Functional dioecy in *Nothapodytes nimmoniana*, a distylous species in the Western Ghats

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Nothapodytes nimmoniana (Icacinaceae) is a subcanopy tree species, commercially exploited for its wood chips that yield an anti-tumour alkaloid. The species displays intriguing sexual variation. Apart from the flowers which are comparable to typical thrum and pin morphs of distylous species, some flowers show intermediate features. We studied functional gender of the different flower types to assess the sexuality of individual plants in two populations in the Biligiri Rangan Hills Wildlife Sanctuary, Western Ghats, India. The species shows functional/cryptic dioecy. Thrum flowers are functionally males, pin flowers are females and intermediate flowers are bisexual with functional pollen and stigma. Apart from male and female plants, a small proportion of plants which are essentially males produces a small number of female and bisexual flowers. We refer to the sexual system in Nothapodytes as subdioecy and it appears to be a transitory stage in the evolution of dioecy from heterostyly.

Keywords: Distyly, functional/cryptic dioecy, functional gender, heteromorphy.

NOTHAPODYTES NIMMONIANA (Graham.) (family Icacinaceae), formerly *Mappia foetida*, is a small, spreading, sub-canopy tree distributed in the Indo-Malaysian region and China. In India, it is distributed in moist and dry deciduous forests and sometimes evergreen forests of the Western Ghats, as well as in the eastern states of West Bengal and Assam. Camptothecin, an active component with anti-cancer properties is extracted from the wood chips^{1,2}, and the herbal extract carrying the trade name 'ghanera' is traded intensively. Natural populations of *Nothapodytes* are vulnerable to indiscriminate harvest for trade and an estimated 20% of the population of this species is believed to have declined over the last decade³. The species has a threat status of endangered/ vulnerable⁴.

Hombegowda *et al.*⁵ reported that the population of N. nimmoniana growing in Dandeli Wildlife Sanctuary, Karnataka, shows a wide array of sexuality with male, female, hermaphrodite, monoecious, andromonoecious and gynomonoecious individuals, indicating that the species is polygamodioecious. Sexual differentiation in dioecious flowering plants is often incomplete⁶. Work on angiosperm breeding systems involving two, sometimes variable sex forms, has emphasized the value of a functional rather than a morphological approach to their definition⁷⁻¹². During our intensive studies on the reproductive ecology of the species in two populations in the Western Ghats, we identified three floral types - pin and thrum comparable to distylic systems^{12,13}, and an intermediate type with stamens and pistil of intermediate length. This led us to further investigate aspects of functional gender of the three floral types and sexual fidelity of individuals in the populations. The study essentially involved: (i) recording the phenology and sexuality of flowers on the basis of their morphology, their distribution on different individuals in the population, and any transitions in sex across the years, (ii) verifying the functional gender of the flower, (iii) establishing the breeding system of trees that produce male, female and bisexual flowers, and (iv) quantifying fruit set.

The study was conducted in the moist deciduous forests of Biligiri Rangan Hills Wildlife Sanctuary in the central Western Ghats of India, for three years (2004–2006). Our observations were concentrated in two populations (Doddasampige 11°57′79″N, 77°09′32″E and Malkibetta 11°57′21″N, 77°08′88″E) separated from each other by a distance of approximately 4 km, and data collected from the two sites were pooled for analyses. The altitude of the study area is 1210 m, with a mean annual rainfall of 1800 mm. Trees ranged from a height of 10 to 14 m.

A bamboo ladder was used for accessing flowers in the canopy. Thirty flowers of each of the three types (thrum, pin and intermediate) were scored for measuring floral parts using digital callipers. Ovaries were carefully dissected under a stereomicroscope (N = 20 from each type) to determine the number of ovules in an ovary. We used one-way ANOVA to compare mean stamen and mean pistil lengths of the intermediate flowers with those of thrum and pin flowers.

To study inflorescence phenology, we tagged 8–10 inflorescences each on ten randomly selected trees, covering individuals bearing all three floral types. The number and type of flowers opening everyday on each inflorescence were scored. This gave us data not only on the intensity and pattern of flowering, but also helped us know if there was a shift in the sex expression of individual trees during the flowering season. To establish the consistency of sex expression of individual trees, we marked 40 trees with metal tags and their sexuality was recorded each year during the three-year study by scanning flowers from several branches. Each tree was

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scored as 'male', 'female' or 'polygamous' (trees that bore mostly male flowers, but also a small proportion of bisexual and female flowers).

Male sexuality of flowers of different types was verified by estimating pollen fertility by staining them with a drop of acetocarmine. To estimate pollen production, mature but undehisced anther (N = 15 thrum, N = 15 pin, N = 12 intermediate from four individuals of each sex type) was taken on a slide in a drop of safranin and teased with a needle to extrude pollen. After removing the debris, the number of pollen grains was carefully counted under a compound microscope. Pollen viability was assessed on alternate days beginning with the day of anthesis, until there was no viable pollen left, using fluorescein diacetate test¹⁴. Pollen performance was assessed by carrying out manual pollination on the stigma of pin, thrum and intermediate types and observing pollen germination and pollen tube growth following aniline blue fluorescence technique^{15,16}

Female function of flowers was analysed by studying its ability to support pollen germination and pollen tube growth in manually pollinated pistils. We also assessed female function on the basis of fruit set. Many enzymes, particularly non-specific esterases, have been shown to be present on the surface of the functional stigma¹⁷. To test if there are any differences between flowers of three types in stigma surface esterases, we conducted a cytochemical test for non-specific esterases¹⁸.

To study the mating system on female and bisexual flowers developed on the polygamous trees, we carried out hand-pollination experiments using the emasculation (for bisexual flowers) and bagging method. For bisexual flowers we used pollen from the same plant (self-pollination) and from male flower of another plant (cross-pollination). As flowers were too small to be marked or tagged individually, we bagged the whole inflorescence after snipping-off younger buds and older flowers.

To check for natural selfing in inflorescences containing male, female and bisexual flowers in polygamous trees, we bagged some polygamous inflorescences (N =30; after snipping-off open female and bisexual flowers that could have already been pollinated) and opened them after a month to record fruit set. The resulting fruit set would be an evidence for natural self-pollination in polygamous trees. To estimate percentage fruit set, we tagged 30 inflorescences each on four female trees and four polygamous trees (2005 and 2007) and counted the number of female and/or bisexual flowers. We also bagged female flowers on 25 inflorescences to test for apomixis. These inflorescences were monitored until fruit maturity and the number of fruits on each inflorescence was counted. Natural fruit set in female inflorescences (female trees) and mixed inflorescences of polygamous trees was compared and the difference tested using Student's t-test.

CURRENT SCIENCE, VOL. 99, NO. 10, 25 NOVEMBER 2010

The species flowers from end of May to late July. The flowering period lasts for 4–6 weeks. Flowers are arranged in large terminal corymbose panicles of 20–400 flowers. They are foetid-smelling. Each inflorescence lasted for 2–3 weeks. Petals and sepals were greenish-cream and villous.

Morphologically all floral types appear bisexual with typical dimorphic flowers – thrum type with long stamens and short pistil, and pin type with long pistil and short stamens (Figures 1a, b and 2a, b). The two types are generally borne on different plants. Thrum types have turgid anthers that produce pollen grains, and have small, green stigma on a shortened style. Pin morphs have shrunken, non-dehiscent anthers that contain no pollen, and a prominent stigma. On the basis of these morphological features, we have termed thrum type as male flowers and pin type as female flowers. Both male and female flowers bear ovary with two ovules. Pollen viability in male flowers lasted for about 10 days from the day of opening, starting with 29.3% on day-1 and reducing to 4.3% on the day-11. Further observations on 40 marked trees led to the identification of a third floral type on 35% of them. This type had the lengths of stamens and the pistil intermediate to those of thrum and pin types (Figures 1 c and 2 c). These flowers produce viable pollen grains and bear two ovules in the ovary. We refer to such flowers as bisexual. Bisexual flowers have medium-sized pistil and their stamens and anthers are of varying sizes. Mean stamen length of bisexual flowers was found to be significantly longer than that of female flowers (one-way ANOVA, F-statistic: 22.4 on 2 and 87 DF, P < 0.05) and not significant (P = 0.15) when compared to stamens of male flower. Pistils of bisexual flowers were intermediate in length from male and female flowers, and when tested with one-way ANOVA showed high significance (F-statistic: 63.18 on 2 and 87 DF, P < 0.05). All the three sex types had a disc-shaped nectary between the



Figure 1. Diagrammatic representation of the three morphs of a *Nothapodytes* flower. Mean lengths of stamen and pistil (mm) are given for each morph. a, Male flower with a short style and long stamens; b, Female flower with a long style and short stamen; c, Bisexual flower – the variation in the length of the style and stamen is indicated by dotted lines. Among the five stamens, two or three are usually short and the rest are long.

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Figure 2. Morphology and functional gender of the three floral morphs in *N. nimmoniana*. a, Male flower; b, Female flower; c, Bisexual flower with style and stamens of intermediate length. d-f, Pollen and stigma function in *N. nimmoniana*. d, Male and bisexual flowers produce functional pollen, seen here fluorescing viable with FDA test. e, f, Fluorescent photomicrographs of the stigma and upper part of the style. The stigma of male flowers does not support pollen germination (e). A few hair-like, slightly fluorescing structures on the stylar region are surface trichomes; the stigmas of both female and bisexual flowers support good pollen germination and pollen tube growth (f).

Table 1. Mean lengths (\pm SD; mm) of flower parts of *Nothapodytes nimmoniana* (N = 30 for each morph). Mean stamen length of bisexual flower when compared (paired *t*-tests) to that of female flowers was found to be significantly different (P < 0.05) and when compared to male flowers was found to be insignificant (P = 0.24). Pistil length comparisons showed a significant difference between bisexual and female flowers (P < 0.05) and between bisexual and male flowers (P < 0.05)

Flower type	Diameter	Petal	Stamen	Anther	Pistil	Ovary	Stigma	Number of pollen grains/anther
Male	7.48	6.46	5.39	1.29	5.39	1.57	0.49	1030 (± 156)
	(± 0.96)	(± 0.8)	(± 0.69)	(± 0.22)	(± 0.63)	(± 0.29)	(± 0.8)	
Female	7.33	4.78	2.06	0.98	6.78	1.4	1.22	0
	(± 0.44)	(± 0.47)	(± 0.36)	(± 0.19)	(± 0.27)	(± 0.17)	(± 0.08)	
Bisexual	6.44	5.01	5.18	1.69	6.2	1.68	0.69	448 (± 329)
	(± 0.46)	(± 0.53)	(± 0.66)	(± 0.31)	(± 0.35)	(± 0.22)	(± 0.16)	

petals and the ovary, surrounding the ovary about onethird of the lower part. Floral part measurements of the three types are presented in Table 1.

In polygamous trees, female and bisexual flowers made their appearance in the male tree in a few (<10%) inflorescences towards the second week of flowering. During the entire flowering period of 15–20 days, a total of 4.41% (\pm 3.2%) female flowers and 4.47% (\pm 3.1%) bisexual flowers were produced on an inflorescence (N = 24); majority of the flowers (91.12% \pm (5.8%)) were male. Both male and bisexual flowers produced functional pollen (Figure 2 *d*). The number of fertile pollen grains differed significantly (P < 0.05) between male and bisexual flowers (Table 1). In bisexual flowers, only two or three of the total five anthers, which are longer, produced fertile pollen; the shorter anthers contained a few (32 ± 9.9) viable pollen grains per anther (N = 12) or all sterile pollen. The stigma of male flowers did not support pollen germination (Figure 2 *e*) in manually pollinated flowers and they did not set fruits. Female flowers did not produce functional pollen. The stigmas of both female

Flower type	Pollination using pollen from $(N = 12)$	Average fruit set (%)		
Bisexual	Male flowers of the same plant	33.2		
Bisexual	Male flowers from a different plant	37.5		
Bisexual	Bisexual flowers of the same flower/same tree	33.0		
Bisexual	Self pollen (bagged flowers)	26.7		
Female	Male flowers of the same tree	40.7		
Female	Male flowers from a different tree	39.0		
Female	Bisexual flowers of the same tree	43.4		

Table 2. Results of hand-pollination experiments on bisexual and female flowers borne on polygamous trees

and bisexual flowers supported good pollen germination (Figure 2f) and pollen tube growth, and resulted in fruit set.

To test for differences on the surface of the stigmas of the three types of flowers, we conducted a cytochemical test for esterases on the stigmatic surface. The stigmatic surface of female and bisexual flowers developed a pinkish colour, indicating the presence of esterases. The stigma of male flowers did not develop any colouration, indicating absence or very low amount of esterases.

Hand-pollination of female and bisexual flowers with pollen of male and bisexual flowers of the same tree as well as male flowers of other trees showed good pollen germination and set fruits (Table 2), suggesting that the female and bisexual flowers produced on polygamous trees are self-compatible. Eight of the 30 bisexual and female buds (on mixed inflorescences of polygamous trees) netted to keep out visiting insects set fruit, indicating 26.7% natural self-pollination.

There was great variation in the extent of fruit set between years. In 2005, mean natural fruit set under open pollination in mixed inflorescences (on polygamous trees) was 40.67% (± 28.8) and that in female inflorescences (on female trees) was 46.83% (\pm 15.03). The fruit set in female and mixed inflorescences did not show a significant difference (Student's *t*-test, t = -0.92, df = 28.5, P = 0.36). During 2006, however, fruit set in female inflorescences was as low as 6.91% in comparison to 20.02% in mixed inflorescences with a highly significant difference in the means (Student's *t*-test, t = -3.32, df = 48, P < 0.05). Female flowers that were bagged to test for apomixis did not set fruit. In the second year of study (2006), male trees in the vicinity of five of the seven target female trees did not flower.

Of the 40 trees marked for recording sexuality each year, transitions from one sex type to another during the flowering period in 2005 and 2006 were observed in a few individuals. Male trees remained male across the years. Three out of 14 (21.4%) polygamous individuals became male in the second year. We also recorded in the third year, female to polygamous transition in three out of 17 (17.6%) individuals. Such changes are often referred to as 'sex lability'.

Flowers of *N. nimmoniana* are reported to be bisexual in various floral descriptions^{3,19}. On morphological observation, the flowers are comparable to typical dimorphic

floral morphs. We confirmed the sexuality of the three types of flowers by determining the functional gender of flowers through analyses of pollen and pistil function. The thrum-type flowers consisting of normal ovaries have non-functional stigma, suggesting that they might have developed from female sterilization of bisexual flowers. The pin-type flowers consisting of shrunken anthers lacking pollen have functional stigma which supported pollen germination; the female sex appears to have evolved from male sterilization of bisexual flowers. Thus, *N. nimmoniana* exhibits functional/cryptic dioecy as has been reported in several species^{9,20,21}. Male and female flowers differ functionally, but not morphologically. To our knowledge, functional dioecy has so far been reported in homostylus species. *N. nimmoniana* seems to be the first example of functional dioecy in heterostylus species.

About one-third of the trees in our populations produced a proportion of female and bisexual flowers. The number of female and bisexual flowers in polygamous trees is limited to around 7%. We use the term 'incomplete dioecy' to describe the sexuality seen in *N. nimmoniana*. The bisexual flowers produced on polygamous trees revealed intermediate function for their anthers; only two or three of the total five anthers produced fertile pollen. The rest had a few functional or all sterile pollen in them, thus exhibiting incomplete male sterility. The stigma of bisexual flowers also permitted good pollen germination. Further confirmation of sexuality came from fruit-set data: pin and intermediate floral types set fruits, but thrum types never set fruits.

The surface of functional stigma has been shown to contain a range of components made up of several enzymes and non-enzymic moities referred to as extracellular matrix (ECM)^{17,22}. These ECM components play an important role in pollen germination and pollen tube growth²². In the present study, esterases could be localized on stigmas of female and bisexual flowers, but not on male flowers. It, therefore, appears that non-functioning of the stigma of male flowers is due to the absence of esterases or any other components of ECM, which are required for pollen function. Thus female sterility in male flowers seems to be the result of inactivation of gene(s) involved in the secretion of ECM component(s) required for pollen function onto the surface of the stigma. Further, male sterility in female flowers appears to be the result of inactivation or mutation of gene(s) required for

pollen development, as has been reported in several species^{23,24}.

In a population of *N. nimmoniana* studied in northwestern Karnataka⁵, the species has been reported to show a wider range of variation in sexuality. This population has been experiencing regular physical disturbances such as chopping of branches (for trade of wood chips). Perturbations and flexibility in sexuality may possibly be magnified when the plants are subjected to severe disturbances²⁵.

Marked individuals monitored for sexual fidelity over three flowering seasons show that males remain males and some females become polygamous. Some polygamous individuals became males in the second year, not producing female and bisexual flowers. Lloyd¹⁰ has used the term 'inconsistent males' for this condition. The system seems similar to the report on *Fraxinus excelsior*²⁶, where there are (i) trees predominantly male, but always a few female and hermaphrodite flowers on some branches, and (ii) trees purely male in some years, but in other years producing some female and hermaphrodite flowers.

As many as five pathways have been suggested for the evolution of dioecy⁶. Among these, evolution of dioecy from distyly is one²⁸⁻³⁰. In dioecious species derived from distyly, long-styled plants are typically female and shortstyled plants are male. Although the present study does not focus on the evolutionary pathways leading to dioecy, it is tempting to suggest that in N. nimmoniana dioecy has evolved from distyly by sterilization of anthers in female and stigma in male flowers, as has been documented in several other families like Rubiaceae²⁸, Boraginaceae³¹ and Menyanthaceae²⁹. We suggest that the sexual system in Nothapodytes is a stage of evolution between heterostyly and dioecy. A major difficulty in considering the origin of dioecy in Nothapodytes from distyly has been the absence of any distylous taxa in Icacinaceae. But Icacinaceae is not a monophyletic family; it includes several evolutionary lineages³². There are however three instances where a reference is made to unisexual flowers in Icacinaceae - Leretia sp. where flowers are described as 'perfect, rarely unisexual by abortion'; Discophora sp. where flowers are 'possibly unisexual', and Citronella sp. where there are fertile and sterile stamens, and fertile and sterile pistils³³.

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CURRENT SCIENCE, VOL. 99, NO. 10, 25 NOVEMBER 2010

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