Journal of Tropical Forest Science 38(1): 40–47 (2026)
https://doi.org/10.26525/jtfs2026.38.1.40
ISSN: 0128-1283, eISSN: 2521-9847

SEXUAL SYSTEM OF *EURYCOMA LONGIFOLIA* JACK (SIMAROUBACEAE)

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Submitted February 2025; accepted September 2025

Hermaphroditism is common in flowering plants, but the presence of unisexual flowers across many taxa, coupled with the combined influence of genetic and environmental factors, makes determining sexual systems challenging. Specifically, androdioecy, where both male and hermaphroditic individuals coexist in the same population, has often been confused with gender diphasy, a strategy in which plants alter their sex expression between seasons. In this study, we investigated the reproductive function of male and hermaphroditic flowers in *Eurycoma longifolia* (Simaroubaceae) to clarify its sexual system and assess whether it exhibits gender diphasy. Our findings revealed that, while male flowers were present in a notable proportion of individuals relative to hermaphrodites, they also offer significant reproductive advantage in terms of pollen contribution for siring success. Furthermore, *E. longifolia* displayed low self-pollination rate suggesting predominant outcrossing. The occurrences of sex changes in three individuals in the 2024 flowering season suggests that *E. longifolia* may not be an androdioecious species, but a possible rare example of gender diphasy.

Keywords: gender diphasy, androdioecy, gene flow, outcrossing, Tongkat Ali

INTRODUCTION

Eurycoma longifolia Jack is a common tropical medicinal plant which belongs to the family Simaroubaceae. The plant is distributed in the forests of Malaysia, Indonesia, Thailand, Vietnam, Myanmar and Cambodia. *Eurycoma longifolia* is known locally as Tongkat Ali (Malaysia), renowned for its male aphrodisiac properties to improve libido and energy (Gimlette & Thomson 1977). Traditionally, the root of this plant is used for the treatment of a variety of ailments such as bleeding, cough, fever, anti-malarial treatment, ulcer, high blood pressure and fevers (Bhat & Karim 2010).

Eurycoma longifolia was first described in 1822 by Sir William Jack, based on his observations in Tappanuly and Bencoolen in Sumatra, as well as Singapore (Jack 1977). Jack reported that hermaphroditic and male flowers occurred on different *E. longifolia* plants (Figure 1), a breeding system later recognised by Darwin (1877) as androdioecy, a rare sexual system in which male individuals coexist with hermaphrodites within the same population (Richards 1997). Subsequent theoretical and empirical studies have suggested that for an invader to successfully establish and sustain a hermaphroditic population, male individuals must disperse more pollen and sire more than twice as many offspring as the hermaphrodites. Indeed, unisexual individuals face considerable challenges in gaining a reproductive advantage through male function alone, particularly when compared to the seed production of hermaphrodites (reviewed in Pannell 2002a, 2002b). This may help explain why androdioecy is so rare in nature, occurring in only a few plant taxa. However, androdioecy could be more prevalent than it seems, as plant sex is often determined using morphological traits that may not accurately reflect reproductive function. For instance, some plants that appear androdioecious based on their morphology may actually be functionally dioecious, with their morphologically hermaphroditic flowers functioning solely as females (Mayers & Charlesworth 1991).

Androdioecy is often mistakenly identified as gender diphasy, or "gender switching." For example, some perennial plants (e.g., *Panax trifolius*) can alter their sex expression from year to year, functioning as either a male or a hermaphrodite in a given flowering season. When observations are limited to a single season, these plants may be incorrectly classified as androdioecious (Schlessman 1991, Manicacci & Despres 2001).

Figure 1 Floral morphology of *Eurycoma longifolia*: (A) hermaphroditic, and (B) male

This study aims to clarify the sexual system of *E. longifolia* through four-year phenological observations and to assess the proportion of siring success of pollen from hermaphroditic and male flowers using genetic paternity analysis. Given that the current propagation method for commercial planting of *E. longifolia* relies primarily on seeds, understanding its reproductive biology is crucial for improving seed production, ensuring reliable fruit set, and enhancing plantation success. As such, this research holds significant value not only for *E. longifolia* planters but also for the broader scientific community.

MATERIALS & METHODS

Flowering phenology was carried out at two *E. longifolia* planted sites at the Forest Research Institute Malaysia (FRIM) campus, namely Ethnobotanical Garden and Bukit Hari (Field 48) with 88 and 28 trees, respectively (Table 1). The distance between the two sites is estimated to be approximately 2.7 km. The diameter at breast height (dbh) of the trees at both sites ranged from 2 to 18 cm. *Eurycoma longifolia* flowering usually occurs annually between January and April from 2021 to 2024.

To determine the proportion of siring success of pollen from hermaphrodite and male flowers, leaf samples were collected from all 116 individuals. Specifically, mature fruits (~50 fruits from each tree) were collected from 10 mother trees (hermaphrodite). The DNA samples were extracted from leaves using modified 2x CTAB method (Murray & Thompson 1980) and purified using High Pure PCR Template Preparation Kit (Roche). DNA was extracted from the embryos of each mature fruits using DNeasy Plant Mini Kit (Qiagen).

All DNA samples were genotyped for eleven microsatellite markers: *Elo*005; *Elo*025; *Elo*026; *Elo*044; *Elo*055; *Elo*066; *Elo*099; *Elo*104; *Elo*199 (Tnah et al. 2011); *Elo*T050; *Elo*T058; (Lee et al. 2018). Multiplex PCRs were performed in 8 µL reaction mixture, with about 10 ng of template DNA, 0.032 µM of each forward and reverse primer, and 1X master mix of Type-it Multiplex PCR Kit. The PCR programme included 5 min at 95 °C, 35 cycles of 95 °C for 30 s, 50 °C for 90 s, and 72 °C for 30 s, followed by a 30 min final extension at 60 °C. The PCR products were then subjected to fragment analysis using an ABI 3500xl Genetic Analyser (Applied Biosystems). Allele sizes were assigned according to the internal size standard and individuals were genotyped using Genemarker v2.6.4.

Paternity analysis was carried out using CERVUS version 3.0.7 (Kalinowski et al. 2007). The number of alleles, observed and expected heterozygosity, null allele frequency and exclusion power (with the known mother) were analysed. Knowing the mother genotype, the exclusion power was the probability that an unrelated male, randomly sampled, would be excluded as a pollen donor in a paternity analysis.

Paternity analysis for each offspring was performed using the maximum likelihood method (Meagher 1986), complemented by a simulation of parentage analysis to assess the confidence in parentage assignments. The haplotype of the putative male parent for each offspring was inferred by subtracting the

Table 1 Four years flowering phenology data for *Eurycoma longifolia* from two locations in the Forest Research Institute Malaysia

Location	No. of plant monitored	2021			2022			2023			2024		
		No. of plant flowered	No. of hermaphrodite (fruiting)	No. of male plant	No. of plant flowered	No. of hermaphrodite (fruiting)	No. of male plant	No. of plant flowered	No. of hermaphrodite (fruiting)	No. of male plant	No. of plant flowered	No. of hermaphrodite (fruiting)	No. of male plant
Ethnobotanical Garden	88	56	25 (24)	31	5	0	5	4	0	4	37	12	25[a]
Bukit Hari	28	21	11 (10)	10	1	0	1	0	0	0	6	2	4[b]
Total	**116**	**77 (66%)**	**36 (34)**	**41**	**6 (5.2%)**	**0**	**6**	**4 (3.5%)**	**0**	**4**	**43 (37%)**	**14**	**29**

[a]Two flowering male plants found to exhibit hermaphrodite flowers
[b]One flowering male plant found to exhibit hermaphrodite flowers

female's genotype from the offspring's multilocus genotype. This male haplotype was then compared with all possible haplotypes from the 116 trees. The highest log-likelihood ratio (LOD score), derived from the genotypes of the offspring and each potential parent, was used to statistically distinguish non-excluded candidate parents. To quantify the confidence in the parentage assignment, a delta statistic was employed, which reflects the difference in LOD scores between the most likely and the second most likely candidate parents. Paternity confidence was further evaluated through a simulation step, comparing the likelihood distributions of random (false) offspring assignments with the true assignments. The simulations were conducted with 100,000 randomly generated offspring, and the confidence level wasset at 95%.

RESULTS AND DISCUSSION

To ascertain the sexual system of *E. longifolia*, we present the flowering phenology observation data carried out for four-years period (2021, 2022, 2023, and 2024) at two sites within FRIM (Bukit Hari and Ethnobotanical Garden).In 2021 (from February to May), a total of 116 trees were monitored —88 trees from Ethnobotanical Garden and 28 trees from Bukit Hari (Table 1). Of the 116 trees, 77 trees (66%) were flowering with 41 trees bearing only male flowers and 36 trees bearing only hermaphrodite flowers.

The observation of *E. longifolia* flowering phenology (Figure 2) was conducted at monthly intervals until the formation of axillary buds (Stage 1). Followed by weekly intervals for reproductive shoots and panicles formation (Stage 2). Elongation of panicles and panicles branching (Stage 3). Flowering bud's formation (Stage 4) and blooming (Stage 5); a perfect flower, containing both stamens and carpels, is described as bisexual or hermaphroditic (Figure 1A), while male flowers have stamens that produce pollen (Figure 1B). Early stage of fruits formation with developing cotyledon (Stage 6). Comparison of fruits maturity with no obvious embryo (Stage 7) and with present of embryo (Stage 8). Overall, it took approximately 112 days from the initiation of the axillary bud formation to fruits maturity.

Our 2021 flowering phenology observation was consistent with the species sexual description by Jack in 1822 confirming androdioecy (Jack 1977). In 2022 and 2023 the percentage of flowering trees was notably low, at 5.2% and 3.5%, respectively (Table 1). This was related to changes in weather pattern, with frequent rainfall observed during this period in contrast to 2021. Nonetheless, sexual orientation of those individuals that were flowering remain unchanged. For the 2024 flowering season, percentage of flowering trees were higher (37%) compared to 2022 and 2023. Our observation discovered three of the 41 plants previously identified as male produced male flowers at the beginning of the flowering period but after the initial male flowers have withered, these plants undergone sexual changes and produced hermaphroditic flowers on different branch. No sexual changes were observed in any of the plants previously identified as hermaphrodite. This observation suggests that *E. longifolia* may not be an androdioecious plant but characterises a rare case of gender diphasy.

To investigate the siring ability of both flowers, genetic analyses was carried out. All loci used in the study were polymorphic, with the number of alleles per locus ranging between 6 and 19 with mean number of alleles being 12.45 (Table 2). The observed and expected heterozygosities ranged from 0.513 to 0.882 (mean = 0.749) and 0.561 to 0.907 (mean = 0.762), respectively. The small average variation between observed and expected heterozygosity values suggest the presence of null alleles is minimal (mean F_{null} = 0.0097). More than 70% of the selected markers exhibited high PIC values (mean = 0.732), suggesting a high degree of informativeness for evaluating genetic diversity. The variability of these markers resulted in an exclusion power of 0.999999 for identifying the second parents of the offspring in the paternity analyses. With this high exclusion power, the markers were able to exclude offspring with multiple candidate fathers. Of the 499 half-sibs from 10 mother trees, the proportion of siring success of pollen contributed by hermaphroditic and male flowers was 15.83% and 81.36%, respectively (Table 3). This suggests that male flowers were more successful in siring seeds and play a crucial role in ensuring the reproductive success of the species. This difference in male fitness was likely driven by increased pollen export from male flowers, which resulted from higher pollen production and reduced self-pollen deposition. The production of male flowers in andromonoecious breeding systems

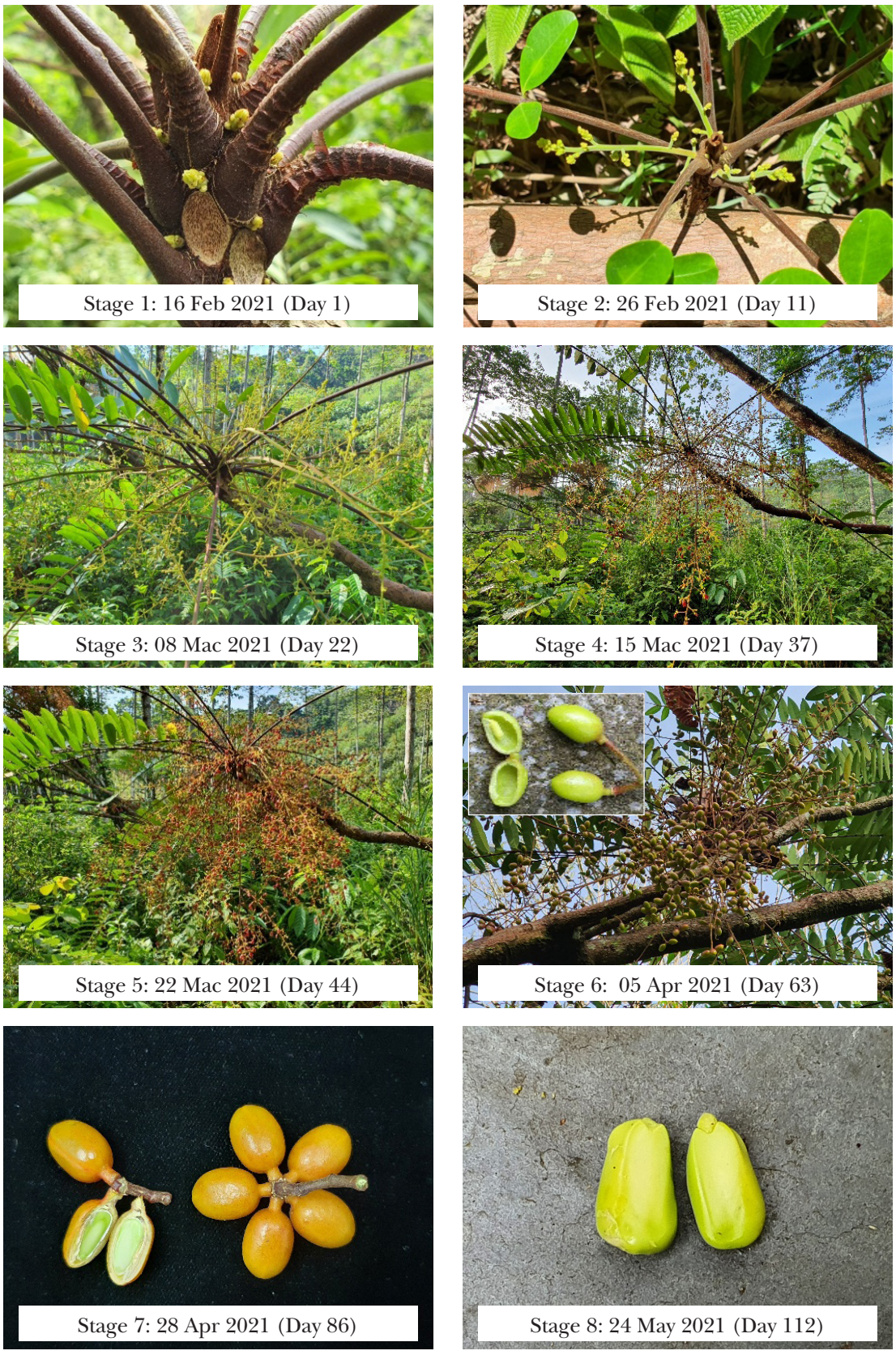

Figure 2 Flowering phenology observation of *Eurycoma longifolia*. Stage 1: axillary buds' formation (Day 1); Stage 2: reproductive shoots and panicles formation; Stage 3: elongation of panicles and panicles branching; Stage 4: flowering buds formation; Stage 5: blooming flowers; Stage 6: fruits formation with growing cotyledon; Stage 7: fruits with no obvious embryo; and Stage 8: mature fruit with present of embryo

Table 2 Genetic diversity values and exclusion indices for 116 individuals of *Eurycoma longifolia*

Locus	No. of alleles	Heterozygosity		PIC	Exclusion probability		F_{null}
		Observed	Expected		First parent	Second parent	
*Elo*025	6	0.702	0.689	0.634	0.728	0.562	-0.0115
*Elo*026	19	0.863	0.907	0.899	0.317	0.188	0.0245
*Elo*044	16	0.882	0.866	0.851	0.425	0.269	-0.0097
*Elo*055	9	0.715	0.762	0.729	0.626	0.445	0.0360
*Elo*199	12	0.631	0.704	0.656	0.702	0.531	0.0573
*Elo*T050	7	0.789	0.773	0.737	0.617	0.439	-0.0114
*Elo*005	15	0.860	0.812	0.796	0.515	0.341	-0.0317
*Elo*066	16	0.769	0.733	0.690	0.667	0.492	-0.0264
*Elo*099	9	0.689	0.769	0.737	0.616	0.436	0.0572
*Elo*104	12	0.829	0.808	0.782	0.549	0.374	-0.0137
*Elo*T058	16	0.513	0.561	0.536	0.813	0.634	0.0359
Mean	**12.45**	**0.749**	**0.762**	**0.732**	**0.598**	**0.428**	**0.0097**
Total exclusion power (first parents) = 0.999971							
Total exclusion power (second parents) = 0.999999							

*PIC – polymorphic information content; F_{null} – null allele frequency

Table 3 Proportion of siring success of pollen contributed from hermaphrodite and male flowers

Sex	Pollen donor	No. of half-sibs	Percentage (%)
Hermaphrodite	12	79	15.83
Male	32	406	81.36
Selfing (Hermaphrodite)	2	2	0.41
Unknown orientation	10	12	2.40
Total		**499**	

may also enhance an individual's male fitness, a concept known as the 'pollen donation hypothesis' (Bertin 1982, Elle & Meagher 2000). Male flowers could be more effective than hermaphroditic flowers at donating pollen through several mechanisms. First, male flowers may produce more pollen than hermaphroditic flowers. In studies of aquatic *Sagittaria*, male flowers were found to produce up to four times the amount of pollen as hermaphroditic flowers (Huang 2003). Similarly, in this study, *E. longifolia* male flowers sired five times as many seeds as hermaphroditic flowers. Second, male flowers may produce higher-quality pollen. Research has indicated that pollen quality can be greater in male flowers, hermaphroditic flowers, or equal in both types (Emms 1993, Cuevas & Polito 2004, Vallejo-Marin & Rausher 2007). However, studies examining seed siring success suggest that male flowers often exhibit equal or higher siring ability compared to hermaphroditic flowers (Cuevas & Polito 2004, Sunnichan et al. 2004, Zhang & Tan 2009). Lastly, male flowers may experience less sexual interference, thereby exporting more pollen than hermaphroditic flowers. Although direct tests of this hypothesis are rare, one study found that male flowers primarily serve as pollen donors, while hermaphroditic flowers act as pollen recipients (Quesada-Aguilar et al. 2008). To overcome the reproductive disadvantage, it is crucial that male individuals exhibit higher fitness than hermaphrodites. This advantage can be achieved either by producing a greater number of offspring (resource compensation) (Geber et al. 1999).

On the other hand, the siring ability of hermaphroditic pollen indicates its viability and suggests that hermaphroditic flower's function both as pollen donors and recipients. A small proportion of self-pollination (0.41%) was observed (Table 3), implying that the species exhibits a self-incompatibility mechanism that promotes cross-pollination. In addition, our preliminary observation indicates that the frequent visitors to *E. longifolia* flowers is stingless bees (*Trigona* sp.) (personal observation). We suspect that nocturnal fauna may also play an important role in facilitating the pollination of the species. This was evident from the paternity analysis, which revealed long-distance pollination with an estimated distance of 2.7 km (Figure 3).

In conclusion, continuous monitoring of *E. longifolia* flowering patterns, with an emphasis on evaluating plant size associations, will further clarify and confirm its sexual system, offering valuable insights into its reproductive dynamics.

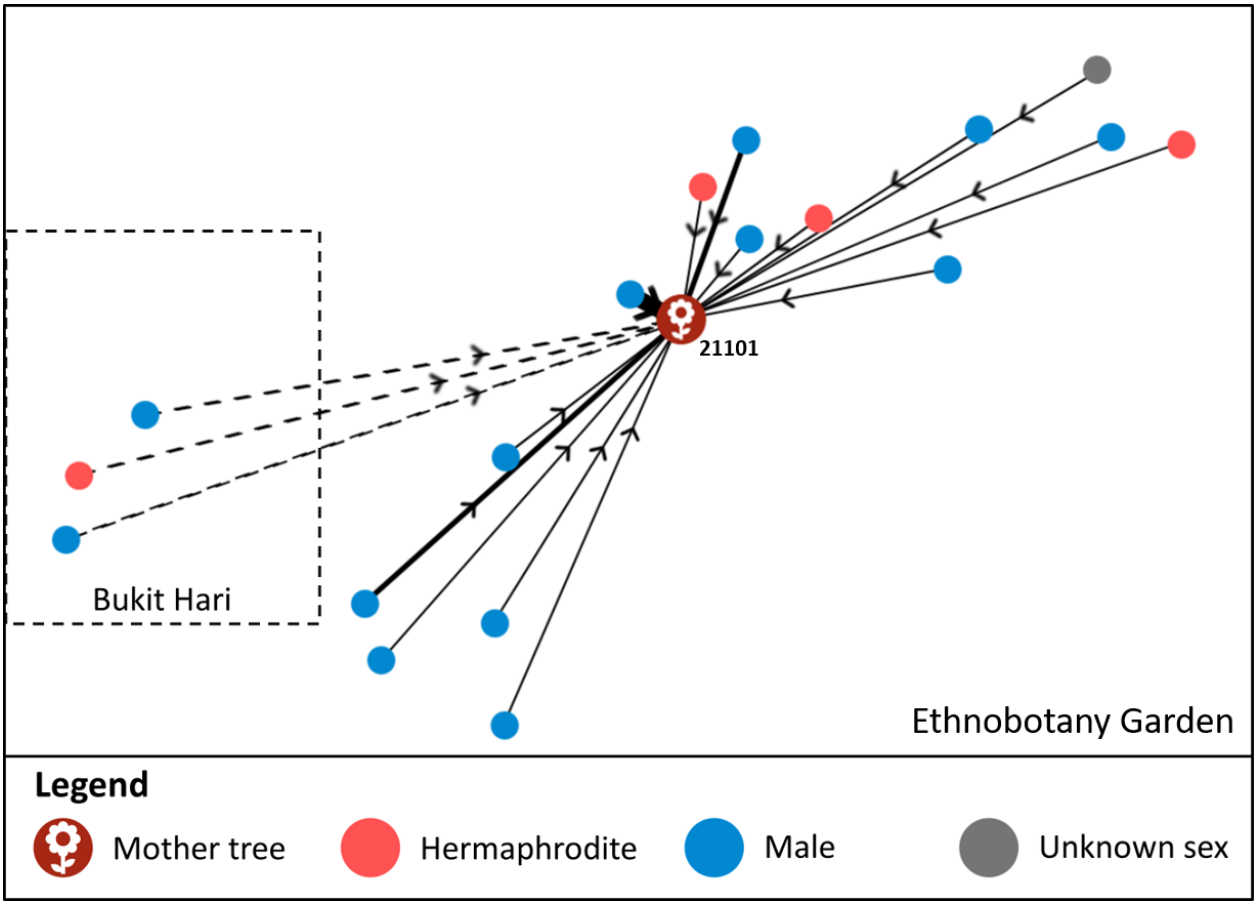

Figure 3 An example of pollen flow contribution to mother tree 21101. The thickness of the lines connecting pollen donors to mother trees indicates the number of successful pollinations by each donor. The dashed lines represent pollen contribution from Bukit Hari, located 2.7 km away

ACKNOWLEDGEMENTS

We would like to thank Yasri Baya and Ramli Ponyoh for their help in sample collection, Sharifah Talib, Ghazali Jaafar, Yahya Marhani and Mohamad Izham Mohd Ariffin for their help in DNA extraction. This study was supported by the Government of Malaysia under the 12th Malaysia Plan 2021–2025.

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