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Communication

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Overcoming the pollination barrier through artificial pollination in the Wild Nutmeg *Knema attenuata* (Myristicaceae), an endemic tree of the Western Ghats, India

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Abstract: The barrier to pollination and pollinator assemblage were investigated in *Knema attenuata*, a dioecious tree species endemic to the Western Ghats of India. It occupies an intermediate canopy stratum of the low and mid-elevation wet evergreen forests. In order to observe floral display, insect foraging and fruit development, four populations of *K. attenuata* were selected. The population diagram of each population was constructed by marking one female tree as the centre and male trees available at different radii from the female tree. Direct observations and swap net trapping were used to sample insects in the canopy during the flowering season of 2016 and 2017. *Knema attenuata* exhibited generalised pollination through diverse insects: thysanopterans (thrips), coleopterans (beetles), halictid bees, and dipterans (syrphid and phorid flies), where thrips played the major role. On analysing the floral display, it was found that the male flowers provided no rewards and thus attracted less pollinators than the female flowers. Among the four populations studied, three showed more than 70% fruit setting and the rate of abscission in flowers and young fruits were negligible. One population was without fruit setting and trials on artificial pollination resulted in fruit setting. A very low frequency of seed germination was observed in natural conditions which was enhanced by a seed germinator.

Keywords: Fruit setting, seed germinator, syrphid flies, thrips.

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Author details: M.G. Govind, Research scholar (University of Kerala), Plant Genetic Resource Division JNTBGRI perusing PhD entitled chemotaxonomic studies on the family Myristicaceae from the Western Ghats. Research involves complete taxonomic revision as well as phytochemical profiling of all Myristicaceae members of the Western Ghats. Dr. K.B. Rameshkumar, Senior Scientist, Phytopharmacology Division actively working on phytochemical profiling endemic plants of the Western Ghats especially with high medicinal potential. Currently Principal Investigator of SERB funded project 'Phytochemical studies on Indian Cyperaceae'. Dr. Mathew Dan, Head and Senior Scientist Of Plant Genetic Resource Division, JNTBGRI actively involved in conservation and charecterisation medicinal plants research and a leading plant taxonomist specialized in Zingeberaceae. Currently principal investigator of SERB funded project 'Revision of Indian Piperaceae' .

Author contribution: First author carried out Research work as part of PhD program under combined guidance and supervision of second and third authors.

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INTRODUCTION

Myristicaceae R.Br. has a pantropical distribution, represented by about 21 genera and 520 species (Christenhusz & Byng 2016). The members are well represented in the moist evergreen forests of the Western Ghats by three genera — *Knema* Lour., *Myristica* Gronov. and *Gymnacranthera* Warb. Genus *Knema* has 93 species in total (Mabberley 2018) and the distribution ranges from southern India through southeastern Asia to southern China and Indo-China, and throughout Malaysia (Wilde 1979). In India, eight species and two sub species of the genus *Knema* were reported. In the Western Ghats, *Knema attenuata* (Wall. ex Hook.f. & Thomson) Warb. is the only representative which is also endemic (Nayar et al. 2014). *Knema attenuata* is a Least Concern (World Conservation Monitoring Centre 1998) riparian, dioecious, medium-sized tree species. It mainly inhabits forest river basins and low–mid elevation of forest areas of the Western Ghats. It is one of the ingredients of 'Ashwagandadhi nei' a medicated ghee used in Ayurvedic treatment (Ravikumar et al. 2000). The plant has anti-bacterial, anti-fungal, anti-oxidation, anti-larval, and insecticidal properties (Vinayachandra et al. 2011; Vinayachandra & Chandrashekar 2014). *Knema attenuata* is known as 'Chora pine' in Malayalam due to its blood coloured exudates from its bark. Because of its regularly whorled axial branching pattern, people just cut them down and use it as a cloth stand.

There are many conflicting reports on the pollination of the family Myristicaceae. Many researchers have pointed out the presence of a specialized beetle pollination syndrome (Armstrong & Drummond 1986; Armstrong & Irvine 1989; Armstrong 1997; Momose 2005). In *Knema*, different beetles belonging to Curculinoids, Staphylinids, and Chrysomelids were reported as pollinators (Momose 2005). The floral morphology of this family might also host non-beetle pollinators. Thrips have always been found on Myristicaceae and have been established as pollinators in *Horsfieldia grandis* in Sarawak (Momose et al. 1998) and *M. dactyloides* in Western Ghats (Sharma & Armstrong 2013).

The present study was an attempt to determine the pollinators, to conduct artificial pollination trials, to confirm the pollination barrier, and to find out the germination efficiency of seeds in *Knema attenuata*.

MATERIALS AND METHODS

Population study

Four populations of *K. attenuata* were selected from different localities of Agasthyamala Biosphere Reserve of the southern Western Ghats and GPS (with Garmin etrex 30) coordinates were recorded. The sites were Ponmudi forest area (Population 1), Kallar eco-tourism area (Population 2), Shendurney Wildlife Sanctuary (Population 3), and Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) campus in Palode (Population 4), and the map of the study area was prepared using QGIS software (Fig. 1). The study was carried out during peak flowering and fruiting seasons, November–February, in the consecutive years 2016 and 2017. In each population, one healthy female tree with girth at breast height (GBH) of more than 30cm was spotted and marked. The male trees within a 100m radius of the central female plant were marked and a population diagram was drawn ("RADAR model"). The perimeter from female tree was divided into six classes and the number of male trees in each class was marked indicating the vicinity of male plants (Fig. 2).

Morphological characterisation

Quantitative morphological characters (length and diameter of fruits) as well as qualitative characters of (colour, taste, smell, and texture of aril and rind) were recorded (Table 1). The flower exhibition and enumeration were also recorded. The number of flowers in 30 inflorescences each on four female trees and four male trees representing different populations were evaluated using standard arithmetic mean and standard deviation. The female flowers were dissected and examined under a stereo microscope (Carl Zeiss Stemi DV4) to observe gynoecium characters. The nectar measurements were made using graduated microcapillaries. The pollen grains from 10 flowers of male trees in each population were taken and the viability was assessed on alternate days after anthesis using acetocarmine staining technique. The viability was calculated as per the standard procedures proposed by Shivanna & Rangaswamy (2012) using a Leica DM 2500 microscope.

$$\text{Pollen viability (\%)} = \frac{\text{Number of stained pollen}}{\text{Total number of pollen}} \times 100$$

Observations on pollination

The insects near the inflorescence were caught using a sweep net, immobilized using chloroform vapour and

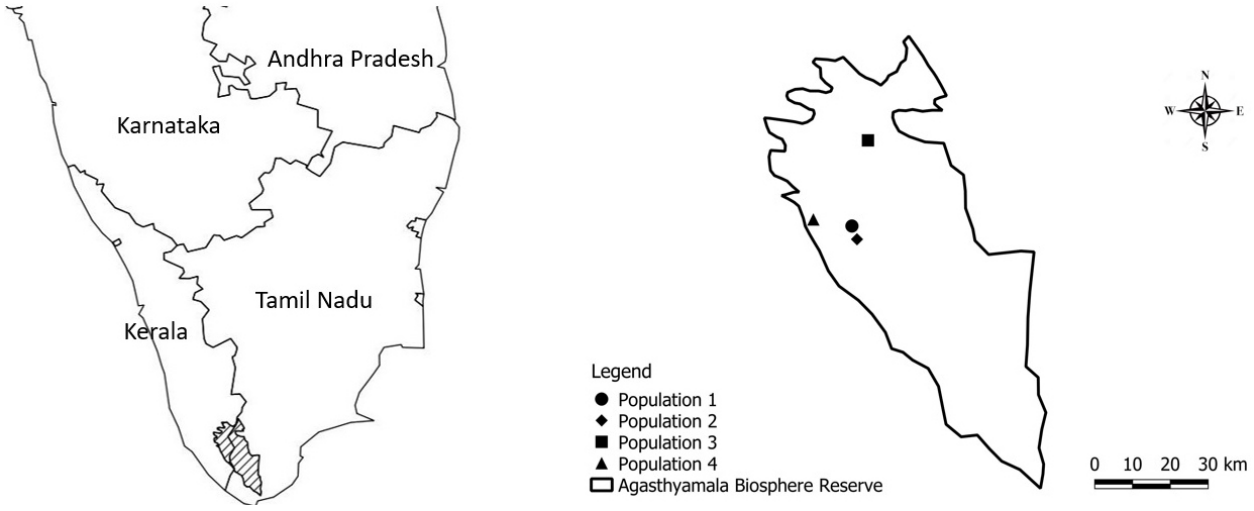

Figure 1. Population 1—Ponmudi Hills (8.443^{0}N, 77.071^{0}E) | Population 2—Kallar eco-tourism area (8.423^{0}N, 77.080^{0}E) | Population 3—Shendurney Wildlife Sanctuary area (8.565^{0}N, 77.093^{0}E) | Population 4—Jawaharlal Nehru Tropical Botanic Garden and Research Institute campus (8.452^{0}N, 77.014^{0}E).

scanned under a stereo microscope (Carl Zeiss Stemi DV4). Those that contained any trace of pollen grain on their bodies were considered as potential pollinators. Identification of insects up to generic level was carried out by matching with standard references on the pollinators of Myristicaceae. The stigma of five female flowers from each population was observed under the stereo microscope to assess pollen deposition.

Though profuse flowering, receptive stigma, and the presence of suspected pollinators were observed in the female plant of *K. attenuata* in JNTBGRI campus (Population 4) during 2016 and 2017, fruit setting was totally nil. Therefore, artificial pollination was carried out. Fifty flower buds from 20 inflorescence were tagged (minimum 2 in each inflorescence) and bagged. The flowers were completely opened, after 16 days and a sticky exudate was found in the stigma. Using the pollen of male flowers collected from population 4, 48 flowers were artificially pollinated by softly rubbing the anther disc directly on to the stigma of the flower and the remaining two were set as control to examine the number of days taken for abscission of non-pollinated flowers (Image 1e). The pollinated and control flowers were re-bagged.

Germination

For germination study, 38 seeds obtained through artificial pollination (population 4) and 70 seeds each from normal fruit setting (populations 1, 2, 3) were selected. Each batch of seeds was wrapped in wet acid free paper towels, labelled, and placed in a seed germinator (Kemi Seed Germinator) in darkness, maintained at 30 ± 2^{0}C, and 80% relative humidity (RH). The vigour calculation was done using standard protocol (Czabator 1962).

Germination vigour = Mean daily germination (MDG) x Peak value.

$$\text{MDG} = \frac{\text{Final germination percentage}}{\text{Days for complete germination}}$$

$$\text{Peak value} = \frac{\text{Highest see germination}}{\text{No. of days for germination}}$$

Ten seeds from artificially pollinated population (population 4) and 20 each from naturally pollinated populations (populations 1, 2, 3) were sown in the experimental plot providing same edaphic conditions to evaluate germination.

RESULTS

Floral Morphology

An Inflorescence of both male and female contain 3–5 flowers. Male flowers were comparatively smaller than female flowers (Image 1a,b), both with three-lobed perianths. The androecium was stalked with 13 stamens arranged on dark red staminal disc. The gynoecium was with two stigmas, thick and short style and ovoid ovary. Maximum viable pollen grains were recorded

Table 1. Quantitative and qualitative characters of *Knema attenuata* mature fruits.

Characters	Population 1	Population 2	Population 3	Population 4*
Length (cm)	3.9 ± 0.05	3.5 ± 0.02	3.6 ± 0.03	3.6 ± 0.02
Diameter (cm)	2.50 ± 0.40	2.5 ± 0.38	2.6 ± 0.32	2.5 ± 0.23
Odour of aril	Pleasant	Pleasant	Pleasant	Pleasant
Taste of aril	Slightly sweet	Slightly sweet	Slightly sweet	Slightly sweet
Texture of rind	Tomentose	Tomentose	Tomentose	Tomentose

*-Artificially pollinated fruits. Population 1—Ponmudi Hills | Population 2—Kallar eco-tourism area | Population 3—Shendurney Wildlife Sanctuary area | Population 4—Jawaharlal Nehru Tropical Botanic Garden and Research Institute campus.

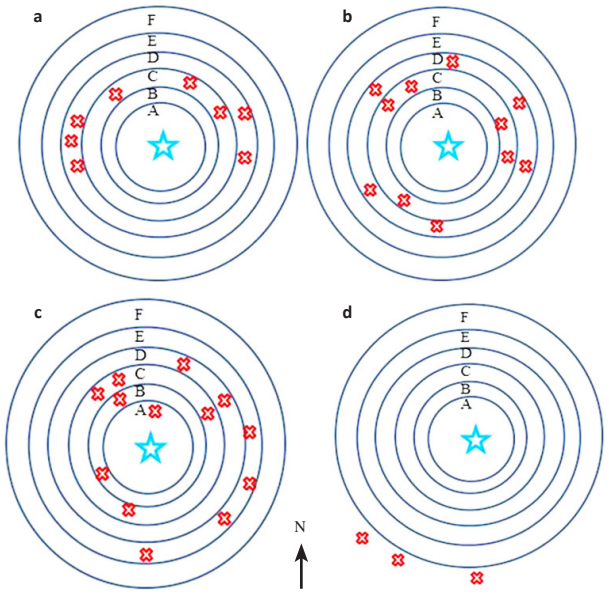

Figure 2. Population diagrams showing male distribution around female trees: a—population 1 | b—population 2 | c—population 3 | d—population 4. A—0–10 m | B—10–30 m | C—30–50 m | D—50–70 m | E—70–90 m | F—90–100 m.
☆—***Knema attenuata*** **female tree** ⊠—***Knema attenuata*** **male tree**

on the second and third day (66.4 ± 5.3% & 64 ± 5.8%, respectively) after anthesis. Morphologically the flowers in all the four populations were identical.

Proximity of male trees and pollination efficacy

Among the four populations studied, population 3 from Shendurney Wildlife Sanctuary showed the maximum success in pollination; that is about 70% of the female flowers produced fruits, which directly correlated to more number of male trees nearer to female tree, i.e., 13 male trees within 100m radius. Populations 1 and 2 showed comparatively lesser success rate of fruit setting, about 50% and 60%, respectively. It was in accordance with the lesser number of male trees, i.e., eight and 11 trees respectively within 100m radius, where the nearest one was 50–70 m from the female tree. There was no fruit setting in population 4 where the nearest male tree was beyond 100m from the female (Fig. 2d). Out of 60 female flowers examined from each population (populations 1, 2 & 3), 55–60 pollen grains were spotted on stigma in 3–4 day old flowers which showed 77.3 ± 4.6 % viability. Whereas, the female flowers of population 4 showed no traces of pollen grains, clearly indicating the remote occurrence of male trees (Fig.s 2a,b,c). The length of mature fruit was found to be 3.9 ± 0.05 cm and diameter (just before splitting) 2.5 ± 0.04 cm. The rind of fruits was golden brown in colour and tomentose in texture. The average fresh weight of the fruit was 15.05 ± 0.67 g. The bright red aril fully covered the shiny brown seed (Image 1f).

The observations on insect visits revealed that thrips and syrphid flies were frequent visitors to both male and female flowers. Thrips were observed more and about 60% of them carried pollen. Some syrphid flies were also spotted with pollen grain. Non-pollinating visitors like ants and wasps were also observed. Though the presence of insects was observed in population 4, insects with pollen grains were not observed.

Artificial pollination

All artificially pollinated flowers in population 4 produced fruits and early stage abscission was not observed during fruit development and thus the success rate was 100%. The dimension of each fruit was recorded at an interval of five days of growth stage and compared to the data of naturally pollinated fruits, and no significant variation was found. The morphology of fruits in both types were also found identical (Table 1).

Seed germination

Seeds from both naturally as well as artificially pollinated flowers exhibited a similar period of dormancy, about 30 days in the seed germinator. Germination on both were observed from the 31st day onwards and

Image 1. *Knema attenuata*: a, b—male and female flowers | c—development stages of female flower | d—thrips bearing pollen | e—hand pollination | f—fully matured fruit | g, h—seed germination stages of natural and artificially pollinated fruits. © M.G. Govind.

the plumule appeared on the 46th day (Images 1g,h). The germination vigour of naturally pollinated seeds was slightly more than that of artificially pollinated seeds, 0.44 and 0.37, respectively. The percentage of germination in both naturally and artificially pollinated seeds was almost similar (82–84 %). The seeds of both groups were much delayed (> 80 days) to germinate when sowed in the experimental plot.

DISCUSSION

Though specialised beetle pollination syndrome was reported in Myristicaceae (Armstrong & Drummond 1986; Armstrong & Irivin 1989; Armstrong 1997), in *Knema* there were no previous reports on pollination. The present study throws some light on pollinators, the pollination barrier, and the possibility of artificial pollination in *K. attenuata*. During the study, pollen bearing thrips were detected in three populations. Earlier studies on Myristicaceae also established the presence of thrips with pollen (Armstrong & Drummond 1986; Armstrong & Irivin 1989; Williams et al. 2001). Another interesting observation was the occurrence of nymphs of thrips inside the urn-shaped flowers (Image 1d); this correlated with the observation by Moog et al. (2002) that the *Myristica* flowers appeared to be the hatching sites of thrips.

A high concentration of thrips was observed in the female tree, where male trees were at close proximity. Armstrong (1997) also specified that close vicinity of plants could result in effective movement of thrips from male flowers causing pollen export to female flowers. Syrphid flies (hover flies) were also clearly observed on both male and female plants showing scavenging activity and pollen shipment. Sharma & Shivanna (2011) reported the presence of hover flies in *Myristica dactyloides* and discovered them as the major pollinators. Due to wider male flowers and exposed stigma, the pollinators gain easy access to the flowers of *K. attenuata*.

Some beetles execute utilisation of female flowers as suitable sites for agonistic and mating activities (Gottsberger 1977, 1988; Goldblatt et al. 1998) and certain beetles prefer the temperature inside the flower (Bay 1995; Seymour & Schultze 1997; Bernhardt 2000), whereas others show forage activity on sticky exudation on stigma or petals (Momose 2005). Sharma & Shivanna (2011) observed the same phenomenon in *M. dactylodes* from Western Ghats, and stated that wet stigma and urn-shaped flower provide food and shelter for the beetles. The observations perfectly matched with that of the present study in *K. attenuata* where typical semi urn-shaped flower and sticky stigmatic exudate gave some reward for the pollinators. In *K. attenuata* floral exhibition was higher in male trees than female because of the attractive bright red-coloured staminal disc.

The investigations by Armstrong & Drummond (1986), Sharma & Shivanna (2011), and Sharma & Armstrong (2013) revealed that thrips as well as beetles are pollinators in certain species (*Myristica fragrans, M. dactyloides, M. fatua*, and *Gymnacranthera canarica*) of Myristicaceae. Sharma & Shivanna (2011) stated that majority of the loss in fruit set in *M. dactyloides* was due to flower abscission and the rest by the fruit abortion. But in *K. attenuata* it was observed that, floral abscission was comparatively very low and more than 70% flowers produced fruits and the rate of fruit abscission during maturation was also very low. Howe & Westley (1997) stated that high pollination efficiency and normal fruit set combined with observations on flower abscission and fruit abortion indicates no pollination limitation in the population. In spite of the receptive stigma in healthy female flowers and presence of pollinators and fertile pollen in the available male plant, pollination was absent in population 4 of *K. attenuata,* just because of the remoteness of the male plant. All the other populations studied (populations 1, 2, 3) showed >50% success in fruit setting. In all the four populations studied, abiotic and biotic components were almost similar except the proximity of male plants to female plants. All the data offers clear evidence that in *K. attenuata*, in spite of all favourable parameters, the distance of the male plant from the female plant affects successful pollination.

Trials on seed germination showed that seeds from both artificially pollinated and naturally pollinated flowers, expressed almost similar patterns in the germinator. No signs of germination were observed in field trials. This result correlates with the observation of locating only less than five seedlings within a radius of 100m around the female tree of *K. attenuata*. In order to confirm the shortage of seedlings, further explorations were conducted on different populations of *K. attenuata* (Coorg, Pathanamthitta, Wayanadu & Vazachal) in the southern Western Ghats, and the result was similar. The distance of the male plant from the female one could be a barrier for fruit setting and very low seed germination adversely affects the establishment of viable populations. Vigorous deforestation practices along with loss of habitat, utilisation for trade and natural calamities like flood and landslides are threats to the existence of species (Howe & Westley 1997). Ex situ

production of seedlings and its reintroduction may aid establishment of the population of *K. attenuata,* one of the best approaches to safeguard this endemic species from extinction.

CONCLUSION

The observations on pollination in *K. attenuata* revealed that the pollinators are thrips and syrphid flies. The remoteness of male trees from female trees is a pollination barrier. For effective insect pollination, the optimum distance between male and female trees is 40–50 m. Artificial pollination was found to be effective in the successful production of viable seeds. Since the species is dioecious; the findings have great importance towards the conservation of this species.

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