Analytical results lead us to summarize that the similarity in isotopic composition of the thermal springs and non-thermal waters in both the study areas indicate that the thermal springs are primarily attributed to meteoric origin. The nature of isotopic composition implies that, the thermal springs are mostly recharged by local groundwater. Signature of oxygen isotope ratios does not endorse the contribution from water–rock interaction at great depths. The obvious $\delta^{18}O$ shift suggests that geothermal waters, particularly at 3–5 km from the geothermal springs, have continuity with shallow and surface waters.

1. Aguilera, E., Cioni, R., Gherardi, F., Magro, G., Marini, L. and Pang, Z., Chemical and isotope characteristics of the Chachimbiro geothermal fluids (Ecuador). *Geothermics*, 2005, **34**, 495–517.
2. Chatterjee, S. D. and Ghose, D., Variations in the isotopic abundance of emanating gases from some radioactive Indian thermal springs. *Indian J. Phys. A*, 1984, **58**, 345–349.
3. Ayling, B., Molling, P., Nye, R. and Moore, J., Fluid geochemistry at the Raft river geothermal field, Idaho: new data and hydrological implications. In Proceedings, 36th Workshop on Geochemical Reservoir Engineering, Stanford University, USA, 2011, SGP-TR-191.
4. Majumdar, R. K., Majumdar, N. and Mukherjee, A. L., Geoelectric investigations in Bakreswar geothermal area, West Bengal, India. *J. Appl. Geophys*., 2000, **45**(3), 187–202.
5. Mukherjee, A. L., Utilization of geoheat and water from Bakreswar thermal springs, Birbhum district. In *Mineral Development in West Bengal* (ed. Banarjee, A. K.), Oxford University Press, Oxford, 1982, pp. 131–136.
6. Majumdar, N., Majumdar, R. K., Mukherjee, A. L., Bhattacharya, S. K. and Jani, R. A., Seasonal variations in the isotopes of oxygen and hydrogen in geothermal waters from Bakreswar and Tantloi, Eastern India: implications for groundwater characterization. *J. Asian Earth Sci*., 2005, **25**, 269–278.
7. Nagar, R. K., Vishwanathan, G., Sagar, S. and Sankaranarayanan, A., Geological, geophysical and geochemical investigations in Bakreswar–Tantloi thermal field, Birbhum and Santhal Parganas districts, West Bengal and Bihar, India. In Proceedings of the Seminar on Geothermal Energy in India (eds Pitale, U. L. and Padhi, R. N.), Geological Survey of India Special Publication No. 45, 1996, pp. 349–360.
8. Sharma, S. K., Geothermal: a sustainable energy outlook for India. In Proceedings World Geothermal Congress, Bali, Indonesia, 25–29 April 2010.
9. Das, N. K., Choudhury, H., Bhandari, R. K., Ghosh, D., Sen, P. and Sinha, B., Continuous monitoring of ^{222}Rn and its progeny at the remote station for seismic hazard surveillance. *Radiat. Meas.*, 2006, **41**, 634–637.

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Real-time PCR technique-based detection of coconut root (wilt) phytoplasma

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Phytoplasmas are unculturable, phloem-limited plant pathogens and are associated with coconut root (wilt) disease. Recently, real-time polymerase chain reaction (PCR) methods were employed for the detection of plant pathogens. In the present study, a specific and efficient real-time PCR-based detection system for coconut root (wilt) phytoplasma was developed using double-stranded DNA intercalating dye, SYBR Green. Primers were designed to specifically amplify a 218 bp fragment from the 16S ribosomal DNA region. A melting curve analysis was programmed at the end of the cycling reaction. A unique melting peak at 80.71°C was observed for infected coconut samples. The healthy and no-template control had a lower or no melting peak. The PCR products were also sequenced and results were analysed using Blastn. The sequences showed similarity with sugarcane white leaf disease phytoplasma, with the nucleotide identities being more than 98%. This technique is highly sensitive for the detection of phytoplasma in coconut.

Keywords: Coconut root (wilt), phytoplasma, real-time PCR, SYBR Green.

THE root (wilt) disease is a major threat to coconut cultivation in South India, especially in Kerala and is the major reason for low coconut productivity in the state[1]. The disease is non-lethal but debilitates the productive capacity of coconut palms. The diagnostic symptoms include flaccidity of leaflets, yellowing of older leaves, necrosis of leaflets and deterioration and decay of the root system[2]. The leaflets bend inward to produce the typical ribbing symptom so that the whole frond gives a cup-like appearance. Abnormal shedding of buttons (female flowers) and immature nuts are also noticed. The root (wilt)-affected palms become susceptible to diseases like leaf rot and pests like rhinoceros beetle and red palm weevil.

Phytoplasma belonging to 16SrXI group is established to be associated with coconut root (wilt) disease[3]. Phytoplasmas are phloem-bound, unculturable, cell wall-less prokaryotes bound by a trilamellar unit membrane. They spread from plant to plant by the sap-sucking insect vector (*Proutista moesta*) and have a dual host cycle in which they can replicate in their insect vectors as well as in the sieve tubes of their plant hosts[4].

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The current management strategy for coconut root (wilt) is to remove severely infected palms. Hence a sensitive and reliable diagnostic method for quick and early detection is of immense use. Detection and identification of phytoplasma are necessary for accurate disease diagnosis[5]. Earlier methods of diagnosis were dependent on characterization of plant symptoms and observation of phytoplasmas in ultrathin sections of diseased plants[6]. Sasikala *et al.*[7] developed a DAC indirect ELISA protocol for early detection of coconut root (wilt) phytoplasma. Molecular techniques for phytoplasma detection have proved to be more accurate and reliable than the conventional methods. Polymerase chain reaction (PCR) amplification of the 16S rDNA using phytoplasma-specific universal and group-specific primers has been employed widely in diagnostics[8]. Detection of coconut root (wilt) phytoplasma using the universal primer pair P1/P7 gives inconsistent results[3]. Usually the conventional PCR method necessitates nested PCR because of the low titre of phytoplasma in the host plant, especially in woody plants like coconut. Because the concentration of phytoplasma in the nucleic acid preparation obtained from infected plants is less and one-step PCR cannot produce detectable amounts of products, nested and semi-nested PCRs have to be followed wherein the products generated using the first round of PCR are again amplified using the phytoplasma group-specific primers in the subsequent PCRs. For coconut root (wilt) phytoplasma, semi-nested primer pairs were designed from the sequencing of a 1.8 kb fragment amplified by primers P1/P7 (GenBank accession no. FJ794816) from a phytoplasma infected coconut sample[3]. But the detection system requiring PCR and nested-PCR followed by gel electrophoresis is time-consuming. Recently, real-time PCR methods have been employed for the detection of plant pathogens, including phytoplasma because of the accuracy, specificity, less analysis time and absence of post-PCR manipulations that prevent carry-over contamination. Real-time PCR has been effectively used for the detection of phytoplasma diseases in many crop plants[9–11].

The objective of the present study was to develop a specific and efficient real-time PCR-based detection system for coconut root (wilt) phytoplasma using double-stranded DNA intercalating dye, SYBR Green. Samples from infected palms were collected from two locations in Kerala, namely Kasaragod and Kayamkulam (Table 1). DNA was extracted from 3 g spindle leaf tissue using a modified phytoplasma-enrichment protocol. Grassy shoot diseased sugarcane sample (S4) was used as the positive control. Healthy coconut sample was taken as the negative control.

The 16S rDNA of phytoplasma was selected as the target for amplification. Initially, a 1.8 kb fragment of the 16S rDNA was amplified from the infected sugarcane and a coconut sample using universal primer pair P1/P7. The PCR products were eluted from agarose gel using Gel Elute Kit (Sigma), cloned into pTZ57R/T cloning vector (Fermentas Life Science) and sequenced. Subsequently, clones from the sugarcane sample were screened by appropriate restriction enzymes and positive clones were picked to isolate the purified plasmid. This plasmid sample was used as the positive control. The 16S rDNA sequence obtained from the infected coconut sample (GenBank accession no. FJ794816) was used to design a specific primer pair F1 (CGTCTAAGGTAGGGTCGATGA)/R1 (GGACTTGAACCACCGACCTC) to amplify a 218 bp fragment for real-time PCR (Figure 1). The primer sequence was BLAST-searched against the bacterial sequence database of NCBI. Primers were selected based on no similarity with bacterial sequences in order to have sensitivity in amplification of the phytoplasma sequence.

Real-time PCR was performed with the Stratagene Real Time PCR system and the results were analysed using MxPro software. Fluorescent molecule, SYBR Green, was included in the PCR master mix. Two and a half microlitres of DNA sample (20 ng) was added to a reaction mix containing 0.16 μM of each primer, 6.5 μl of Brilliant II Fast SYBR Green QPCR Mastermix (Stratagene) and 3 μl of sterile MilliQ water to make a final volume of 15 μl. The thermal cycling conditions included a preincubation at 95°C for 10 min (segment 1), followed by 40 cycles of amplification at 95°C for 10 s and 55°C for 30 s (segment 2), and finally the melting curve analysis at 95°C for 10 s, 55°C for 30 s and 95°C for 30 s (segment 3). The *Ct* (cycle threshold) value was automatically calculated by plotting fluorescence intensity against the number of cycles. All reactions were performed in duplicate with positive, healthy and no-template controls (NTC). The PCR products were further checked on agarose gel. The products were eluted using Gel Elute Kit (Sigma) and sequenced to confirm the identity of the amplicons. The sequences were subjected to similarity search using Blastn. Multiple sequence alignment using ClustalW was performed to get the relatedness among phytoplasma sequences from different samples.

Table 1. Details of plant samples used for real-time PCR detection of root (wilt) disease phytoplasma and location of sample collection. Root (wilt) is endemic in Kayamkulam, whereas it is found in isolated pockets in Kasaragod, Kerala

Sample	Location of collection
36	Kasaragod
15cci	Kayamkulam
38ai	Kayamkulam
34ai	Kayamkulam
5bi	Kayamkulam
64a	Kayamkulam
7ai	Kayamkulam
22di	Kayamkulam
Healthy	Kasaragod

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>coconut rwd p1/p7
tacaatggctgttacaaagagtagctgaaacgcaagtttatagccaatctcataaaagcagtctcagttcggattgaagtctgcaactcgacttca
tgaagttggaatcgctagtaatcgcgaatcagcatgtcgcggtgaatacgttctcggggtttgtacacaccgcccgtcaaaccacgaaagttggta
atactcaaaaacggtagcctaacttcttcggaagagggaacCGTCTAAGGTAGGATCGATGAttggggttaagtcgtaacaaggtatccctaccgg
aaggtggggatggatcacctcctttctaaggaataatttttttaaatattcatcttcagttttgaaagacttagttcttataagtttttctttttta
aggaaaagaaaatgggcctatagctcagttggttagagcacacgcctgataagcgtGAGGTCGATGGTTCAAGTCCatttaggcccaccaactgaa
aaaaaggtctttttttctaattcttaaaaaaaaagttctttgaaaagtagataaacaaag
```

Figure 1. Binding site of primers F1/R1 on the phytoplasma 16S rDNA sequence. Red colour indicates F1/R1 primer binding site on the coconut root (wilt) phytoplasma 16S rDNA sequence (GenBank accession no. FJ794816).

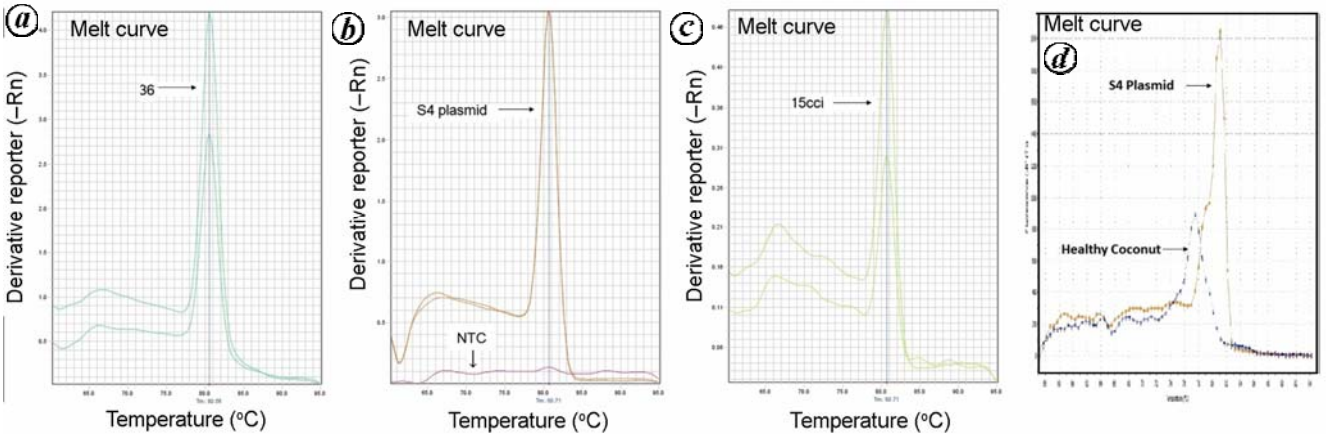

Figure 2. Dissociation (melting) curve analysis. ***a***, Infected coconut sample 36. ***b***, Positive control S-4 plasmid sample and the no-template control (NTC). ***c***, Infected coconut sample 15cci; ***d***, Positive control S-4 plasmid and negative control healthy coconut. Melting peak is obtained at 80.71°C for infected coconut and the positive control. NTC had no melting peak, while the healthy sample has a lower peak indicating primer dimer formation.

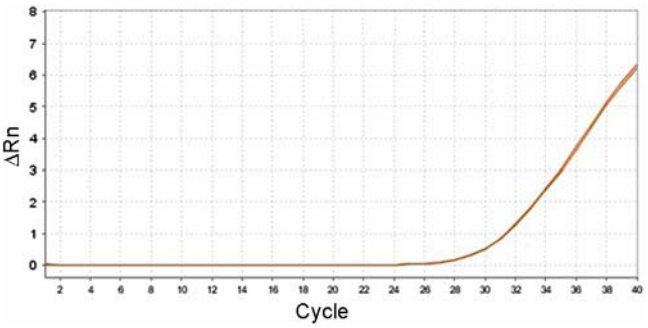

Figure 3. Amplification plot for a diseased coconut sample. The *Ct* value is calculated by plotting the normalized fluorescence against the number of cycles.

The primer pair F1/R1 amplified a fragment of 218 bp from root (wilt) infected coconut samples and the positive control sugarcane sample. However, no amplification was seen for the healthy coconut sample and NTC. All of the eight diseased samples used in this study showed positive result, indicating a PCR efficiency of 100%. The dissociation (melting) curves for infected coconut and sugarcane samples had a single peak at 80.71 ± 0.7°C, indicating that only the target fragment was amplified. The NTC showed no melting peak, whereas the healthy sample showed a lower melting peak (Figure 2). The mean *Ct* value of the samples ranged from 25.56 to 38.24 (Table 2). The sugarcane plasmid sample had a low *Ct* mean of 11.75. The healthy sample and the NTC showed no *Ct*. The amplification plot for a diseased coconut (sample no. 36) is shown in Figure 3. It can be seen that the *Ct* value for the sample is 25.63.

Similarity search using Blastn revealed that sequences were similar with sugarcane white leaf phytoplasma 16S rDNA (GenBank accession no. FM208260.1), with the nucleotide identity being more than 98%. Multiple sequence alignment using ClustalW revealed high conservation between phytoplasma 16S rDNA sequences from different coconut samples (Figure 4). Representative sequences were deposited in NCBI GenBank Database (accession nos JF732990, JF732991, JF732992).

This study presents a SYBR Green-based real-time PCR assay for phytoplasma detection from root (wilt) diseased coconut samples. Detection involving single-step PCR is less sensitive and usually requires nested-PCR. Among the eight diseased samples studied here, only one coconut sample (sample no. 36) could be detected using single-step PCR. Sample 5bi, which could not be detected even in nested-PCR, showed positive result using real-time PCR. Thus, real-time PCR represents a fast and sensitive alternative to conventional nested-PCR used for phytoplasma detection. Among the various detection chemistries

Table 2. Mean cycle threshold (*Ct*) value and melting temperature (*Tm*) of the product. SYBR Green was used as the reporter molecule. Reactions were performed in duplicate and the mean *Ct* was determined. *Tm* of the product was determined to be 80.71 ± 0.7°C. There was only a single peak

Sample	Mean *Ct*	*Tm*
36	25.56	80.71 (Figure 2 *a*)
15cci	36.31	80.71 (Figure 2 *c*)
Sugarcane plasmid	11.75	80.56 (Figure 2 *b*)
38ai	36.21	80.71
34ai	34.60	81.01
5bi	35.65	81.01
64a	27.83	80.71
7ai	37.99	81.01
22di	38.24	80.86
Healthy	–	– (Figure 2 *d*)
NTC	–	–

Total number of samples studied	11
Negative control	2
Samples amplified	9
PCR efficiency	100%

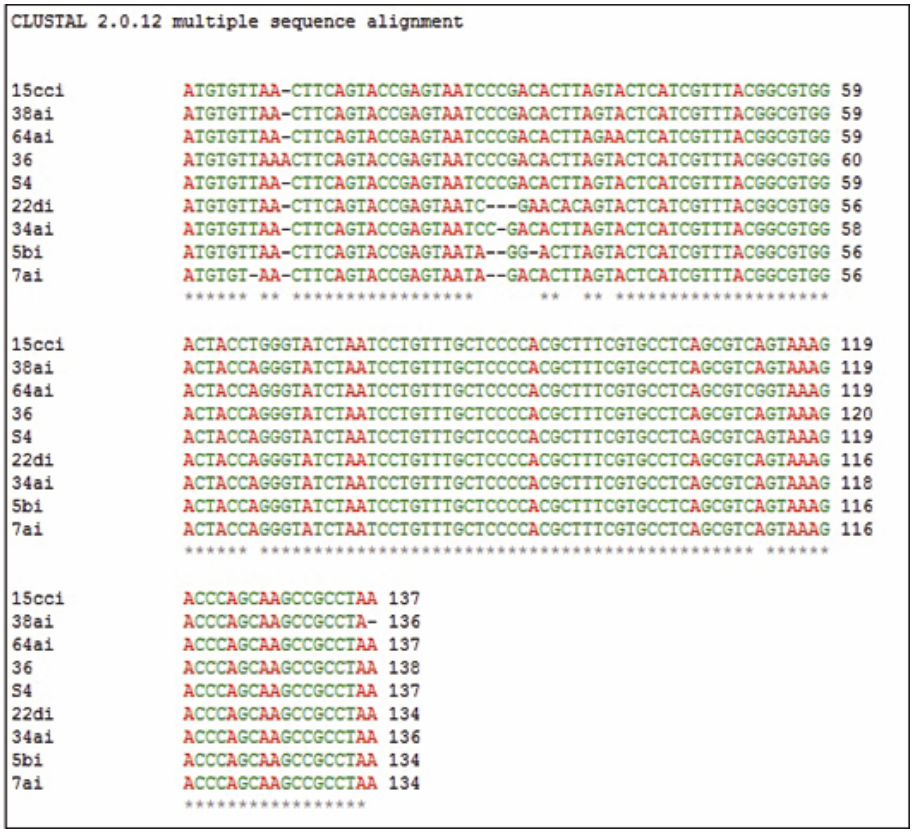

Figure 4. Comparison of partial nucleic acid sequences of phytoplasma 16S rDNA using ClustalW. The asterisk (*) denotes conserved regions.

for monitoring real-time PCR products, the SYBR Green-based method used in the present study is the most economical one. Although the dye binds to the minor groove of double-stranded DNA in a sequence-independent manner, the precision of the PCR amplification can be checked by melting curve analysis[12]. In a previous study, SYBR Green-based real-time PCR was used successfully for the detection of *Candidatus* Phytoplasma prunorum from infected apricot trees and the insect vector, *Cacopsylla pruni*[10]. Three different phytoplasmas from the apple proliferation group (16SrX) were detected and quantified using real-time PCR conjugated with fluorescent SYBR Green[9]. The melting peak characteristic of the phytoplasma was detected and could differentiate the apple proliferation group from the other phytoplasma groups. A diagnostic technique for '*Ca*. P. prunorum',

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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