pattern of gene flow via pollen and seed dispersal strongly influences the genetic structure within a population[2].

Teak (*Tectona grandis* L.f.) which belongs to the family Verbenaceae, is a large tree from the seasonally dry forests in South and South East Asia. It produces a valuable and durable timber. Studies on the breeding system in teak have been conducted in different countries

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which show that teak is almost exclusively out crossed[3]. Teak trees produce several large inflorescences each consisting of hundreds of small flowers, opening over a span of many weeks. In spite of the generally abundant flowering, seed set is low[4–8]. Details on the mechanism of pollination in teak have not been fully understood. The distance of pollen flow and seed dispersal has also not been studied. This information would be essential for formulating genetic conservation measures and for effective management of seed orchards. Inza *et al.*[9] report that modelling simulations with molecular research will improve knowledge of landscape patterns of genetic diversity within species distribution and help in developing resource management plans that enhance conservation of natural teak populations.

Studies on mating systems have benefited from the development of biochemical markers such as isozymes, since 1970s. However, with the development of DNA markers, the analysis of mating systems became more precise. Microsatellite markers present great advantages for determining mating systems since these are co-dominant and multi-allelic markers, which can be reliably scored in a simple assay[10]. Allele frequencies can be estimated directly without making crosses[11]. Power to discriminate among male parents depends on the number of markers, amount of allelic variability and frequency of alleles in the population. Microsatellites are co-dominant markers and generally have high allelic richness and heterozygosity. Hence, they are suitable for paternity analysis as well as pollen flow and seed dispersal studies.

Contemporary pollen gene flow in plant species has been intensively studied using microsatellite markers and paternity analysis approaches in open-pollinated seeds sampled from individual seed-plants[12–15].

The objective of the present study was to examine the natural mating system and contemporary gene flow through pollen and seed dispersal in *T. grandis* using microsatellite markers. Molecular analysis was carried out with eight microsatellite markers in a natural teak population.

The present study was carried out during the period 2004–2006. A continuous patch of 10 ha of disturbed mixed deciduous forest area with teak was selected at Thamaravellachal (Peechi Range) at lat. 10°30′N and long. 76°22′E in the Peechi–Vazhani Wildlife Sanctuary, Kerala, India. There were 190 adult teak trees in the selected plot area during the survey period, but the number of trees was reduced to 174 during the tenure of the study. All the 174 adult teak trees and 100 seedlings on the forest floor in this population were marked, their latitude and longitude recorded with Global Positioning System (GPS), and each tree was located and mapped using the software MapInfo Professional 8.5 (www.mapinfo.com), a desktop mapping software for location information. Developing leaves were collected from all the adult teak trees and the selected seedlings on the forest floor. Genomic DNA was extracted using a modified CTAB method[16].

Seeds were collected from nine randomly selected trees in this population and germinated. The teak fruits generally have four locules. In order to find out the number of seeds present per fruit, they were cut open. As the germination percentage was found to be low, embryos were isolated from the seeds and used for DNA extraction. DNA was extracted from 20 embryos/progenies from each of the nine trees (180 progenies in total) using the DNeasy Plant mini extraction kit (Qiagen).

DNA samples were diluted to 30 ng concentration in a volume of 2 µl. Eight microsatellite markers designed for teak by Hugo Volkaert, Kasetsart University, Thailand, were screened, out of which six markers, namely AC01, AC28, AG04, AG14, AG16 and AC44 (GenBank/EMBL accession nos AJ511746, AJ511764, AJ539416, AJ539417, AJ539419 and AJ515256 respectively) were used for the final analysis.

DNA extracted from all the 174 adult trees, 180 embryos/progenies and 100 seedling progenies on the forest floor was amplified using the eight microsatellite markers. DNA amplification was done in 12 µl (total volume of master mix) with 2 µl of DNA (30 ng), 0.1 µl of *Taq* polymerase (0.3 U), 1.2 µl of primers (12 pmol each of both forward and reverse primers) and *Taq* buffer (10× concentration), 2.4 µl of dNTPs (0.024 mM of each dNTP) and 0.2 µl of (0.02 mM) $MgCl_2$. DNA amplification was performed in a programmable thermal cycler (PTC-200, MJ Research, USA). The PCR was programmed to denature the DNA at 94°C for 3 min, followed by 32 cycles of denaturing at 94°C for 45 s, annealing at 48°C for 45 s and extension at 72°C for 1 min, and final extension of 3 min at 72°C. The quality of the amplified products was checked on 1.5% agarose.

The fragments were electrophoresed on 4.5% denaturing polyacrylamide gel containing 7.5 M urea. Next, 5 µl of aliquots was loaded on each well in the Sequi-Gen GT Nucleic Acid Electrophoresis system (38 cm × 50 cm × 0.4 mm vertical electrophoresis apparatus, Bio-Rad Laboratories) using 1×TBE as running buffer and a constant power of 90 W for one and a half hours. After electrophoresis, silver staining was done to visualize the bands. The bands were scored for allelic polymorphism as well as for heterozygosity/homozygosity.

The heterozygous and homozygous individuals were identified, as the microsatellite markers are co-dominant. Allele identification was first done in a few samples and these were then used as reference samples during the subsequent electrophoresis. Likewise, alleles were identified and marked in each set of adult trees and their progenies, including seedlings on the forest floor for all the markers.

The genotype data of all the adult trees (174) and progenies (180) were analysed[17] using the software Cervus, Version 3. The genotypic fingerprints of each of the progenies with known maternal parent were compared

with all the adult trees to find out the potential pollen donors. Through the maximum likelihood method, the male parent could be identified. The distance between the known mother parent and the newly identified male parent was measured using Map Info Professional, which gave the structure of pollen dispersal. Following the same method through the analysis of the fingerprints, both the parents of the hundred seedlings on the forest floor were also identified. The closest parent from the seedling was taken as the mother, which is the normal procedure followed usually. If a single parent was identified, it was assumed to be the maternal parent. If two parents were identified inside the population, the closer parent was considered the seed parent[18–20]. When the haplotype of the offspring is compatible with the nearest reproductive tree, it is a probable assumption that the nearest reproductive tree is the seed parent of the offspring[21]. Recent studies in *Shorea leprosula* by Fukue *et al.*[22] indicated that most immigrant seedlings originated from neighbouring mother trees. If the same individual was found to be the maternal and paternal parent, this seedling was considered a self-progeny[23]. Once the parents were identified, the distance moved by the pollen from the male parent to the female parent and by the seeds from the mother trees could be measured; and the contemporary gene flow through pollen and seed dispersal could be evaluated.

The percentage of crossing and selfing was estimated from the data generated through parentage analysis. From the total progenies analysed, the cross-pollinated as well as the self-pollinated progenies were counted to estimate the percentage of selfing. To determine the genetic structure of parent and progeny populations, various genetic diversity measures, namely heterozygosity, gene diversity[24] and inbreeding were estimated using the software FSTAT[25].

Out of the total fruits collected, 47.39% had seeds and the remaining 52.61% were without embryos. Around 38.89% fruits had only one embryo, 6.21% fruits had two embryos, 1.63% had three embryos and 0.65% had four embryos. Experiments in Thailand showed that the number of pollen grains per ovule was too low for good seed set, although high percentage of teak flowers was pollinated[7]. Studies in Kerala also concluded that the low stigmatic pollen load, which is insufficient to fertilize all the four ovules, especially in days of heavy rain is the main reason for the low seed setting in teak[7].

The amplicons of all microsatellite loci produced distinct banding patterns on the denaturing polyacrylamide gels, from which individual genotypes could be deduced (Figure 1). In the present study, out of the total eight markers tested, two (ADH-MS and CPI MS primers), which produced fewer than three alleles and did not give good results for all the samples, were excluded from further analysis.

For parentage analysis, working with a larger number of loci or highly polymorphic markers with more number of alleles is the only way to reduce the probability of more than one non-parent carrying a set of alleles that are compatible with the offspring at all loci.

The number of alleles observed for the six microsatellite markers varied from 2 to 8 in the parental population and 3 to 7 in the progenies. A total of 41 and 38 distinct alleles respectively, were obtained from the parental and progeny populations, and some of the alleles from AC01, AG14 and AC44 in the parental population were found to be lost in the progeny population. As the number of alleles per locus was found to be high, the discriminating power to identify the parents was also high. The estimate for allelic richness was found to be 6.26, and average polymorphic information content (PIC) of the six microsatellite markers was 0.501. Genetic markers showing PIC value higher than 0.5 are normally considered as highly informative in population-genetic analyses[26]. Hence the average PIC value and allelic richness of the selected populations indicated that the resolving power of the loci was sufficient and the output was suitable for unbiased estimation of individual reproductive success and to distinguish its parentage. This was also confirmed by the result of non-exclusion probabilities obtained from the parentage analysis and it was found to be nil.

The mean observed heterozygosity was lower (0.4764) than the expected heterozygosity (0.5293) in the parent population. This might be due to the mating between related genotypes. Gene diversity estimated for the parent population was 0.563, whereas it was 0.484 for the progenies. The difference in gene diversity between parental population and their progenies was less by 0.079, which must be due to loss of alleles or inbreeding. This is also confirmed by the fact that a total of 41 alleles were obtained in the parental population, whereas only 38 alleles were noted in the progenies. Analysis of genetic diversity with eight microsatellite loci in a tropical tree, *Copaifera langsdorffii*, in a small (4.8 ha), isolated population with 112 adult trees and 128 seedlings found in the stand

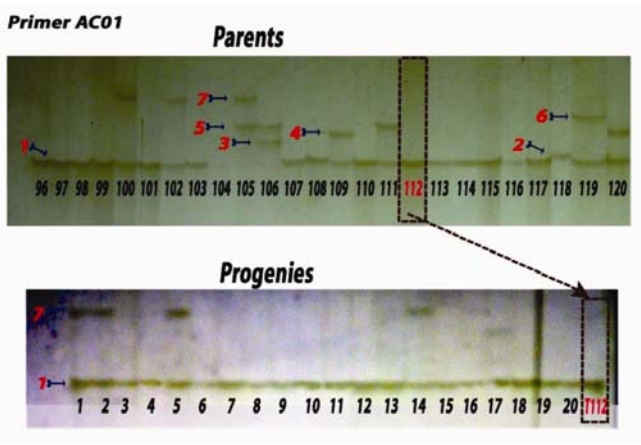

Figure 1. Comparison of alleles from progenies with mother parent.

revealed that the seedlings had significantly lower levels of genetic diversity than the adult trees[23].

In this disturbed population, as expected, significant inbreeding (heterozygote shortfall) of about 0.070 (7%) was estimated. Many factors such as inbreeding, null alleles (non-amplifying alleles) and the occurrence of population substructure (Wahlund effect) have been reported as the reasons for heterozygote deficiency in populations[24].

The main distance of seed dispersal from their mother parents was within a range 51–100 m. Maximum number of seeds (33 seedlings/33%) were dispersed within this distance range. Twenty-three per cent of the seeds were dispersed to 101–150 m range. Four per cent seeds were transferred to the maximum distance range of 251–300 m (Table 1). The maximum distance of seed dispersal observed in this plot was 291 m and the minimum distance was 8 m.

The analysis of 174 adult teak trees and 180 progenies from the population showed that pollen transfer was mainly in the range 151–200 m. The maximum distance of pollen flow was 414 m and the minimum distance, excluding selfing, was 14.4 m. Here, out of the total 180 progenies, 42 have their male parents within the main distance range (151–200 m), which contributes to 23.33% of the total progenies. A total of 17.78% of the progenies had their male parents within a range of 101–150 m. Only one of the progenies had the pollen parent in the maximum distance of 414 m, which represents only 0.56% of the total progenies. Most of the progenies (70%) had their pollen parents below 200 m distance (Table 2). No progenies were produced through pollination from outside the plot.

The pollen dispersal analysis showed that the main distance of pollen flow was below 200 m. It indicates that the pollen dilution zone must be more than 200 m in seed orchards to restrict pollen from outside. The analysis on seed dispersal showed that the dispersal was mainly in the distance range of 51–100 m. This indicated that in natural teak populations, the distance of pollen flow is more than the seed movement. Hence, major contribution in maintaining genetic diversity is from pollen transfer. For partially or fully outcrossed species, gene flow via pollen is generally believed to occur at much greater rates than gene flow via seeds[27]. In most studies on tropical plant species, gene flow through pollen acts over longer distances than through seed dispersal, with most seeds getting dispersed only over a short distance or just dropping under the parent plant[28–32].

The two factors responsible for mating distance in tropical trees are the performance of pollinators and flowering tree density[21]. The behaviour of pollinators partially determines the distance over which pollen can be dispersed[33]. The impact of population density, pollinator abundance and composition change over the range of species on the outcrossing rate and pollen dispersal at a landscape level has been discussed by several workers[34–36]. At an intermediate scale (within populations and in the space between close populations), pollinators are responsible for substantial pollen flow[37]. Higher outcrossing rates may have been promoted by encouraging more pollinators and also reducing the chance of closely related mating[38].

The present study revealed that the pollen and seeds were transferred in all directions of the plots, resulting in thorough mixing of alleles in the teak populations. This mixing may help in spreading an advantageous allele from its localized area or sub-population to the whole population[39,40], resulting in genetically diverse populations. Voigt[41] found that gene exchange via both pollination and seed dispersal influences the genetic structure of plant populations. Thus pollen and seed dispersal (gene flow) are the principal determinants of genetic structure and diversity in tree species[33]. Pollen and seed migration rates and their relative contribution to total gene flow at different spatial scales could be useful for defining strategies for *in situ* conservation[37].

Studies on the mating system revealed that cross-pollination explicitly dominated in the sampled trees. Out of the total 180 progenies, 173 were cross-fertilized (96.11%) and the rest were self-fertilized. The increased percentage of cross-pollination helps teak to be genetically highly diverse. Analysis of the seedlings on the forest floor also showed 97% cross-pollination. So estimation of outcrossing rate was not influenced by different developmental stages such as seeds or seedlings in teak, which confirmed the report of early-acting self-incompatibility during pollen tube entry into the ovule through the micropyle[8]. In few other species inbreds have

Table 1. Seed dispersal in different distance classes

Distance of seed dispersal (m)	Number/percentage of seedlings dispersed in the particular distance range
1–50	8
51–100	33
101–150	23
151–200	16
201–250	10
251–300	4

Table 2. Pollen flow in different distance classes

Distance of pollen flow (m)	No. of progenies having their male parent in the particular distance range	Percentage of progenies
1–50	21	11.67
51–100	31	17.22
101–150	32	17.78
151–200	42	23.33
201–250	17	9.44
251–300	13	7.22
301–350	11	6.11
351–400	5	2.78
401–450	1	0.56

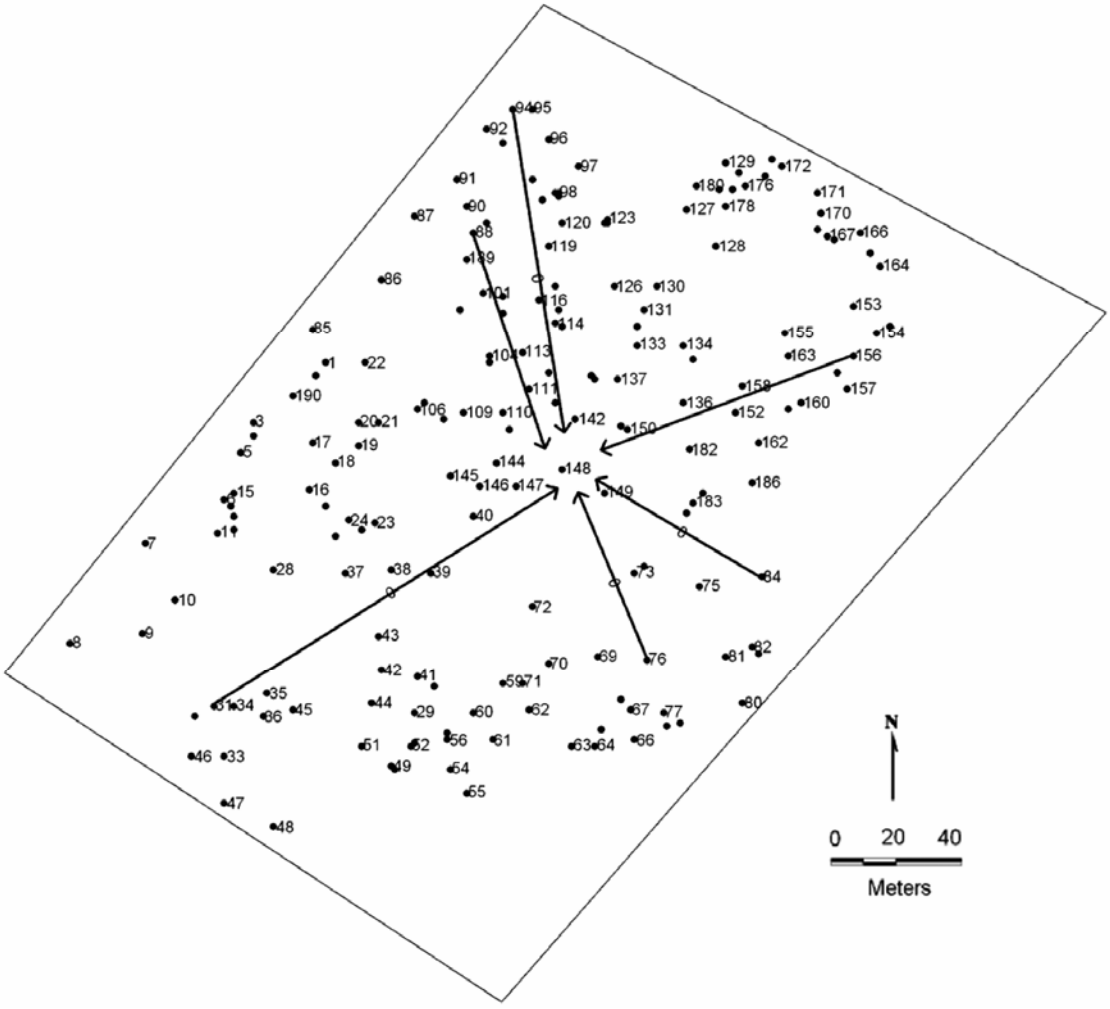

Figure 2. Pollen flow from different pollen donors to the mother tree no. 148 in the study plot.

Table 3. Number of pollen donors to produce 10 seeds by each of the mother trees

Mother tree number	151	112	178	11	148	42	100	20	124
Total number of pollen donors	9	10	9	10	10	8	6	10	7

shown low survival capacity. Bittencourt and Semir[42] documented changes in outcrossing rate with developmental stages of seeds due to late-acting self-incompatibility system in other species. So the mating system analysis proved that teak does not manifest self-incompatibility later in the seedling stage with respect to survival, which confirmed earlier reports even though these were not through DNA marker studies. This high outcrossing rate is in agreement with an earlier report in teak based on isozyme studies, where 89–95% outcrossing was observed[3].

Female fertility pattern in this disturbed plot showed that female parents received pollen from almost different directions (Figure 2). It was also revealed that most of the individual mother trees were pollinated by many pollen donor trees. When ten progenies from each of the nine mother trees were analysed, the number of pollen donors to a mother tree ranged from six to ten to produce ten progenies (Table 3). Out of the total nine mother trees, four received pollen from ten different trees to produce ten seeds, by which all the seeds turned to be dissimilar and diverse. Out of the total 26 multi-seeded fruits, each of the 23 fruits had seeds with different pollen parents, indicating that many of the flowers are pollinated by multiple male parents. Hence seeds even within one fruit are non-identical. There are possibilities for the same pollinators visiting many trees or the same flowers being pollinated by many pollinators. The activity of pollinators was reflected in the proportion of pollen donors. The high proportion of pollen donors indicates active movement of pollinators found in this plot. Data on multi-parental mating in which a female parental tree received pollen from many different pollen donors also supported the above assumption.

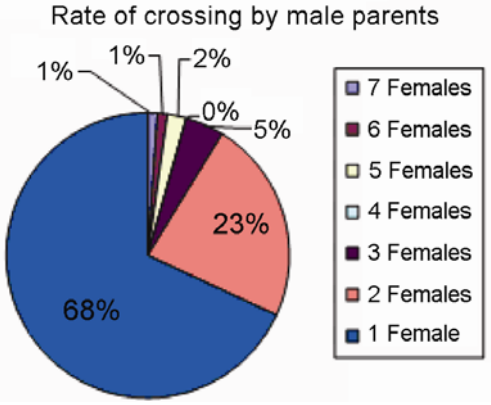

Figure 3. Percentage of male parents crossed with one or more females.

Nason *et al.*[43] found that mother trees in *Ficus dugandii* had numerous pollen donors and observed up to 11 pollen donors to produce 15 fruits and a single male parent donated pollen to 11 female trees, leading to high genetic diversity. But due to disturbance or loss of alleles, the gene diversity was found to decrease in the subsequent generation.

By analysing the male fertility pattern, one tree (no. 165) was found to have donated pollen to seven different female parental trees and produce a maximum of 11 seeds. Two other trees (nos 101 and 106) had produced five and six progenies respectively, by donating pollen to five different females. Likewise, two other trees (nos 98 and 79) produced seven progenies by crossing with six and three different female parents respectively. Tree no. 2 had produced six progenies through crossing with two different females.

Thus, a total of 91 pollen donor trees (52.3%) contributed pollen to produce 180 progenies. Out of the total 91 male parents, 68% trees crossed with only one female parent. Each of the 23% male donors crossed with two female trees, 5% with three female trees, 2% with five female parents, and 1% each with six and seven female parental trees. A maximum of seven different female parents were pollinated by a single male parent (Figure 3). The high rate of multi-parental mating, as seen in the present study, is the main reason for the high within-population gene diversity reported in teak by various authors using biochemical and DNA markers.

1. Wright, S., Systems of mating. *Genetics*, 1921, **6**, 111–178.
2. Schuster, W. S. F. and Mitoon, J. B., Paternity and gene dispersal in limber pine (*Pinus flexilis* James). *Heredity*, 2000, **84**, 348–361.
3. Kjaer, E. D. and Suangtho, V., Outcrossing rate of teak (*Tectona grandis* L.f.). *Silvae Genet.*, 1995, **44**, 175–177.
4. Hedegart, T., Breeding systems variation and genetic improvement of teak (*Tectona grandis* L.f.). In *Tropical Trees, Variation, Breeding and Conservation* (eds Burley, J. and Styles, B. T.), Linnean Society Symposium Series 2, Academic Press, London, 1976, pp. 109–122.
5. Palupi, E. R. and Owens, J. N., Pollination, fertilization, and embryogenesis of teak (*Tectona grandis* L.f.). *Int. J. Plant Sci.*, 1997, **158**, 259–273.
6. Tangmitcharoen, S. and Owens, J. N., Floral biology, pollination, pistil receptivity, and pollen tube growth of teak (*Tectona grandis* L.f.). *Ann. Bot.*, 1997, **79**, 227–241.
7. Indira, E. P. and Mohanadas, K., Intrinsic and extrinsic factors affecting pollination and fruit productivity in teak (*Tectona grandis* L.f.). *Indian J. Genet. Plant Breed.*, 2002, **62**, 208–214.
8. Mohanadas, K., Mathew, G. and Indira, E. P., Pollination ecology of teak in Kerala, KFRI Research Report No. 225, Kerala Forest Research Institute, Peechi, 2002, p. 36.
9. Inza, J. F., Yehili, J. L., Nafan, D., Simon, P. A., N'guetta, Abdourahamane, S. and Daniel, V., Genetic structure and conservation of teak (*Tectona grandis*) plantations in Côte d'Ivoire, revealed by site specific recombinase (SSR). *Trop. Conserv. Sci.*, 2008, **1**, 163–185.
10. Ferreira, M. E. and Grattapaglia, D., *Introdução ao uso de marcadores moleculares em análise genética*, Embrapa-SPI, Cenargen, Brasilia, Brazil, 1996, 2nd edn, p. 220.
11. Hamrick, J. L., Linhart, Y. B. and Mitton, J. B., Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. *Annu. Rev. Ecol. Syst.*, 1979, **10**, 173–200.
12. White, G. M., Boshier, D. H. and Powell, W., Increased pollen flow counteracts fragmentation in tropical dry forest: an example from *Swietenia humilis* Zuccarini. *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 2038–2042.
13. Robledo-Arnuncio, J. J. and Gil, L., Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Heredity*, 2005, **94**, 13–22.
14. Bittencourt, J. M. and Sebbenn, A. M., Patterns of pollen and seed dispersal in a small fragmented population of a wind pollinated *Araucaria angustifolia* in southern Brazil. *Heredity*, 2007, **99**, 580–591.
15. Bacles, C. F. E. and Ennos, R. A., Paternity analysis of pollen-mediated gene flow for *Fraxinus excelsior* L. in a chronically fragmented landscape. *Heredity*, 2008, **101**, 368–380.
16. Doyle, J. J. and Doyle, J. L., CTAB DNA extraction in plants. *Phytochem. Bull.*, 1987, **19**, 11–15.
17. Marshall, T. C., Slate, J., Kruuk, L. E. B. and Pamberton, J. M., Statistical confidence for likelihood based paternity inference in natural populations. *Mol. Ecol.*, 1998, **7**, 639–655.
18. Dow, B. D. and Ashley, M. V., High levels of gene flow in bur oak revealed by paternity analysis using microsatellites. *J. Hered.*, 1998, **89**, 62–70.
19. Bacles, C. F. E., Lowe, A. and Ennos, R., Effective seed dispersal across a fragmented landscape. *Science*, 2006, **311**, 628.
20. Nakanishi, A., Tomaru, N., Yoshimaru, H., Manabe, T. and Yamamoto, S., Effects of seeds- and pollen-mediated gene dispersal on genetic structure among *Quercus salicina* samplings. *Heredity*, 2008, **102**, 182–189.
21. Konuma, A., Tsumura, Y., Lee, C. T., Lee, S. L. and Okuda, T., Estimation of gene flow in the tropical–rain forest tree *Neobalanocarpus heimii* (Dipterocarpaceae), inferred from paternity analysis. *Mol. Ecol.*, 2000, **9**, 1843–1852.
22. Fukue, Y., Kado, T., Lee, S. L., Ng, K. K. S., Norwati, M. and Tsumura, Y., Effects of flowering tree density on the mating system and gene flow in *Shorea leprosula* (Dipterocarpaceae) in peninsular Malaysia. *J. Plant Res.*, 2007, **120**, 413–420.
23. Martins, K., Chaves, L. J., Vencovsky, R. and Kageyama, P. Y., Genetic structure based on nuclear and chloroplast microsatellite loci of *Solanum lycocarpum* A. St. Hil. (Solanaceae) in Central Brazil. *Genet. Mol. Res.*, 2011, **10**, 665–677.
24. Nei, M., *Molecular Evolutionary Genetics*, Columbia University Press, New York, 1987, p. 512.
25. Goudet, J., FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3), 2002; http://www.unil.ch/izea/softwares/fstat.html

26. Botstein, D., White, R. L., Skolnick, M. H. and David, R. W., Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet*., 1980, **32**, 314–331.
27. Levin, D. A. and Kerster, H. W., Gene flow in seed plants. *Evol. Biol.*, 1974, **7**, 139–220.
28. Dick, C. W., Genetic rescue of remnant tropical trees by an alien pollinator. *Proc. R. Soc. London, Series B*, 2001, **268**, 2391–2396.
29. Burczyk, J., Lewandowski, A. and Chalupka, W., Local pollen dispersal and distant gene flow in Norway spruce (*Picea abies* [L.] Karst). *For. Ecol. Manage*., 2004, **197**, 39–48.
30. Difazio, S. P., Slavov, G. T., Burczyk, J., Leonardi, S. and Strauss, S. H., Gene flow from tree plantations and implications for transgenic risk assessment. In *Forest Biotechnology for 21st Century. Research Signpost* (eds Walter, C. and Carson, M.), Trivandrum, 2004, pp. 405–422.
31. Trapnell, D. W. and Hamrick, J. L., Partitioning nuclear and chloroplast variation at multiple spatial scales in the neotropical epiphytic orchid, *Laelia rubescens*. *Mol. Ecol*., 2004, **13**, 2655–2666.
32. Marsico, T. D., Jessica, J. H. and Romero-Severson, J., Patterns of seed dispersal and pollen flow in *Quercus garryana* (Fagaceae) following post-glacial climatic changes. *J. Biogeogr.*, 2009, **36**(5), 929–941.
33. Dick, C. W., Hardy, O. J., Jones, F. A. and Petit, R. J., Spatial scales of pollen and seed-mediated gene flow in tropical rain forest trees. *Trop. Plant Biol.*, 2008, **1**, 20–33.
34. Franceschinelli, E. V. and Bawa, K. S., The effect of ecological factors on the mating system of a South American shrub species (*Helicteres brevispira*). *Heredity*, 2000, **84**, 116–123.
35. Dick, C. W., Etchelecu, G. and Austerlitz, F., Pollen dispersal of tropical trees (*Dinizia excelsa*: Fabaceae) by native insects and African honeybees in pristine and fragmented Amazonian rainforest. *Mol. Ecol*., 2003, **12**, 753–764.
36. Degen, B., Bandou, E. and Caron, H., Limited pollen dispersal and biparental inbreeding in *Symphonia globulifera* in French Guiana. *Heredity*, 2004, **93**, 585–591.
37. Martins, K., Chaves, L. J., Vencovsky, R. and Kageyama, P. Y. Genetic structure based on nuclear and chloroplast microsatellite loci of *Solanum lycocarpum* A. St. Hil. (Solanaceae) in Central Brazil. *Genet. Mol. Res.*, 2011, **10**, 665–677.
38. Lyngdoh, N., Joshi Geeta, Ravikanth, G., Uma Shaanker, R. and Vasudeva, R., Influence of levels of genetic diversity on fruit quality in teak (*Tectona grandis* L.f.). *Curr. Sci.*, 2010, **99**, 639–644.
39. Fisher, R. A., The wave of advance of advantageous genes. *Ann. Eugen*., 1937, **7**, 355–369.
40. Barton, N. H. and Whitlock, M. C., The evolution of metapopulations. In *Metapopulation Biology: Ecology, Genetics and Evolution* (eds Hanski, I. A. and Gilpin, M. E.), Academic Press, San Diego, 1997, pp. 183–210.
41. Voigt, F. A., Influence of pollination and seed dispersal on the genetic population structure of two *Commiphora* species in Madagascar and South Africa. Ph D thesis, Johannes Gutenberg University of Mainz, Germany, 2005.
42. Bittencourt, N. S. and Semir, J., Late-acting self-incompatibility and other breeding systems in *Tabebuia* (Bignoniaceae). *Int. J. Plant Sci.*, 2005, **166**, 493–506.
43. Nason, J. D., Herre, E. A. and Hamrick, J. L., The breeding structure of a tropical keystone plant resource. *Nature*, 1998, **391**, 685–687.

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Receding water levels hasten metamorphosis in the frog, *Sphaerotheca breviceps* (Schneider, 1799): a laboratory study

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Gosner stage 19 tadpoles of *Sphaerotheca breviceps* were exposed to constant or progressively decreasing water levels until metamorphosis with abundant food supply. The tadpoles experiencing constant water levels (column height of 40 mm) reached metamorphic climax (MC) in 35.07 ± 0.44 days and metamorphosed at 39.00 ± 0.43 days at a mean body mass of 409 ± 9.0 mg and 14.64 ± 0.08 mm snout-vent length. In contrast, the larvae experiencing decreasing water levels (from 40 to 12 mm column height) reached MC in 30.93 ± 0.35 days and metamorphosed at 34.73 ± 0.35 days at a significantly smaller body mass and size compared to those reared in constant water levels. In both the treatments survival of tadpoles was 100%. The study reveals that *S. breviceps* tadpoles are capable of developmental plasticity and with progressive decrease in water levels, the trade-off between growth and development is in favour of development, resulting in early metamorphosis at a small size.

Keywords: Metamorphosis, receding water levels, *Sphaerotheca breviceps*, tadpoles.

AMPHIBIANS generally have a complex life cycle that involves an aquatic larval stage. Further, the larval stage is critical as the larvae have to complete development and attain a threshold size before metamorphosis and emergence on land. The important metamorphic traits in anurans are larval period and size at metamorphosis[1,2]. The aquatic stage is designed to exploit the aquatic medium for growth and therefore larval duration has an impact on the size at metamorphosis. The size at metamorphosis, therefore, depends upon the developmental and growth rates during the larval stage. Most anuran amphibians opportunistically breed in temporary water bodies and face many challenges such as crowding, competition for food and space (resources), predator pressure (generally aquatic insects and carnivorous tadpoles of conspecifics and heterospecifics, etc.) and importantly, pond desiccation. All of these necessitate evolution of appropriate strategies for successful completion of metamorphosis and emergence on land[3]. Indeed, several empirical studies on anurans have shown that factors such as kinship and density[4–8], predator pressure[4,9–11], tempera-

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