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Apomixis in *Coscinium fenestratum* (Gaertn.) Colebr.

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Coscinium fenestratum is getting endangered due to the extensive exploitation for medicinal use. Irrespective to the profuse seed set, occurrence of seedlings in the wild is meager that prompted a seed study. As a dioecious liana, female plants bear fruits even in the absence of male plants in the vicinity and also the bagged unopened flower buds develop in to viable seeds. Absence of pollinators and air born pollen grains, development of single seed from tricarpillary gynoecium, all attributed for an enigmatic development. In consequence of curiosity, studies on pollen viability, pollen germination, pollen ovule ratio and embryological studies *etc* were conducted. Embryo formation was observed with 80% of the longitudinal microtome sections taken with 1st to 3rd week aged fruit primordia irrespective to the lack of pollen on the stigma and pollen tube entry into the stylar tube. Comparative molecular studies on mother plant and respective F1 seedlings revealed genetic identity that confirmed apomixis.

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1. INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr. is a highly sought endangered medicinal dioecious liana of the moon seeded family Menispermaceae with its distribution amongst the moist deciduous to evergreen forests of Peninsular India, Sri Lanka, Singapore and Malaysia. Medicinal properties are attributed to the presence of isoquinoline alkaloids like 'berberine' with many biological activities [1,2]. Over exploitation for stem and root, long duration for attaining reproductive maturity, seed dormancy and low seedling survival *etc*. made the plant rapidly vanishing from natural habitat. Over a period, this has become threatened and listed as critically endangered in Kerala, Karnataka, and Tamilnadu states [3], and also as forest products of negative exports [4]. Ministry of Commerce vide notification no, 47 (PN)/ 92-97 dated 30th March 1994 banned the export of *C. fenestratum* [5]. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed this medicinal plant as endangered because of its large scale harvest [6]. *C. fenestratum* is enlisted among the 12 medicinal plants of Kerala which occupy prior position with respect to conservation [7]. The plant is facing genetic erosion [8]. Presently, IUCN Red list of threatened plants (2015) classified *C. fenestratum* in the category "data deficient" [9].

In *C. fenestratum* natural seedling population is found to be limited irrespective of profuse blooming and ample seed set. While studying the nature of pollination and pollinators, it was revealed that in spite of the lack of vicinity of male flowers and also even bagged unopened flowers set fruits. It was observed that out of the 90% viable pollens, only 50% germinated. Absence of pollen grains over the sticky slides hanged on the female plant at basal, middle and host tree canopy levels during the flowering season indicated lack of anemophily. These observations lead to the detailed studies on flower to fruit development and for this microtome sections of flower buds and young fruit primordia at different development stages were observed. Apomixis was further confirmed using comparative molecular studies (DNA isolation) on mother plant and seedlings.

2. MATERIALS AND METHODS

2.1 Phenology

Profusely growing liana at Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) at an altitude of 150 m, amongst the foothills of southern Western Ghats (Lat- $8^{o}45$'and $8^{o}47$'N, Long-$77^{o}1$'and $77^{o}4$'E) were selected for study and phenology was compared with feral plants at Rosemala (Lat- 8.9184^{0}N, and Long- 77.1744^{0}E).The work was carried out in three consecutive years 2018-2019, 2019-2020 and 2020-2021. The events of leaf flushing, flowering (from floral bud initiation to withering of flowers), fruit set (fruit initiation to maturation) and fruit /seed dispersal were observed by approved methods [10,11].

2.2 Pollination Study

To understand the type of pollination, sticky slides were hung on the female plant during the flowering season at basal, middle and host tree canopy levels. Some of the unopened flower buds were also carefully bagged and observed the developmental stages.

2.3 Pollen-Ovule Ratio

The number of pollen grains per flower was calculated as per the approved method suggested by Dafni et al. [12]. For this, anthers from 10 flowers were separately pulled out and macerated with a solution of distilled water and detergent. The pollen suspension (100µl) was placed on a clean micro slide and the total number of pollen grains in the sample was counted under the microscope. The procedure was repeated with 10 samples and the average number of pollen grains was calculated.

For ovule count, young pistils were collected from flowers. The average number of ovules per ovary was determined by dissecting the ovary under the microscope. The procedure was repeated in 20 randomly selected flowers and the pollen-ovule ratio was calculated by the following formula [13].

$$\text{Pollen} - \text{Ovule ratio} = \frac{\text{Mean number of pollen grains/flower}}{\text{Mean number of ovules/flower}}$$

2.4 Pollen Germination Test

Pollen germination test was conducted according to Shivanna and Rangaswamy [14]. Brewbaker and Kwack medium [15] with 10% sucrose were found to be the most suitable medium. Fresh pollen samples were placed with platinum needle into nutrient medium which were placed on petridishes lined with moist filter paper and incubated for 4 hours. After incubation, a drop of cotton blue was added and allowed to disperse. Pollen grains with pollen tubes longer than the diameter of the pollen was counted as viable. The germinated pollen grains and tube elongation were recorded under a Leica DME light microscope at low magnification (10 x 10). The process was repeated with 5 replicates and average values were recorded. The results were calculated as per the following formula:

$$\text{Pollen germination percentage} = \frac{\text{Number of pollen grains germinated}}{\text{Total number of pollen grains observed}} \text{X } 100$$

2.5 Pollen Viability Tests

2.5.1 Acetocarmine glycerin staining technique

Fresh pollen grains collected from different flowers were transferred to a clean slide and 2 drops of acetocarmine – glycerin mixture in 1:1 ratio was added and mixed thoroughly. After 15 minutes the prepared slides were examined under the light microscope (Leica DME, Germany). The number of stained and unstained pollen grains was counted. The stained pollen grains were considered as fertile and the unstained pollen as sterile.

2.5.2 TTC test

Stock solution used was 0.3% of 2, 3, 5 triphenyl tetrazolium chloride (TTC) in 5% sucrose solution. This was to prevent the bursting of pollen grains. Small amount of pollen grains was suspended and distributed uniformly in a drop of solution over a clean microslide. The preparations were incubated in dark humidity chamber (95% RH) under laboratory temperature for 30-60 min. The dark red colored pollen grains were counted as viable [14].

2.5.3 FCR test

The pollen viability by FCR (Flurochromatic reaction) test was made according to the procedure of Heslop-Harrison, [16]. The stock solution was prepared by adding 10mg of fluorescein di-acetate (FDA) in 5 ml of acetone. The test solution was prepared by adding a few drops of stock solution to 1ml 10% sucrose solution until the resulting mixture showed persistent turbidity. Adequate amount of pollen grains was suspended and uniformly distributed in a drop of FDA solution which was then incubated in a humidity chamber (> 90% RH) for 5-10 minutes, before being observed under a fluorescent microscope (Leica DME, Germany). Bright yellowish green fluorescing pollen grains were counted as viable [14]. Control experiments were performed using heat-killed pollen kept at 80°C for 2 hrs [17].

2.6 Microtome Sectioning

Transverse as well as longitudinal sections of 300 flower buds and young fruit primordia were observed to detect the presence of pollen grains on the stigma, chance of pollen tube entering into the nucellus, embryo development, nature of fertilization *etc*.

2.7 Collection of Sample for DNA Isolation

Seeds from the bagged fruits were germinated and seedlings were raised. Fresh leaves were collected from both the mother plant as well as from the seedlings raised from the same mother plant.

2.8 Genomic DNA Isolation and ISSR Analysis

Total genomic DNA from the young leaves was isolated following the method of Murray and Thompson, [18] using cetyltrimethylammonium bromide (CTAB). After ethanol precipitation DNA was resuspended in 0.1cm^3 of 1XTE buffer (pH 8.0).The DNA was quantified spectrophotometrically by taking the absorbance at 260nm. Genetic diversity analysis using ten ISSR primers were carried out in 0.025 cm^3 reaction mixture containing 0.2 mMdNTP's, 10mM Tris-HCL,1.5mM $MgCl_2$ 50 mMKCl, 0.1%Triton X-100, 1.0 U Taq DNA polymerase (Finzymes,Helsinki,Finland), 15 pmol of primer and 50 ng of genomic DNA. Amplification reactions were carried out in an EppendorfMastercycler (Eppendorf, Hamburg, Germany). The thermocycler program used for the reaction as follows: 94^{o}C for 2min (1 cycle); 50^{o}Cto 55^{o}C for 2 min (1 cycle); 72^{o}C for 2 min

Table 1. List of ISSR primers used and their sequences

Sl No.	Primer Code	Primer Sequence 5' ⟶ 3'	Annealing Temperature (^{0}C)
1	820	5'-GTG TGT GTG TGT GTG TC-3'	52
2	823	5'-TCT CTC TCT CTC TCT CC-3'	52
3	824	5'-TCT CTC TCT CTC TCT CC-3'	52
4	826	5'-ACA CAC ACA CAC ACA CC-3'	52
5	834	5'-AGA GAG AGA GAG AGA GYT-3'	50
6	835	5'-AGA GAG AGA GAG AGA GYC-3'	53
7	836	5'-AGA GAG AGA GAG AGA GYA-3'	50
8	840	5'- GAGAGAGAGAGAGAGAYT*-3'	50
9	843	5'- CTC TCT CTC TCT CTC TRA -3'	50
10	848	5'-CAC ACA CAC ACA CAC ARG**-3'	50

Y = C, T

(1 cycle); 94^{o}C for 30 secs (35 cycles); 50^{o}Cto 55^{o}C for 1 min (35 cycle); 72^{o}C for 2 min (35 cycle); and a final extension of 72^{o}C for 7min. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. PCR products were separated on 1.2% agarose gel by electrophoresis and proper amplification was detected by illuminating the gel in UV light. The gel picture thus obtained was properly documented using *UVP* Gel Documentation system. The presence and absence of bands was determined and was converted into binary data and fed into the computer for various software analyses. List of ISSR primers used and their sequences is documented in Table 1.

3. RESULTS AND DISCUSSION

Though this species blooms profusely and set seeds lavishly, natural seedling population is found to be limited. An assessment of flower to fruit ratio was made on selected plants to find out the cause of low natural regeneration.

3.1 Phenology of the Species

Leaf flourishing was observed throughout the year. Flowering period spreads between September- February and fruiting period is during the monsoon months of June- July. Male flowers of September last up to February while the occurrence of female flowers spreads in between October to December. Fully mature fruits were collected during the months of June –July. Fruits are dispersed by bats and civets.

Just after the initiation, green floral buds on the inflorescence of almost same age which were marked for the fruit developmental studies were counted. There was an average of 10.25 ± 1.4 flowers in each inflorescence. So there were a total of 7472 ± 2.7 flowers in the experimental liana. After six months of development number of fruits per infructescence was 7.42 ± 3.2 with a total of 5409 ± 2.7. Hence the assessment of flower to fruit ratio revealed that the approximate ratio per plant is 7:5. All the flowers do not develop into fruits. 20% of fruits were abscised.

A single flower bud took around 15-20 days for its development and subsequent blooming. In male plant anthesis was noticed midway between 06.00 - 8.30 am. Anther dehiscence was noticed for a period of 20 hours. In female plant, peak anthesis was between 07.00 - 10.15 am. Stigma was receptive for a period of 22-25 hours. The flowering period extended for a period of two months in a plant.

3.2 Pollen Germination and Viability Studies

3.2.1 Pollen germination

Pollens collected on the day of anthesis registered maximum germination (Table 2)

Table 2. Pollen germination test

Concentration of solution (%)	Age of pollen	% of germination
10	One day before anthesis	52.5 ± 0.5
10	On the day of anthesis	55.5 ± 1.1
10	Two day after anthesis	30.5 ± 0.9

3.2.2 Pollen viability study

Acetocarmine glycerin staining technique - 96.8% pollens are viable (Table 3).

Table 3. Acetocarmine glycerin staining test

Nature of pollen	No of stained pollen	No of unstained pollen	% of viable pollen
Pollen from unopened flowers	155	7	95.7 ± 0.3
Pollen from opened flowers	165	5	97. 0 ± 0.7
Pollen from fully opened flowers	177	4	97.7 ± 1.4

TTC Test - 90.3% pollens are viable (Table 4).

Table 4. TTC test

Nature of pollen	No of stained pollen	No of unstained pollen	% of viable pollen
Pollen from unopened flowers	135	13	91.2 ± 2.1
Pollen from opened flowers	140	10	92.9 ± 0.8
Pollen from fully opened flowers	147	9	94.2 ± 1.3

FCR test. (Flurochromatic reaction) - 93.3% pollens are viable (Table 5).

Table 5. FCR test

Nature of pollen	No of stained pollen	No of unstained pollen	% of viable pollen
Pollen from unopened flowers	131	11	92.3 ± 0.6
Pollen from opened flowers	139	7	93.6 ± 0.5
Pollen from fully opened flowers	141	9	94 .0 ± 0.8

3.2.3 Determination of pollen-ovule ratio

Pollen –ovule ratio was 323:1 (Table 6).

Table 6. Pollen- Ovule ratio

Number of pollen	Number of ovary	Pollen-Ovule ratio	Log value
968	3	323:1	2.51

Although 90% pollen was found viable, only 50% germinated. Since the number of pollen grains in *C. fenestratum* flower is below 1000, anemophily can't occur [13] and based on the pollen-ovule ratio (Table 6) the breeding system was of facultative autogamy [13]. This indicates that mainly selfing (autogamy) occur in such plants but outcrossing (xenogamy) may also be possible. Pollen- ovule ratio can be considered as an indicator of breeding system in flowering plants. Flowers of facultatively autogamous plants tend to self-pollinate prior to or during flower opening and the exposure of receptive stigmas to potential pollinators occurs after self-pollination has occurred.

Plate - 1

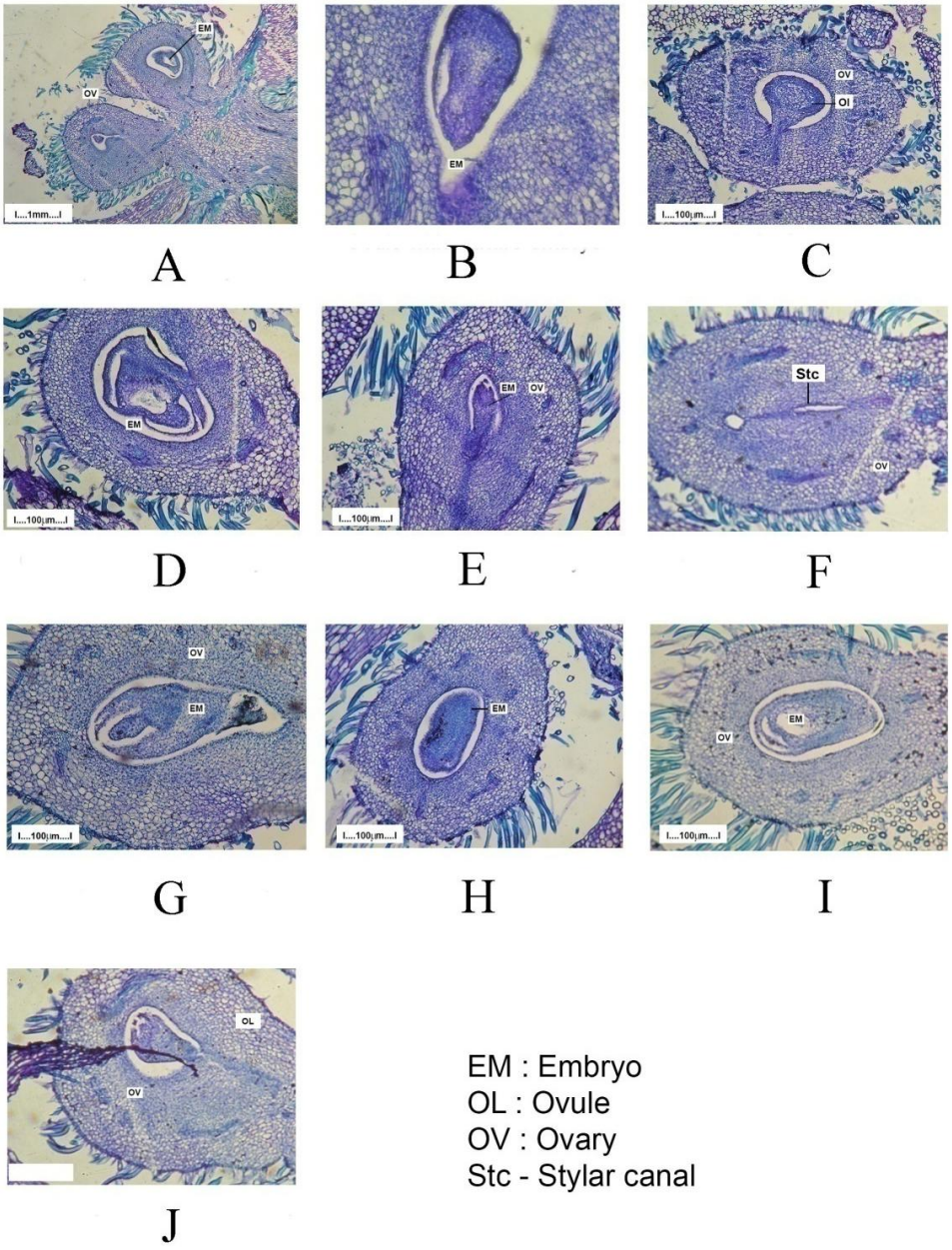

Plate 1. Legend for the

A. L.S. of female flower showing 2 free carpels with embryo sac.
B. Ovary with mature embryo
C. L.S. of ovary
D. Ovule with mature embryo
E. *T.S. of ovule with embryo*
F. *Ovary with stylar canal*
G. *Ovary with mature embryo*
H. *L.S. of ovary with embryo*
I. *L.S. of ovary with vertical embryo*
J. *Ovule with immature embryo*

Table 7. Genetic Similarity matrix of *Coscinium fenestratum*

	M P	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10
M P	1										
S 1	0.97	1									
S 2	0.96	0.99	1								
S 3	0.95	0.98	0.99	1							
S 4	0.95	0.98	0.99	0.98	1						
S 5	0.95	0.98	0.97	0.96	0.96	1					
S 6	0.95	0.98	0.97	0.96	0.96	1	1				
S 7	0.95	0.98	0.97	0.96	0.96	1	1	1			
S 8	0.92	0.95	0.97	0.95	0.95	0.98	0.98	0.98	1		
S 9	0.92	0.96	0.94	0.96	0.93	0.98	0.98	0.98	0.98	1	
S 10	0.91	0.94	0.93	0.94	0.92	0.97	0.97	0.97	0.96	0.99	1

3.3 Microtome Sections of Flower Buds and Fruit Primordia

When microtome sections of flower and young fruit primordia of different developmental stages were analyzed, no evidences were seen regarding the entry of pollen tube into the stylar tube. In sections of some mature ovules even no embryo formation was seen. No pollen on the stigmas of the carpel or any pollen-tubes penetrating the nucellus of the ovules were seen in ovules examined for the purpose (Plate 1). This was in accordance with our flower to fruit ratio and also to the finding that 20% of fruits were abscised. These observations also supported the prevalence of apomixis (Plate 1).

3.4 Genomic DNA Isolation and ISSR Analysis

To authenticate the above results, DNA isolation was done using the leaves of mother plant and seedlings raised from the same plant. High quality DNA was obtained from all the explants using the Modified CTAB method. The samples were diluted and ISSR analysis was carried out using 10 ISSR primers. A total of 50 bands were obtained, of which 10 were found to be polymorphic. The similarity matrix developed (Table 7) using the WINDIST software showed that the similarity index ranges from 0.91- 0.99 with mean of 0.97 thereby suggesting low level of genetic variability in the species. This result confirms the occurrence of apomixis in *C. fenestratum* by virtue of seedling similarity to mother plant.

Apomixis is an alternative method of seed production in plants. Apomictic process occurs in the ovule and progenies are usually genetically exact copies of the female plant.

Both male and female plants in *C. fenestratum* flowers in almost same season but the flowering of male plant (September) is much earlier than that of female (October-November). An assessment of flower to fruit ratio among selected plants revealed the approximate flower to fruit ratio per plant as 7:5. All the flowers do not develop into fruits since 20% of fruits were abscised.

C. fenestratum flowers are not attractive. Outer whorls of the flowers are grayish green, haven't got any striking color. Flowers do not possess any aroma. The pilose nature of floral parts and sticky fluids of filiform stigma akin to anemophilic plants may aid in receiving pollen. Since the pollen doesn't have any specialized morphology for anemophily except the small size, chances for yet other pollinators were also probed in vain.

Lack of pollen grains on the sticky slides which were hanged on the female plant at different levels during the flowering season ruled out the chance of anemophily. In spite of the absence of male flowers in the vicinity and even the bagged unopened flowers set fruits and also sets seeds which germinated normally hinted that there is no true fertilization in *Coscinium fenestratum.*

Regarding post – pollination behavior it was observed that the plants are having plentiful supply of fruits. From a single inflorescence, each flower has got the capacity to produce three fruits, *i.e.,* from each ovule a fruit can be developed. Ovary is tricarpellary, with one ovule in each locule, however only one normally develops while the others get aborted. This may be due to the less chance of effective fertilization of all flowers in an inflorescence, or due to the difference in maturation period of each ovule in a flower, that all the flowers do not develop into fruits.

Large number of fruits at different stages of development was observed from the fruit primordial stage. Microtome sections of young flower buds of different developmental stages (Plate -1) were analyzed and the following interpretation were made

- No evidence was seen regarding the entry of pollen tube into the stylar tube.
- In sections of some mature ovules no embryo formation was seen.
- No pollen on the stigmas of the carpels or any pollen-tubes penetrating the nucellus of the ovules were seen in ovules examined for the purpose.

This is in accordance with the flower to fruit ratio and also to the finding that 20% of fruits were abscised. These observations also support the prevalence of apomixis.

When DNA isolation was done using the leaves of mother plant and seedlings raised from the same plant the similarity matrix developed using the WINDIST software showed that the similarity index ranges from 0.91- 0.99 with mean of 0.97 thereby suggesting low level of genetic variability in the species. This outcome proves the incidence of apomixis in *C. fenestratum* because the seedlings are more or less very similar to the mother plant.

Moderate degree of genetic diversity among seven populations in *C. fenestratum* was reported by Narasimhan et al. [19] and stated that variation within population was slightly higher than between populations. Similarity between individuals within and between populations was also found.

Genetic markers are widely used either alone or in combination with morphological markers to obtain more consistent information on existing genetic diversity with a number of species.

Joshi and Raman Rao [20] while studying the fruit and seed development in *Tinospora cordifolia*, another member of the same family Menispermaceae, stated that presence of embryo is not found in all the specimens of different developmental stages. Like *C. fenestratum*, in *T.cordifolia* also no pollen on the stigmas of the carpels or any pollen tubes penetrating the nucellus of the ovule have seen even in more than two hundred ovules examined for the purpose. The growth of the endosperm on the ventral side of the carpel is very irregular and consequently becomes ruminate. Endosperm in *C. fenestratum* seed is also ruminate and this may be the cause of it. Joshi and Raman Rao [20] added that it was not unlikely that in *T. cordifolia*, the necessary stimulus for the development of the seed and fruit may be coming from the formation of endosperm itself inside and its formation without fertilization is not an unusual thing even in the Ranales.

But Seshagiriah [21] stated that seeds of *Tinospora cordifolia* have a viable embryo and it germinate in the normal way, though develop perhaps by the influence of foreign pollen. The embryo can be seen even without the help of a hand lens after dissecting out the ruminate endosperm like *C. fenestratum*. Later Bahadur Singh [22] also reported the same observation. He also opinioned that in *Tinospora cordifolia* male plants are very rare and the chances of effective pollination are meager. Parthenogenesis is also reported in *Disciphania ernstii* Eichler, [23] another member of the family Menispermaceae.

A current survey in Kerala while studying the population status and distribution of *C. fenestratum* proved that in *C.fenestratum* male plants are fewer in number than females [24] which reduces the chance of effective pollination. When examined, the pollen of the male plant is found normal and is viable up to 90%. But pollen germination was only 50%. The lack of pollination may be due to the distance between the male and female plants. In general, dioecious plants are especially liable to failure of fertilization owing to the distribution of their sexes on separate individuals growing often wild apart [25]. Yet another fact is that *C. fenestratum* is a dioecious liana normally growing on other trees with dense foliage like *Aporusa lindleyana*, *Gmelina arborea*, *Tectona grandis etc.* which may reduce the chance of successful pollination. These particulars account for the chance of development of embryo without fertilization. Asker and Jerling, [26] reported that apomixis is common throughout the plant kingdom, from lower forms of algae to higher angiosperms. In about 300 plant species of more than 35 families apomixis is reported with a distribution pattern that indicates a polyphyletic origin. Recent reviews regarding the developmental details of various apomictic processes have been made by many researchers [27-29].

The seedlings raised from the *C. fenestratum* mother plant showed very narrow genetic

diversity and hence the process of apomixis can be recognized here.

4. CONCLUSION

Based on the pollination studies which included pollen viability and germination, pollen- ovule ratio, development of single ovule (although gynoecium is tricarpellary), microtome sections of young flower buds, DNA comparison between the mother plant and F1 generation, the process of apomixis was clearly attributed in *Coscinium fenestratum*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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