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RESEARCH ARTICLE

Reproductive Phenology and Pollination Biology of *Madhuca neriifolia* in wet evergreen forest of Western Ghats, South India

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Abstract

Pollination syndromes are suites of phenotypic traits hypothesized to reflect convergent adaptations of flowers for pollination by a specific type of animal. *Madhuca neriifolia* is an endangered plant species occurring in the Southern Western Ghats of India. The species is sub-annual with an event of two consequent flowering periods per year. The morphological traits of the flowers are evolved in such a way that it can support mixed type of pollination syndromes such as anemophily, entomophily, ornithophily, and chiropterophily. Flowers offer sugar rich nectar present in the flesh corolla and also provide pollen for the visiting pollinators as a pollination reward. The flowers are self-compatible, pollinates both self and cross. Highest mean percentage of fruit set was observed with manual hand cross pollination during November 2011. The high outcrossing index (=4) indicates the necessity of pollinator species for the cross pollination which is supported by the breeding results obtained with manual hand cross pollination. The paired *t*-test between controlled (open pollination) and the treatments (manual self and cross pollinations) were significantly different.

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Introduction

The tropical plant communities display conspicuous seasonal pattern in vegetative and reproductive phenologies at both community and species levels (Sundarapandian *et al.*, 2005). The information on phenological patterns of endemic tree species in tropical forests of the Western Ghats is limited (Bhat, 1992, Murali and Sukumar, 1994). The pollination ecology of plant communities can provide information on whether some plants/pollinators are 'keystone' species, providing disproportionately important ecosystem services (Bond and Philips, 1999). Several researchers working in the tropics and the species rich temperate floras of the southern hemisphere are documenting pollination systems that are remarkably specialized, often involving a single pollinator species (Johnson and Steiner, 2000). The longstanding notion that most angiosperm flowers are specialized for pollination by a particular animal type such as birds or bees, has been challenged on the basis of apparent widespread generalization in pollination systems (Johnson and Steiner, 2000, Devy and Davidar, 2003)

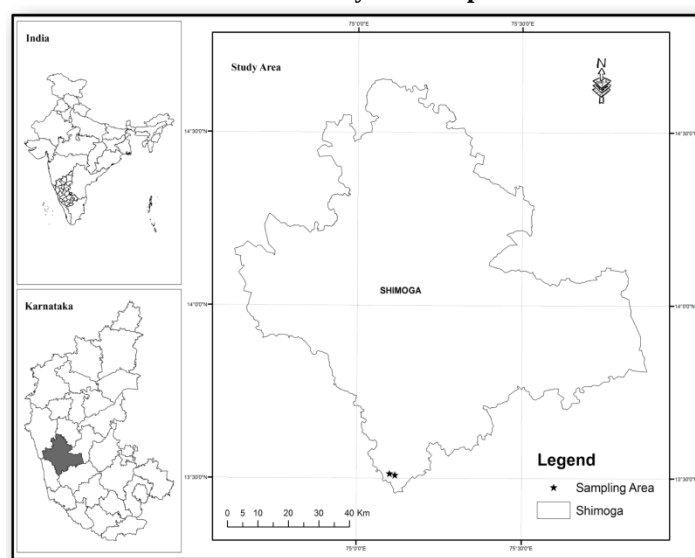
The concept of the pollination syndrome has the implicit notion that pollination systems tend towards specialization, however, empirical evidence indicates that moderate to substantial generalization is the rule rather than the exception (Waser *et al.*, 1996). Animals, primarily insects, are known to pollinate over 90% of plants in tropical forests, and a high proportion of plant species are obligate out crossers (Bawa, 1974, 1990). This high degree of dependence on animals for reproduction brings many tropical plants at risk from anthropogenic disturbance such as loss of habitats and changes in land-use patterns (Kearns and Inouye, 1997). A minimum flower abundance may be necessary to attract pollinators and such an inception may be attained through temporal synchrony (Augspurger, 1981, Schaik *et al.*, 1993). Variation in floral traits including odor, color and morphology, demonstrate the selective pressures imposed by specific pollinator taxa, such as insects and birds. Floral traits are signals that animal pollinators use to locate important food resources (Riffell *et al.*, 2008) and the flower phenotypes are believed to be nonrandom combinations of colors, odors and morphologies hypothesized to reflect selective pressure imposed by certain classes of pollinators (Zych, 2007, Schiestl and Schluter, 2009, Jager *et al.*, 2011).

The genus *Madhuca* (Sapotaceae) is comprised of economically important plants and is represented by five species in the Indian sub-continent. The flowers are exceptionally sugar rich (66-72 % of dry weight) and are used in folk medicine and for the preparation of strong intoxicating liquor (Awasthi, 1975). *Madhuca neriifolia* is a medium sized, evergreen tree with a dense spreading canopy and distributed in semi-evergreen to evergreen forests of Southern India. This is common along the streams and the rivers and occur up to an altitude of 600-1200 meters above mean sea level (MSL) with the current threat status as vulnerable (Ved and Goraya, 2010) at local level and endangered at global level (IUCN, 1994). The information on the phenology, pollination biology and the breeding systems of *Madhuca neriifolia* is limited. The current study reveals the pollination associated syndromes and breeding systems of *M. neriifolia* growing in the Southern Western Ghats regions of India.

Study Site

The study was carried out at Agumbe forest range of Western Ghats, situated in Shimoga district of Karnataka (Fig 1). These forests are classified as tropical wet evergreen forests of the *Dipterocarpus indicus* - *Humboldt brunonis* - *Poeciloneuron indicum* type (Pascal *et al.*, 1988). Champion & Seth (1968) have classified them as 'west coastal tropical evergreen forest' (Arjun and Parthasarathy, 2000). Agumbe region falls in 13°30'9.64"N and 75° 5' 25.15"E with elevation ranges 400-600 meters above mean sea level (MSL) and the forest is composed with rich endemic flora (Pascal *et al.*, 1988). Agumbe is the wettest region in Karnataka, with a mean average annual rainfall of 5000 – 8000 mm and is credited as one of the highest rainfall spots in India after Cherrapunji.

Study area map



Materials and Methods

Vegetative and reproductive phenology

The phenological events were studied by randomly selecting 25 trees from undisturbed areas of the study region. The observations were made on four phenophases, namely (i) leaf sprouting, (ii) leaf and fruit drop (iii) flowering and anthesis, and (iv) fruit setting, during two successive years between January 2011 to January 2013. The phenological records were made every week during high activity period of the flowering season from October to March till fruit maturation and dispersal, and three week intervals during the rest of the year. A particular phenophase was considered to have started or terminated when at least 20% of the selected individuals were in a particular vegetative or reproductive phenophase (Sharma and Khanduri, 2007).

The floral phenology of the species was studied by tagging two inflorescences each of ten randomly selected trees and the flowers were observed from bud stage until senescence. Flowers were checked every three hours to determine the timing of anther dehiscence and to document the specific time of pollen release (Marten and Fenster, 2008). The period of stigma receptivity of selected flowers was observed regularly on flower opening day and the successive days (Kalinganire *et al.*, 2000, Douglas and Freyre, 2010). The receptive stigmas were found to

be wet glistening and turgid, and the stigma was observed through a hand lens of **10x** magnification to determine the duration of their receptivity. After a specific period of time stigma becomes dry and blackish indicating the loss of receptivity (Tidke and Thorat, 2011).

Pollinator observations and Breeding systems

Pollinator observations were carried out over 24 hours per flowering period and totally 96 hours for all the four cycles of flowering during the two year study between 06h00-18h00 and the duration of time spent by each pollinator and floral visitor species was noted. Nocturnal observations were also made because of the cream colored, nectar rich flowers and the observations were done throughout the day and the pollinators were differentiated from floral visitors by keen observation of their type of foraging activity and landing site on the trees (Fenster *et al.*, 2004). The "Outcrossing index" (OCI) was determined from the fresh flowers, which is the sum of assigned values for three characteristics of the flower includes the diameter of the flower, temporal spatial separation of anthers and stigma and temporal separation of anther dehiscence and stigma receptivity (Cruden, 1977, Waites and Agren, 2006).

Manual hand-pollination experiments were undertaken to test the breeding systems of the species during the two subsequent years with two flowering seasons each. Inflorescences from each mother tree was marked separately for open/natural pollination, hand self-pollination and for hand cross pollination respectively. The natural fruit set was assessed on the tagged inflorescences which were left open for the pollinator visitation and the number of fruits upon maturity was noted. The hand-cross-pollination was made by emasculating the anthers from the matured flower buds and manually pollinating the stigma with pollen collected from another individual or a mixture of pollen from one to five individuals followed by bagging the inflorescence to prevent the self-pollination. The hand self-pollination was conducted by transferring the pollen from the anthers of the same flower to the stigmatic surface or the pollen collected from different flowers of the same plant. Apomixis was studied by selecting the mature buds followed by emasculation and bagged immediately in order to control pollination and observed for any possibility of fruit set (Sharma and Khanduri, 2007, Borges *et al.*, 2009, Nayak and Davidar, 2010). Several floral buds were bagged in advance for the purpose of providing fresh and uncontaminated pollen for controlled pollination.

The Index of self-incompatibility (ISI) was calculated as the percentage of the fruit set resulting from hand-self-pollination over that from hand-cross-pollination. A species with ISI ratios <0.25 was considered as self-incompatible and those with ratios >0.25 as a self-compatible (Bawa, 1974, Andrade *et al.*, 1996, Nayak and Davidar, 2010). The natural/open pollination results have been considered as a control to compare the breeding results of hand self-pollination and hand cross pollination as treatments. The results were computed using the paired Student's *t*-tests and the overall breeding results for all the treatments of all flowering seasons with one sample *t*-test by using the statistical software packages PAST v.2.17 (Hammer, 2001) and SPSS v.11.5 (SPSS, 2004).

Pollen count, germination, viability, pollen-ovule ratio and nectar measurements

Pollen production was recorded on five individual trees during the two consequent years of flowering seasons between November 2011 and January 2013. The pollen count was made considering 20 randomly selected closed matured flower buds (Nanda *et al.*, 2006). The numbers of ovules were counted by taking the cross section of ovary (Cruden, 1977, Pias and Guitian, 2001, Griffin and Barrett, 2002). *In-vitro* germination of the pollen studied using Brew-Baker media (Brewbaker and Kwack, 1963) and different concentrations of TTC solutions with concentration ranging from 0.1-0.5 % were used to determine the pollen viability. Ten different flowers were selected each time for extracting the fresh sugary nectar directly from the fleshy corolla four times in different hours of the day. The concentrations of the nectar were measured for each flower the using *refractometer*. The temperature was also recorded each time to obtain the correction factor from the table values given for the instrument (Wunnachit, 1992, Nicolson, 2007).

Results

Phenology and floral biology

The flowers were solitary, complete, campanulate and hermaphrodite with sweet nectary corolla born on the branches in clusters with a persistent style. The stigma was spatially separated from the anthers by an extended growth of style with an outsourcing index four. The peak flower anthesis occurred during evening hours between 05h0-06h30. The anthesis of all the flowers in an inflorescence synchronized with the blooming period within 48hrs

after the first flower anthesis. Anther dehiscence was observed to be maximum during the dusk between 18h00-20h00.

The leaf fall was observed throughout the year, but the peak fall was during the last week of September and the peak leaf initiation occurred during the second week of October. The tree is sub-annual which flowers twice subsequently, with the first round of flowering initiation during the second week of October and the flowers matured and bloomed by the last week of November. This was followed by a second round of floral initiation by the end of November and the flowers matured during the second week of January followed by fruit set during the last week of January and early March respectively (**Fig.2**). This is reported for the first time in *M. neriifolia*. The average number of days taken by the flowers to mature was 34-38 and the fruit set to fruit maturation was about 55-60 days.

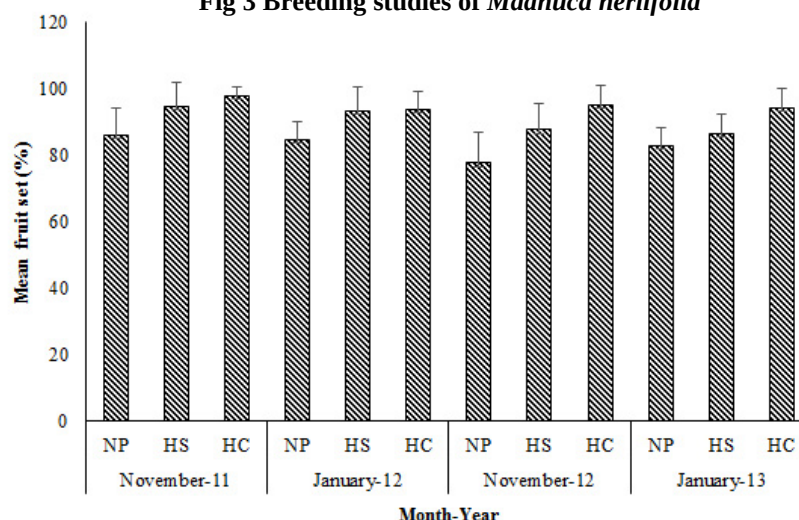
Fig.2 Phenological studies on *Madhuca neriifolia*

Month/Activity	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Vegetative phase												
Leaf initiation												
Leaf fall												
Flowering												
Fruiting												

Breeding systems

The manual breeding experiments results (**Fig 3**) confirmed the highest mean percentage of fruit set with hand cross pollination during November 2011 ($N=10$, $\mu=97.96$, $\alpha=0.05$, $p \leq 0.001$) followed by fruit set with hand cross pollination during November 2012 ($N=10$, $\mu=94.96$, $\alpha=0.05$, $p \leq 0.001$). The least fruit set was observed with open pollinated during January 2013 ($N=10$, $\mu=82.98$, $\alpha=0.05$, $p \leq 0.001$) followed by fruit set with open pollination during November 2012 ($N=10$, $\mu=77.65$, $\alpha=0.05$, $p \leq 0.001$, $CI=95\%$). The paired t -tests between open/natural pollination (control) with the other treatments i.e., Hand self-pollination and hand cross-pollination show a significant mean difference ($N=10$, $p < 0.001$, $\alpha=0.05$, $CI=95\%$) with each treatments except the paired t -test between open pollination and hand self-pollination during January 2013 was found to be non-significant ($N=10$, $p=0.18$, $\alpha=0.05$, $CI=95\%$) (**Table 1**). A mean ISI ratio with the values >0.25 in each case indicates the absence of self-incompatibility in *M. neriifolia*. The emasculation of the flowers followed by bagging without allowing internal or external pollination does not yield any fruit set confirmed the absence of apomixis.

Fig 3 Breeding studies of *Madhuca neriifolia*



Pollen count, germination, viability and pollen-ovule ratio and nectar concentration

The data on pollen production was collected for the two consequent years with two flowering periods each. The mean pollen production per flower was highest during January-2013. Mean *in-vitro* germination of the pollen was found to be 66.86 ± 18.72 % (mean $\pm$ SD). The maximum pollen viability observed to be 95.48 ± 4.98 % (mean $\pm$ SD) in 0.4% TTC solution. The number of ovules per flower was six with a maximum pollen-ovule ratio of 74043.7 observed during January 2011. Among six ovules only one was developed into mature seed. The mean pollen count, pollen-ovule ratio of each flowering cycle shown in **Fig 4**. The nectar concentrations ranged between 22.26 ± 2.52 to 34.47 ± 13.91 % measured during different timings of the day (**Fig 5**).

Fig 4 Pollen count of *Madhuca neriifolia*

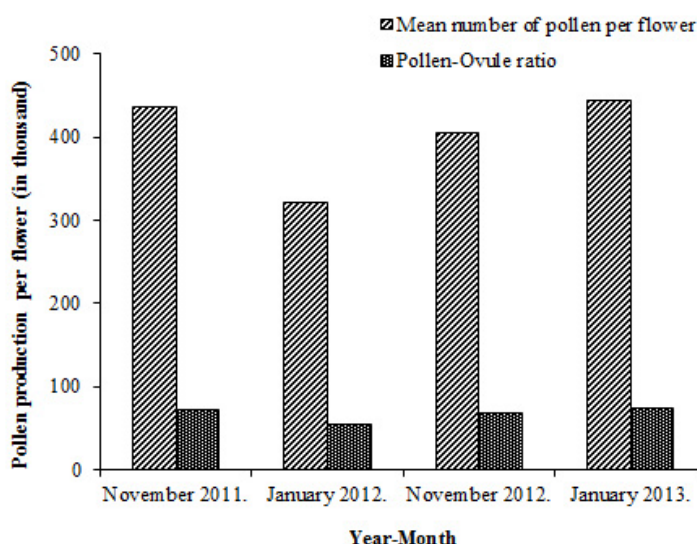
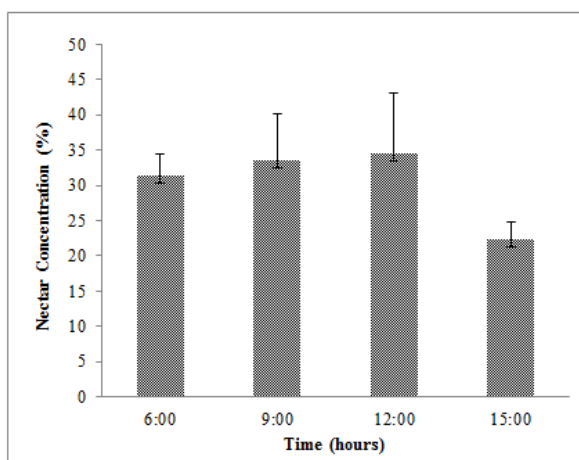


Fig 5 Floral nectar concentration of *M. neriifolia* during different hours of the day



Pollinators and floral visitors

Madhuca neriifolia showed mixed pollination syndromes includes ornithophily, anemophily, melitophily, and chiropterophily. Ants were the most frequent visitors but failed to show much significance with the pollination mechanism, as they only collect the sugary nectar from the external parts of the corolla and usually do not contacting the stigma. *Apis dorsata* and *Apis procera* were also frequent visitors of the flowers and the peak activity was observed between 06h00-12h00 hours. The pollinators include avian species of *Psittacula krameri*, *Pycnonotus jocosus*, *Iole indicia*, and *Pycnonotus cafer* (**Fig 6**). The frequency of visitation of the avian species was maximum during the morning as well as evening hours (**Fig 7**). The corolla is removed and completely eaten by bird species which enhanced the chances of self-pollination. The pollen released from the flowers on the drooping inflorescences

were transferred to the stigmatic surface by means of anemophily was an effective means of natural pollination. *Macacca mulata* was also a frequent among the floral visitors which visits many times a day. They pluck the flowers or even the whole inflorescence causing damage but the physical activity of monkeys on the trees enhanced the self-pollination. The *Funambulus palmarum* are the frequent floral visitors which although remove corolla from the flowers actively participate in the self-pollination without damaging the other parts of the flower. *Cynopterus sphinx* starts foraging activity during the late evening hours and continued during the night, but the frequency of visitation could not be assessed (Table 2).

Fig 6 Pollinators of *Madhuca neriifolia* a) *Macaca mulatta* b) *Pycnonotus jocosus* c) *Psittacula krameri* d) Formicidae (Ants) e) *Iole indica* f) *Apis dorsata*

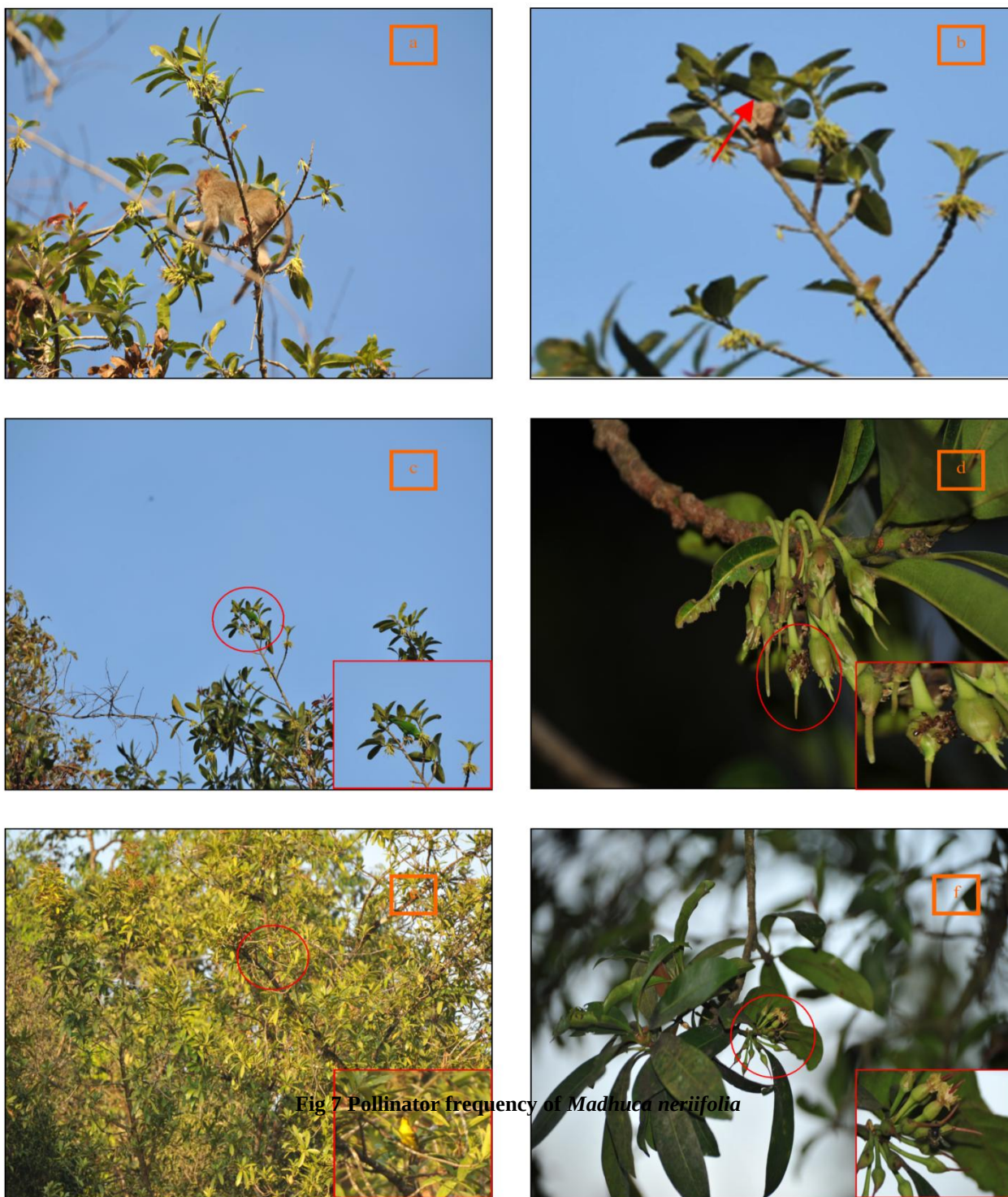


Fig 7 Pollinator frequency of *Madhuca neriifolia*

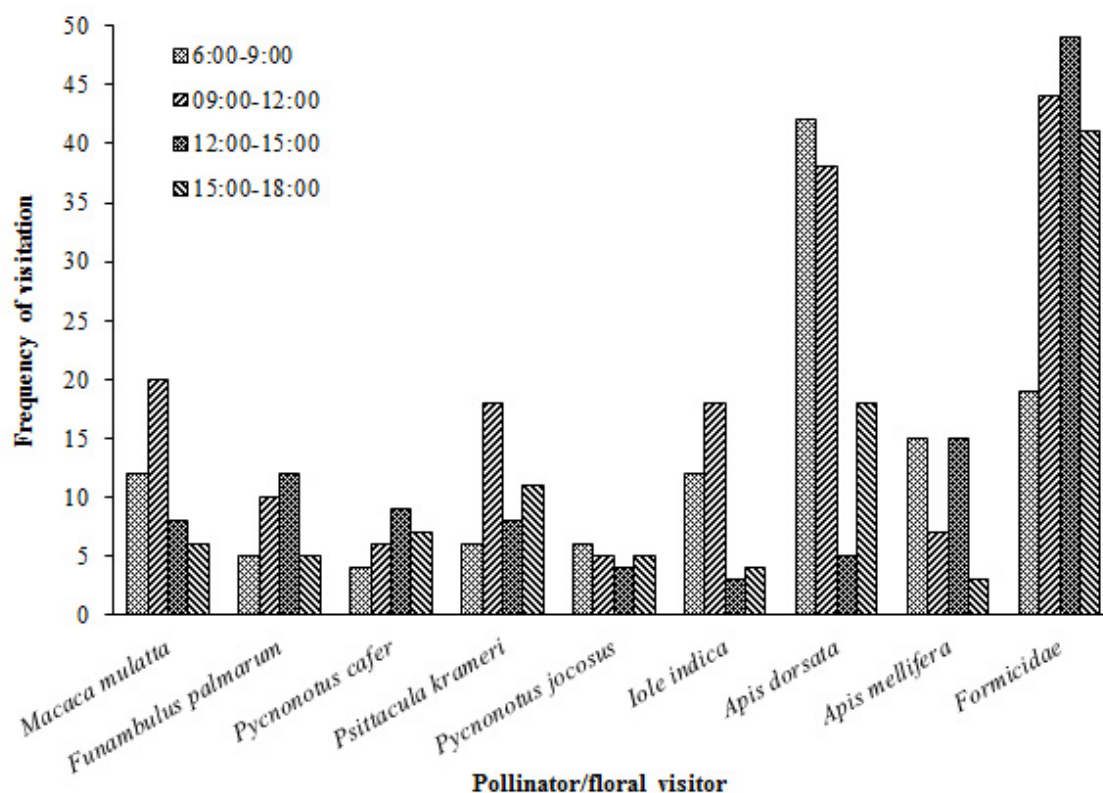


Table 1: Paired *t*-tests between control (natural/open pollination) and different treatments (hand-self and hand cross pollination)

Month-Year	Paired Samples	T	df	Significance (2-tailed) $\alpha=0.05, CI=95\%$
November-11	NP & HS	-3.84	9.00	0.0040
	NP & HC	-4.18	9.00	0.0024
January-12	NP & HS	-2.74	9.00	0.0229
	NP & HC	-3.42	9.00	0.0076
November-12	NP & HS	-2.30	9.00	0.0473
	NP & HC	-4.99	9.00	0.0008
January-13	NP & HS	-1.42	9.00	0.1883
	NP & HC	-4.93	9.00	0.0008

NP-Open/Natural pollination, HS-Hand self-pollination, HC-Hand cross-pollination

Table 2: Pollinator and floral visitors of *Madhuca neriifolia*

Name of floral visitor/pollinator	Pollination reward	Length of visit (Sec)	Visitation Frequency
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<i>Apis dorsata</i>	P*	15-45	VF
<i>Apis mellifera</i>	P*	15-20	VF
<i>Cynopterus sphinx</i>	P*,N**	Unknown	VF
<i>Xylocopa pubescens</i>	N**	Short period	VF
<i>Iole indica</i>	P*,N**	30-40	F
<i>Macaca mulatta</i>	P*,N**	Very long period	VF
<i>Psittacula krameri</i>	P*,N**	20-35	VF
<i>Pycnonotus cafer</i>	P*,N**	15-20	R
<i>Pycnonotus jocosus</i>	P*,N**	20-60	F
<i>Funambulus palmarum</i>	P*,N**	15-45	R
Formicidae (unknown species)	N**	Very long period	VF

P*- Pollen, N**-Nectar, F-Frequent, R-Rare, VF-Very frequent

Discussions

Vegetative and reproductive phenology

The two year phenological observations showed not much significant difference between the occurrence of vegetative and floral phenological events. The peak leaf fall was observed during the September followed by the fresh leaf emergence during wet rainy season of October instead of the dry season as recorded earlier in the tropical forests of India and the La Selva (Rai and Proctor, 1986, Bawa *et al.*, 2003). Episodic or sub-annual flowering was observed in the case of *M. neriifolia* where the species flower two times in a year (Bawa *et al.*, 2003). Although subsequent flowering is reported in *M. neriifolia*, it has not been reported in *Madhuca longifolia* and *M. latifolia*. These phenological responses are not driven by any single factor, which are probably as the result of a compromise between a range of selective forces (Bullock *et al.*, 1983) and mechanisms behind these actions are still ambiguous. They include abiotic factors such as number of dry months, temperature, photoperiods and precipitation (Reich and Borchert, 1982, Prasad and Hegde, 1986, Schaik *et al.*, 1993). Two kinds of biotic processes may also lead to clumped phenologies, firstly variations in the seasonally available biotic agents and secondly they may either attract pollinators and dispersers or quench predators by doing so (Schaik *et al.*, 1993). Phenological schedules may in turn be affected by biotic factors through competition, herbivory, pollination, and seed dispersal, in addition to various climatic variables (Alison, 1997, Rathcke and Lacey, 1985).

Pollination biology

The floral morphology of *M. neriifolia* is evolving in such a way that it attracts a different group of pollinators and showed a mixed pollination syndromes. Plant-pollinator interactions are good examples of mutualistic interactions because nearly three quarters of all extant flowering plants receive pollination services from animals (NRC, 2007). Animal pollination is considered as the ancestral form of pollination in angiosperms (Hu *et al.*, 2008). *M. neriifolia* showed a mixed type of pollination with a diversified group of pollinators such as anemophily, entomophily, ornithophily, as well as chiropterophily. *Macacca mulata* as a more frequent visitor of the flowers is also involved in the foraging activity destructing the flowers, but their physical activity enhanced open pollination. The anthesis during the evening hours enhanced the pollination in *M. neriifolia* as a different group of pollinators facilitate the activity during this time. The pollen transfer rate is maximized when anthesis occurs at dusk and not at dawn, even when diurnal pollinators show a larger pollen removal rate and are more abundant at the flowers (Miyake and Yahara, 1998, 1999). Plants opening at dusk can use these diurnal flower visitors as well as less frequent nocturnal visitors enabling long distance cross-pollination and are more effective in pollen deposition on conspecific stigmas (Miyake and Yahara, 1998, 1999). In this sense, anthesis at dusk would not be a specialization to pollination by nocturnal agents, but a strategy to use a broader set of distinct pollinators (Miyake and Yahara, 1998, 1999). This mixed and more generalist pollination system may be common in other species of *Madhuca* includes *M. longifolia* and *M. latifolia* of Sapotaceae (Nathan *et al.*, 2009, Tidke and Thorat, 2011). The concept of pollination syndrome has the implicit notion that pollination systems tend toward specialization. However, empirical evidence indicates that moderate to substantial generalization is the rule rather than the exception (Waser *et al.*, 1996). Bird pollination or ornithophily is known from a variety of plant species (Knox *et al.*, 1985) and nectarivory or nectar feeding is found in many bird families (Fleming and Nicolson, 2003). But actually many interactions between plants and pollinators occur in semi-disturbed habitats (Kearns *et al.*, 1998).

Most of the fascicles available at the terminal branches and flowers that emit characteristic odors to attract bats (Nathan *et al.*, 2009) was also noticed. The pistils extending the style outside the flower possibly help receiving

the pollen from different individuals thereby enhancing the cross pollination and improved genetic vigor of the seed. The blooming of all the flowers in an inflorescence in a short period of time also enhanced the pollinator visitation. The numerous anthers produce large amount of pollen, which is the main reward for a most frequent insect visitor like bees. The birds removes the sweet corolla without damaging the pistils of the flowers indicating same kind of natural apathy and unexplainable relationship. The bat species *Cynopterus sphinx* also removes only the fleshy corolla and leaves the pistil intact promoting the possibility of transfer of pollen grains of these flowers deposited on the stigma of intact, untouched and neighboring flowers during the detachment by bats (Nathan *et al.*, 2009) is also observed in *M. neriifolia*.

In *Madhuca neriifolia* the flowers are small, borne in dense clusters at the ends of the branches which provides an opportunity for floral visitors to collect the maximum reward in a single visit. Many features of Ornithophily, such as large, robust flowers, abundant nectar, and anther and stigma positions are ideal for foraging and pollination the similar results have been observed in *M. longifolia* and *M. latifolia*. The duration of stigma receptivity varies from a few hours up to ten days (Dafni, 1992). However, it was found to be lasting for 24 to 48 hours in the *M. neriifolia*. Bird-attracting flowers are typically large, showy and robust flowers (Faegri, 1979) and the existence of clear cut syndromes has been challenged (Waser *et al.*, 1996) and the character of robust flowering and the flowers in clusters attracts the birds. Variation in nectar sugar concentration and the amount of energy reward is also to influence attraction, foraging behavior and energetics of different animal pollinators (Feinsinger *et al.*, 1987, Baker *et al.*, 1998, Marten and Fenster, 2008). Thus high sugar concentration of the nectar in the corolla of *M. neriifolia* flowers influence the visitation of many animal pollinators.

Breeding systems

The breeding system determines the spread of genetic variability in the population and this in turn is usually reflected to some extent in the floral morphology of the population. The results of breeding studies suggest that the trees are self-compatible and the percentage of fruit set in both self and cross-pollination are high. The results suggest that the high pollinator abundance and interaction account for the high rate of natural fruit set in *M. neriifolia*. Besides the floral adoptive floral trait also places a role to attract a different group of pollinators. The flowers showed high pollen-ovule ratios and among six ovules per flower only one ovule was developed into functional seed, reflecting poor ovule-seed ratio. Controlled pollination experiments also suggest no change in the ovule seed ratio and only one seed formed out of six ovules in both control and open pollinated flowers indicate the genetic conservative nature of the species. Thus aspects of pollen delivery are not responsible for low seed: ovule ratios and that resource might influence their variation as opined by Griffin and Barrett (2012). The paired *t*-test with a hypothesis of equality of statistical means between the open pollination and treatments (manual hand-self and cross pollination) suggest a significant difference with the fruit set throughout all the flowering seasons with the exception in case of open pollination with hand self-pollination as enunciated by paired *t*-test during January 2013, in which the statistical means were significantly not-different from each. This however suggests that an equal chance of fruit set in both the cases. The reason being high pollinator abundance and the morphological traits or the adaptations of the flower to the diverse group of pollinators. High Outcrossing Index (=4) in *Madhuca neriifolia* with suggest that this species is adapted for outsourcing and requires a pollinator (Cruden, 1977, 2009) and the adopted floral trait supports the mixed type of pollination syndromes.

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