Molecular sexing reveals ontogenetic shifts in sex ratios and underlying processes in a dioecious tree species

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Abstract

Most dioecious plants are trees. However, because of the difficulty in determining sex from vegetative morphology, previous investigations of the sex ratios of dioecious trees were limited to flowering individuals, leading to inadequate and potentially unreliable data on patterns of sex ratios and the underlying mechanisms driving their variation. Here, we applied sex-specific molecular markers to investigate the sex ratio of a fully mapped population of the dioecious tree *Diospyros morrisiana* (Ebenaceae) in a subtropical forest. We also investigated the sexual dimorphism of life-history traits and spatial association between male and female trees to determine potential processes shaping the sex ratio at different life stages. Molecular sexing revealed a female-biased population sex ratio for this *D. morrisiana* population, contrasting with the male-biased operational (i.e., flowering) sex ratio. The sex ratio of *D. morrisiana* shifted from female-biased to male-biased over older life stages. We found that reproduction had a larger impact on the growth of female trees, which may account for the ontogenetic shift in sex ratio. There was no evidence of spatial segregation of the sexes beyond a scale of 2 m. Through molecular sexing of all individuals across all life stages, our work revealed for the first time a shift from a female- to a male-biased sex ratio in a huge population of a dioecious tree species. To better understand variation in sex ratios and the underlying mechanisms in dioecious trees, the sex of non-flowering and juvenile individuals should be included in future studies.

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Keywords

Dioecy, operational sex ratio (OSR), sex-specific molecular marker, reproduction cost, sex allocation theory, spatial point pattern analysis

Abstract

Most dioecious plants are trees. However, because of the difficulty in determining sex from vegetative morphology, previous investigations of the sex ratios of dioecious trees were limited to flowering individuals, leading to inadequate and potentially unreliable data on patterns of sex ratios and the underlying mechanisms driving their variation. Here, we applied sex-specific molecular markers to investigate the sex ratio of a fully mapped population of the dioecious tree *Diospyros morrisiana* (Ebenaceae) in a subtropical forest. We also investigated the sexual dimorphism of life-history traits and spatial association between male and female trees to determine potential processes shaping the sex ratio at different life stages. Molecular sexing revealed a female-biased population sex ratio for this *D. morrisiana*population, contrasting with the male-biased operational (i.e., flowering) sex ratio. The sex ratio of *D. morrisiana* shifted from female-biased to male-biased over older life stages. We found that reproduction had a larger impact on the growth of female trees, which may account for the ontogenetic shift in sex ratio. There was no evidence of spatial segregation of the sexes beyond a scale of 2 m. Through molecular sexing of all individuals across all life stages, our work revealed for the first time a shift from a female- to a male-biased sex ratio in a huge population of a dioecious tree species. To better understand variation in sex ratios and the underlying mechanisms in dioecious trees, the sex of non-flowering and juvenile individuals should be included in future studies.

1 Introduction

Dioecious plants are phylogenetically widespread among angiosperms (Renner 2014) and often play crucial roles in various ecosystems, especially forests (Chazdon et al. 2003, Lin et al. 2020, Wang et al. 2020). Dioecy prevents selfing and potentially improves reproductive efficiency, but also raises a greater risk of population decline driven by an imbalance in the sex ratio (Charlesworth and Charlesworth 1981, Henry et al. 2018), which may be exacerbated by climate change (Eckert et al. 2010, Petry et al. 2016, Hultine et al. 2016). Thus, it is imperative to undertake more thorough investigations into variation in the sex ratios of dioecious plants as well as the underlying mechanisms that drive variation in maturity and other life-history traits that differ between male and female individuals.

The majority of dioecious species are trees with a long life-span (Renner and Ricklefs 1995, Wang et al. 2021b), but studies of the sex ratios of these taxa suffer from several difficulties. One primary challenge is that the sex of immature or non-flowering individuals is difficult to identify through vegetative traits (Lloyd and Webb 1977, Barrett and Hough 2013). Most previous studies have therefore been able to use only flowering individuals (i.e., the operational sex ratio, OSR) in order to estimate the population sex ratio (Barrett and Hough 2013, Field et al. 2013). However, using the OSR could generate unreliable and biased results because the sex ratio of all non-flowering individuals is not necessarily the same as the sex ratio of flowering individuals. This problem is especially acute for long-lived tree species, since their populations often include a high proportion of these immature and non-flowering plants (Nanami et al. 1999, Ueno et al. 2007, Queenborough et al. 2007). To address this issue, sex-specific molecular markers can be used to provide more accurate and reliable information on population sex ratios, because they are not restricted to flowering individuals (Eppley 2001, Stehlik and Barrett 2005, Shelton 2010). These markers have mostly been developed for applied research in agriculture, forestry, horticulture, and medicine, and few studies have applied molecular approaches to provide accurate and comprehensive assessments of the sex ratios of

dioecious trees in natural field environments (Ling et al. 2003, Zhang et al. 2016, Liao et al. 2017, Zhou et al. 2018).

Interestingly, sex ratios can change across plant life stages from juveniles to adults, the study of which is critical for addressing when and how sex ratio biases are established (Eppley 2001, Stehlik and Barrett 2005, Stehlik et al. 2007). Because trees are often most vulnerable to competition, herbivory, and pathogen attack at juvenile stages (Moles and Westoby 2004, Comita et al. 2014), the population sex ratio could change dramatically at these stages if one sex is more likely to survive than the other. However, the OSR cannot be used across a number of these significant stages, because the sex of juvenile trees (seeds, seedlings, and saplings) and other non-flowering individuals are not included in the OSR. Several studies have applied the molecular sexing approach to reveal shifts in the ontogenetic sex ratio (Taylor 1994, Eppley 2001, Stehlik et al. 2007, Shelton 2010). For example, Stehlik et.al (2007) found that the perennial herb *Rumex nivalis* had a slightly female-biased sex ratio using flow cytometry, which shifted to be even more female-biased in later life stages (Stehlik et al. 2007). However, these studies have been limited to herbaceous plants with a relatively short life-span, and variation in the sex ratios across the life stages of trees with a long life-span has not been investigated. Sex allocation theory predicts that the juvenile sex ratio is negatively correlated with the adult sex ratio in species with overlapping generations like trees (West 2009). In species with overlapping generations, individuals not only compete for mates within their own generation but also with individuals from the previous generation. For example, if females are less available among adult individuals, female offspring will face less competition for mates compared to males, resulting in an increased reproductive value for females. Consequently, when the sex ratio of adult individuals is biased towards males, a primary sex ratio biased towards females would be preferred (Werren and Charnov 1978, López and Domínguez 2003, Booksmythe et al. 2018). But, to our knowledge, this hypothesis has not obtained any empirical support from tree species.

Understanding the mechanisms that drive variation in sex ratio is critical, and can be broadly grouped into two categories: genetic and ecological (Stehlik and Barrett 2005, Che-Castaldo et al. 2015). A variety of genetic mechanisms could drive divergence from a 1:1 sex ratio at the seed stage, including segregation distortion, Y chromosome degeneration, and sex-selective abortion (Taylor 1994, Stehlik and Barrett 2005, Stehlik et al. 2007). The early life stages of dioecious trees, such as seedlings, can better reflect such gene-determined sex ratios. Variation in sex ratio at later life stages is mainly influenced by ecological mechanisms involved in sex-related differences in resource allocation trade-offs towards growth, reproduction, and defense (Queenborough et al. 2007, Timerman and Barrett 2019). For example, females generally allocate more energy to reproduction, resulting in later maturity, lower flowering frequency, and higher mortality of female individuals and thus a male-biased operational sex ratio (Lloyd and Webb 1977, Delph 1999, Barrett and Hough 2013, Field et al. 2013). Furthermore, if one sex has a different environmental preference compared to another due to different reproductive inputs, a biased sex ratio could occur in heterogeneous environments (Freeman et al. 1976), and may even lead to spatial segregation of the sexes (Nanami et al. 1999). It should be noted that spatial segregation between two sexes would also occur when inter-sexual competition is stronger than intra-sexual competition (Bierzychudek and Eckhart 1988). Multiple studies have compared the growth pattern and spatial distributions between sexes to explore mechanisms influencing the operational sex ratios in dioecious trees based on flowering individuals (Nanami et al. 1999, Queenborough et al. 2007, Jácome-Flores et al. 2016, Timerman and Barrett 2019). However, as stated above, these results might be biased because of the understandable exclusion of non-flowering and immature individuals.

To address this gap in current studies of the sex ratios of dioecious trees, we applied a molecular sexing approach to investigate variation in the sex ratio across life stages and to determine the underlying mechanisms in the dioecious tree *Diospyros morrisiana* (Ebenaceae) in the Heishiding 50-ha forest dynamics plots located in southern China. Specifically, we identified the sex of all 2,255 tagged individuals in the plot and 349 germinated seedlings through sex-specific molecular markers. Combined with analyses of growth dynamics and the spatial patterns of tagged individuals, we address the following questions:

(1) Does the field-observed OSR accurately reflect the overall population sex ratio of *D. morrisiana* , and

how does the sex ratio change across life stages? We hypothesize that OSR differs significantly from the overall population sex ratio. Additionally, we expect considerable changes in the sex ratio of *D. morrisiana* across life stages. Specifically, the adult trees are more likely male-biased, but juvenile trees are more likely female-biased.

(2) What processes are responsible for the shift in the sex ratio of*D. morrisiana* in later life stages? We hypothesize that the sex-specific difference in the growth–reproduction trade-off will lead to a slower growth rate and higher mortality in female trees, resulting in a sex ratio that is increasingly male-biased in later life stages; We also tested whether male and female trees are spatially separated and discussed the potential cause of spatial pattern.

2 Materials and Methods

2.1 Study site and study species

The study was conducted in a 50-ha (1000 × 500 m) forest dynamics plot within the Heishiding (HSD) Nature Reserve, Guangdong Province, southern China. The region has a subtropical moist monsoon climate with an average annual temperature of 19.6°C. The mean annual precipitation is 1743.8 mm, the majority of which falls from April to September. The HSD 50-ha plot was initiated in 2011 and all woody stems with diameter at breast height (DBH, 1.3m) [?] 1 cm were tagged, identified, measured, and georeferenced following the field protocols of the ForestGEO network (Condit 1998). A second census was conducted from January 2016 to March 2017, and all living stems and new recruits were surveyed as above.

Diospyros morrisiana Hance ex Walp. (Ebenaceae) is a dioecious deciduous shrub or small tree inhabiting slopes, ravines, or streamsides in East Asian monsoon subtropical forests between 100 and 1000 m a.s.l (Wu and Raven 1997). It is a dominant species in early and mid-successional subtropical forests in southern China and can grow up to 20 m in height (Abbas et al. 2021). The flowers are small (about 7mm long), white, and urn-shaped. Male plants have axillary cymose inflorescence with 2–3 flowers, whereas female flowers are solitary. The fruits are fleshy berries, yellow when ripe, with a sweet flavor. The flowering season is from May to June, and fruits are ripe in November. The flowers are bee-pollinated, whereas seeds are dispersed by civets (*Paguma larvata* and *Viverricula indica* , Viverridae) (Corlett 1996, 2001).

2.2 Phenotypic sexing and molecular sexing

In May–June and September 2017, all tagged individuals of *D. morrisiana* in the plot were censused. Flowering individuals were recorded and leaves of all individuals were simultaneously collected for molecular sexing. Phenotypic sexing process in the field was undertaken by visual identification, based on flower structure and pedicels or fruit stalks remaining on the branches. Flowering individuals were revisited in May–June 2018 and their reproductive condition was recorded.

To obtain the sex ratio at the seedling stage, we collected seeds from the canopies of 16 female *D. morrisiana* in the HSD 50-ha plot in December 2017. After soaking in distilled water for 1 h and then sterilizing with 25% perhydrol for 1 h, a total of 2,202 seeds from 16 female trees were planted in plastic seedling trays (28 cells, 7 x 7 x 14 cm each) filled with peat soil in March 2018. The plants were routinely irrigated with Hoagland solution. After 3 months, we recorded the number of germinated seedlings for each parent tree, collected the leaves of all seedlings for molecular sexing.

We used a molecular marker to determine the sex of each individual tree and seedling as follows. Total genomic DNA of *D .morrisiana* was extracted from the silica-gel dried leaves using DNA Plantzol (Lifefeng, Hangzhou, China) according to the manufacturer's protocol with slight modifications. Silica-dried leaves (0.3 g) were ground by a high-through tissue grinder (Scientz-48, Ningbo, China) with magnetic beads and 1%

PVP for 400 s, and mixed with 850 μL Plantzol. After incubating at 65°C for 30 min at 600 rpm in MTH-100 Shaker Incubator (Thermo Scientific, Waltham, USA), an equal volume of phenol:chloroform (24:1) was added, and the tubes were turned upside down for 80 times and centrifuged (12,000 rpm, 20 min, 4°C). DNA was precipitated by mixing the aqueous phase with 520 μL isopropanol, turned upside down gently for 100 times and centrifuged (10,000 rpm, 20 min, 4°C). The DNA pellet was washed with 800 μL 75% ethanol, dried and dissolved in an 80 μL TE buffer [10 mM Tris-HCl (pH 8.0), 1mM EDTA]. After isolation, DNA quality was checked by gel electrophoresis and qualified samples were stored in a -20°C refrigerator.

For molecular sexing, we used the primers *OGI-candF1*(5'-CACAGTAGTCATATATTTTTAGC-3')/ *OGI-spR*(5'-CTGGCACACAAAATATTTTCAACCCT-3') developed by Akagi (Akagi et al. 2014). The PCR reaction mixture contained a total volume of 20 μL including 1 μL template DNA, 1 μL forward and reverse primers (TsingK, Beijing, China), and 10 μL 2 × EasyTaq PCR SuperMix (+dye) (Transgene, Guangzhou, China). Amplifications were performed using the following program: initial denaturation step at 94°C for 3 min; 30 cycles consisting of denaturation step at 94°C for 30 s, primer annealing step at 58°C for 30 s and primer extending step at 72°C for 90 s; and final extending step at 72°C for 7 min. Additionally, we designed and used the primers *OGI_500-F* (5'-ACATACAACCAAGCGGAACTG–3') and*OGI_500_R* (5'-AACAGTGCCACCTTCCTTGA -3'), specific to the homologous regions of the *OGI* and *MeGI* genes. Notably, *MeGI* is an autosomal homolog of *OGI* and present in both male and female individuals. The reaction mixture and program were the same as described above except for the temperature of the primer annealing step (52°C) and extension time (60 s). The PCR amplification products were examined on a 1.0% agarose gel contaning ethidium bromide. The gels were imaged using a Tangunon 4100 Gel Image Analysis System (Tanon Science49 & Technology Co., Ltd.). The PCR products of *OGI-candF1/OGI-spR* showed an approximately 1000bp band only for males, while the PCR products of*OGI_500_F/OGI_500_-R* showed bands for both sexes. Using two primer sets simultaneously limits false negative results and thus improves the credibility of the sex identification. We examined the results of molecular sexing for reproductive individuals, and found it was in full agreement with field observation, which proved the feasibility of our molecular approach.

2.3 Sex ratios and sexual dimorphism in life-history traits

To track the ontogenetic shift in the sex ratio of this population of*D. morrisiana* , we chose 5 and 10 cm as cut-off points to divide the tagged individuals of *D. morrisiana* alive in the second census into three DBH classes, and trees [?] 2 cm DBH were further divided out because most flowering individuals are > 2 cm DBH (98.7%), resulting in the following five stages: seedlings, saplings (1 < DBH [?] 2 cm), small trees (2 < DBH [?] 5 cm), medium trees (5 < DBH [?] 10 cm), and large trees (DBH > 10 cm).

Following the results of determining the sex of individuals using phenotypic and molecular methods, we calculated the OSR and population sex ratio for the whole population and for each size class. Sex ratios were expressed as the proportion of male plants to all individuals. To infer the sex ratios in population of *D. morrisiana* , we used Bayes' theorem to estimate the posterior distribution of sex ratios based on binomial likelihood and a uniform prior. We calculated the mean value, standard deviation, and 95% credible interval to summarize the posterior distribution and determine whether each sex ratio is biased. Calculations were performed with the statistical software R (R Core Team 2020) in the package *rethinking* (McElreath 2020).

To assess for sex-related differences in flowering, growth, and survival, we compared the flowering proportion, DBH, relative growth rate (RGR, log difference of DBH in two censuses divided by 5 years), and mortality between the two sexes. The flowering proportion was expressed as the number of flowering individuals as a proportion of all individuals of a given sex. For flowering individuals, we compared flowering proportion in 2017 between the sexes. In addition, we used a generalized linear model to evaluate the effects of sex and DBH on whether individuals flowered or not. Further, we compared DBH and RGR for males and females among flowering individuals and molecular-sexed individuals, as well as RGR between flowering and non-flowering individuals using a Kolmogorov–Smirnov test. We also compared RGR of males and females in each size classes and used a generalized linear model to evaluate the effects of sex and DBH on RGR.

2.4 Spatial association between the sexes

To infer processes responsible for the sex ratio pattern of *D. morrisiana* population from its spatial distribution, we performed spatial point pattern analyses for all tagged individuals. We first tested if there was spatial segregation between male and female trees through a mark connection function. We calculated the mark connection function *p(r)* , which gives the probability that two trees separated by a distance r are of the same sex or opposite (Illian et al. 2008). It follows that the sum of all mark connection functions equals one. If the sexes are randomly distributed over the trees, the mark connection functions are constants derived from the proportions of male, p_M , and female, p_F , and $p_{MM}(r) = p_M$,$p_{FF}(r) = p_F$,$p_{MF}(r) = p_{FM}(r) = p_M * p_F$. To test if the observed pattern deviated from the expected value, we compared the mark connection functions for the observed pattern to a random labeling null model which fixed the locations of all trees and shuffled the sexes. It indicates the existence of spatial segregation of the sexes if$p_{MF}(r)$ is smaller than the expected value of null model. To detect small-scale spatial structure more precisely, we additionally used a local random labeling null model that switched only sexes of trees located within 30 m, a scale somewhat larger than the typical range of plant–plant interactions that is widely affirmed in tropical forest and our study sites (Harms et al. 2001, Uriarte et al. 2004, Wiegand et al. 2007, Wang et al. 2021a).

To determine whether the observed spatial patterns deviated from the null models, we calculated point-wise simulation envelopes with a significance level $\alpha = 0.05$ from the 25th highest and 25th lowest values of 999 Monte Carlo simulations of each null model. To avoid the Type I error inflation caused by simultaneous inference, we also constructed a global envelope to jointly evaluate whether observed pattern deviated from the null models significantly (Myllymäki et al. 2017). All spatial point pattern analyses were performed in the software*Progamita* (Wiegand and Moloney 2013).

3 Results

3.1 Sex ratio shifted from female-biased to male-biased during ontogeny

In 2017, a total of 297 *D. morrisiana* trees on the plot flowered, 181 males and 116 females, giving a male-biased operational sex ratio (OSR) of 0.61 (95% CI: 0.56–0.65). Among flowering trees, 45.3% of males and 34.5% of females were observed flowering again the following year. In contrast, among the 2,255 tagged individuals >1 cm DBH, the molecular sexing technique identified 1,068 male and 1,187 female trees (Figure 1), giving a significantly female-biased population sex ratio of 0.47 (95% CI: 0.46–0.49) (Figure 2a).

The flowering OSR of *D. morrisiana* was male-biased in each size class from small to large trees (Figure 2a). Although more male saplings (1–2cm) flowered, the number of total flowering individuals (n = 4) at this stage was too small to give a significant result (Table S1). In contrast, molecular sexing revealed that the population sex ratio shifted from female-biased in saplings to male-biased in large trees (Figure 2b).

The sex ratio of seedlings followed this same general pattern. Of the 2,203 seeds we collected from 16 mother trees, only 359 seeds from 12 mother trees germinated and survived to produce leaves. We were able to determine the sex of 349 seedlings. The overall sex ratio of seedlings from all the mother trees was strongly female-biased (0.38, 0.34–0.43) (Table S2). Because of the low numbers of seedlings from each mother tree, most sex ratios were statistically insignificant, except for seedlings of one mother tree (C) which were significantly female-biased.

3.2 Sexual dimorphism in life-history traits

We found significant differences in flowering proportion, tree size (DBH), and growth (RGR) between the two sexes (Figure 3). Male trees exhibited a higher flowering proportion than females (Figure 3a). Model results also showed that male trees had higher probability of flowering than female trees (Figure S1). When comparing all individuals together, male trees had a larger DBH than females, although this difference was

not statistically significant ($D = 0.05$, $p= 0.07$) (Figure 3b, S2b). In contrast, the DBH of flowering female trees was slightly, but not significantly, larger than the DBH of flowering males ($D = 0.07$, $p = 0.92$) (Figure 3b, S2a).

Across all individuals, there was no significant difference between the RGR of males and females ($D = 0.03$, $p = 0.70$) (Figure 3c, S2d). However, in flowering individuals only, RGR was significantly higher in males than in females ($D = 0.19$, $p < 0.01$) (Figure S2a). The RGR of flowering females was significantly lower than that of non-flowering females ($D = 0.20$, $p < 0.01$), whereas the RGR of flowering and non-flowering males did not differ significantly ($D= 0.07$, $p = 0.46$). In the comparison of RGR at each life stage for flowering individuals, flowering male trees had higher RGR than flowering females at the medium-sized stage ($D = 0.27$, $p< 0.01$), while there was no significant difference between RGR of males and females in small flowering trees ($D = 0.18$, $p= 0.78$) (Figure S3). The RGR of male trees was also higher than that of females in flowering large-sized trees, but it was not significant ($D = 0.20$, $p = 0.45$) (Figure S3). Model results also showed that the RGR of male trees was higher than that of females only in flowering individuals but not in all individuals pooled (Figure S4).

During the period between the two censuses, 465 trees died, but only 43 of these trees were sexed, 21 of which were females and 22 of which were males, yielding a mortality rate of 1.77% for females and 2.06% for males.

3.3 Spatial segregation of sexes

For all tagged individuals of *D. morrisiana* in the HSD plot, we found that males and females were spatially segregated only at small scales (Figure 4, S5). The mark connection functionp_{MF} (r) was below the simulation envelopes within 2 m, which indicated that the probability of both female and male trees appearing together at this scale was lower than the expectation of the random labeling null model (Figure 4b). On the other hand,p_{MM} (r) andp_{FF} (r) lay well inside the global simulation envelopes for all distances r , exhibiting no repulsion or attraction within the sexes.

4 Discussion

To our knowledge, the present study represents a significant contribution to understanding variation in sex ratios across life stages and the underlying mechanisms in the dioecious trees. Sex-specific molecular markers were applied for the first time to the study of sex ratios in a fully mapped population of a dioecious tree species and revealed a female-biased sex ratio among all tagged and mapped individuals of *Diospyros morrisiana* in a 50-ha forest plot, which was significantly different from the male-biased flowering sex ratio (or OSR). More importantly, *D. morrisiana* was distinctly female-biased in early life stages, but gradually shifted to be male-biased in later life stages. We also tested several ecological mechanisms known to shape sex-specific differences in both life-history traits and spatial pattern. We found that the onset of reproduction had a larger impact on the growth of female trees than that of males, which may account for the ontogenetic shift in sex ratio. Moreover, there was no spatial segregation between male and female trees, except for the scale smaller than 2 m.

The OSR of flowering individuals of *D. morrisiana* in the HSD 50-ha plot was significantly male-biased (Figure 2a), consistent with previous findings in congeners (House 1992, Somanathan and Borges 2000, Venkatasamy et al. 2007). A systematic review also reported that the mean sex ratio based on flowering individuals of 88 dioecious tree species was significantly male-biased (Field et al. 2013). However, in our study we also identified the sex of all tagged individuals in the plot through sex-specific molecular markers and found that the population sex ratio (including all juvenile, immature, and non-flowering trees > 1 cm DBH) was actually female-biased (Figure 2a), confirming for the first time that the OSR does not represent the true overall population sex ratio in dioecious tree population. The disparity between the sex ratios of the OSR and the full population stemmed from sex-specific differences in flowering probability. We found that male

trees were more likely to flower than females (Figure 3a, S1), which resulted in the *D. morrisiana*population with a female-biased population sex ratio displaying a male-biased OSR. In particular, saplings had a low flowering probability and were therefore extremely underrepresented in the OSR, but saplings were the most female-biased of all life stages. The gap between OSR and population sex ratio in *D. morrisiana* supports the results and inferences of previous studies on sex ratios in dioecious trees, showing male-biased flowering sex ratios and the effects of a greater reproductive investment by females in long-lived growth forms such as trees (Lloyd and Webb 1977, Field et al. 2013). Even so, it would be beneficial to apply sex-specific molecular markers more extensively when investigating sex ratios in dioecious plants, especially in trees.

Another important finding was that the sex ratio of *D. morrisiana*shifted from female- to male-biased through later life stages (Figure 2b). The sex ratio in the seedling stage and for large trees was biased in opposite directions, which is consistent with predictions of sex allocation theory for species with overlapping generations (Werren and Charnov 1978, West 2009). Our study provides the first observational evidence supporting this prediction in dioecious trees. The proportion of female individuals gradually declined across life stages, likely resulting in more intense mate competition occurring among males than females. Thus, it could be suggested that the female-biased primary sex ratio might have been favored by selection. Through variation in the sex ratio across life stages, we could postulate how genetic and ecological mechanisms have jointly driven the ontogenetic shift of sex ratio. A distinctly female-biased sex ratio in seedlings may potentially suggest the involvement of genetic mechanisms at the early stage. As the genus*Diospyros* has an XY sex-determination system, restricted recombination between X and Y chromosome would lead to the accumulation of deleterious genes in Y chromosome, which could potentially affect the survival rate of malesn (Charlesworth and Charlesworth 1981, Ming et al. 2011, Akagi et al. 2014, Pilkington et al. 2019). Multiple studies have found that Y-bearing pollen would be less produced in meiosis or be inferior than X-bearing pollen in certation, while male ovules may also have a lower probability of development and germination (Correns 1922, Błocka-Wandas and Sliwinska 2007, Stehlik et al. 2007).

Different costs of reproduction between male and female trees likely explains much of why the population sex ratio was increasingly male-biased in later life stages (Figure 2). Female *Diospyros*produce large fruit, with a greater investment of carbon, nitrogen, and other nutrients than the male individuals invest in flowers and pollen. This differential cost prohibits many females from maturing at the same small size as males (e.g., Figure 3a, Figure S1), from flowering as frequently as males, and also leads to lower growth rates in larger mature females compared to similar-sized males (Figure S3). Specifically, in flowering individuals, male trees at the medium-tree stage that experienced more reproduction events had a significantly larger RGR than females at the same stage, while there was no difference in RGR between male and female saplings or small trees that seldom flowered (Figure S3). Our results are in line with previous studies of dioecious animal-dispersed trees in which females exhibit a greater reproductive investment (Cipollini and Whigham 1994, Obeso 2002, Queenborough et al. 2007, Barrett and Hough 2013, Field et al. 2013). Among flowering individuals, male trees had a higher probability of reflowering, so it was quite likely that males reproduced more times than females during the five-year census interval. However, the lower flowering frequency of female trees did not offset their higher reproduction costs, which suggested that the difference in the costs of one single reproduction event between males and females would be greater.

We may have underestimated the effects of this difference in the costs of reproduction on the population dynamics in this species. Mature females had lower growth than males, but a higher cost of reproduction in females could also lead to lower survival. Unfortunately, due to the timing of our sampling was too close to the second census, there were insufficient molecular-sexed individuals died during the intervening period. Therefore, we were unable to draw definitive conclusions about potential mortality differences between the sexes. Both lower growth and survival rate of females caused by higher reproductive costs would result in a decreasing proportion of females as size increases, and subsequent plot census will help us decouple the influence of sex-specific growth and survival on the shift in sex ratio. Besides, there are signs that sex-specific mortality unrelated to reproductive investment could be also responsible to the shift of sex ratio (Shelton 2010). We found that the sex ratio of individuals became less female-biased from saplings to small trees (Figure 2b), a period when flowering individuals was scarce and RGR between males and females were not

significantly different. This shift suggests that female saplings (i.e., juveniles) may incur higher mortality than males, which was not driven by reproduction cost per se. Further monitoring of the saplings is needed to confirm a sex-specific difference in mortality at this stage.

Sex-based differences in life-history traits could lead to sex-specific habitat preferences, and may be manifested as sexual segregation in spatial pattern (Bierzychudek and Eckhart 1988). However, we did not detect any evidence of sexual spatial segregation among the tagged individuals, except for distances <2 m (Figure 4, Figure S1). Generally, because the scale at which environmental factors operate is always broader than the scale at which individuals interact, this small-scale spatial segregation is less likely to be attributed to sex-specific preferences for microhabitats, but was instead more likely caused by inter-sexual competition (Harms et al. 2001, Valencia et al. 2004, Wiegand and Moloney 2013, Timerman and Barrett 2019). Specifically, for *D. morrisiana* trees, the crown and underground root system spread far beyond 2 m, which indicates that environmental variation occurring only within 2 m would not lead to a difference in the response of male and female trees. In support of this inference, He et al. (2021) demonstrated that seedlings of *D. morrisiana* were more competitive when grown with opposite-sex neighbors than with same-sex ones in a greenhouse experiment, and suggested that differences in root exudates between the sexes may mediate the inter-sexual competition (He et al. 2021).

Conclusion

In summary, through the novel application of molecular sexing of all individuals in a large forest plot, our study revealed an ontogenetic shift in the sex ratio of a dioecious tree population for the first time, and shed light on the factors driving this change. A higher cost of reproduction for females likely leads to an increasingly male-biased sex ratio in later life stages, and Y-chromosome degeneration may account for the pronounced female-biased sex ratio at early stages. The opposite sex ratios of seedlings and large trees were in accordance to the expectations of sex allocation theory. The difference between OSR and true population sex ratio supported previous results based on the sexing of flowering individuals that males invest less in reproduction, and so can flower at smaller sizes, more frequently, and may grow faster than females. Further studies of sex ratio in a wider variety of dioecious tree species using sex-specific markers are needed to obtain more comprehensive knowledge for sex ratio patterns and process of dioecious trees, and to improve our understanding of the ecology and evolution of breeding systems in plants.

Authors' contributions

C.J. Chu, Y.H. Zhang and W. Lin conceived and designed the study. Y.H. Zhang and W. Lin performed the experiments and data analysis. B.H. Li conducted the forest census. Y.H. Zhang and W. Lin contributed materials collection. W. Lin wrote the paper. C.J. Chu, S.A. Queenborough, M. Ni, Q. He, and Y.H. Zhang revised the paper.

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Data Availability Statement:

Data are provided as private-for-peer review via the following link https://datadryad.org/stash/share/S153-14snQidELyJ-Uy3wr2_SwNIfenljDIbyIelj0A and will be permanently archived in Dryad (https://doi.org/

10.5061/dryad.vt4b8gtzj, not active yet) once the manuscript is accepted.

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Figure 1 Spatial point pattern for male (black points) and female (red points) individuals of *Diospyros morrisiana* at the HSD 50-ha plot. Opaque points represent flowering individuals, while the transparent points represent non-flowered individuals that have been molecular-sexed. The grey scale represents the elevation.

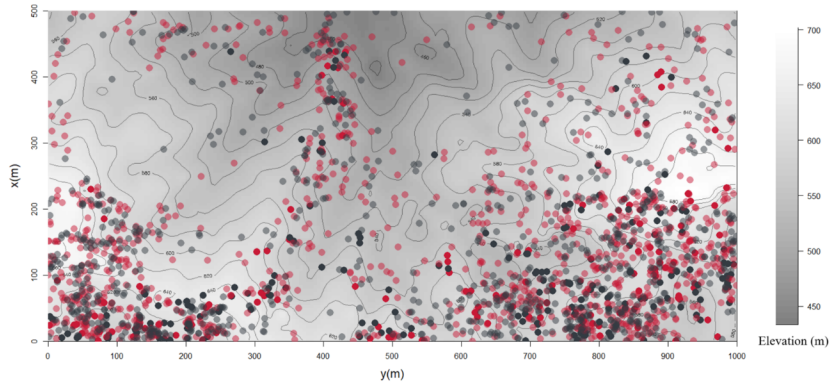


Figure 2 Posterior probability distribution of the operational sex ratio (OSR) and sex ratio for all tagged individuals (a) and sex ratio at each life stage (b) of *Diospyros morrisiana* at the HSD 50-ha plot. The dashed line and shaded area in (a) refer to the mean value and 95% credible interval of distribution. Values plotted in (b) are the sex ratio at different life stages (with 95% credible interval). Intervals on the x-axis in

(b) are the range of diameter at breast height (DBH) for each life stage in cm. The long dark dashed line indicates an unbiased ratio.

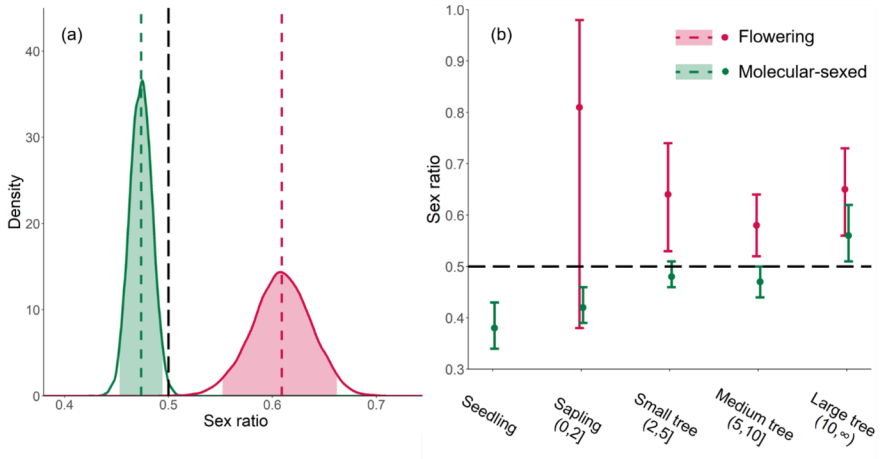
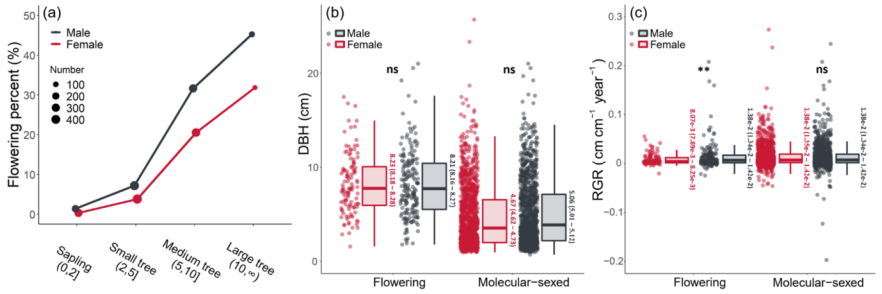


Figure 3. The sex-based difference in life-history traits including flowering proportion (a), diameter at breast height (DBH) (b), and relative growth rate (RGR) (c) of *Diospyros morrisiana* at the HSD 50-ha plot. The size of points on (a) represents the number of molecular-sexed individuals in each size class. Notably, flowering individuals were also molecular-sexed. The boxplots show difference in DBH (b) and RGR (c) between males and females in flowering and molecular-sexed individuals, with each individual represented as a dot. The thick horizontal line indicates the median, the box represents interquartile range (IQR), and the whiskers reach the maximum and minimum value within 1.5 * IQR. p -value significance level: not significant (ns) > 0.05, * < 0.05, ** < 0.01.



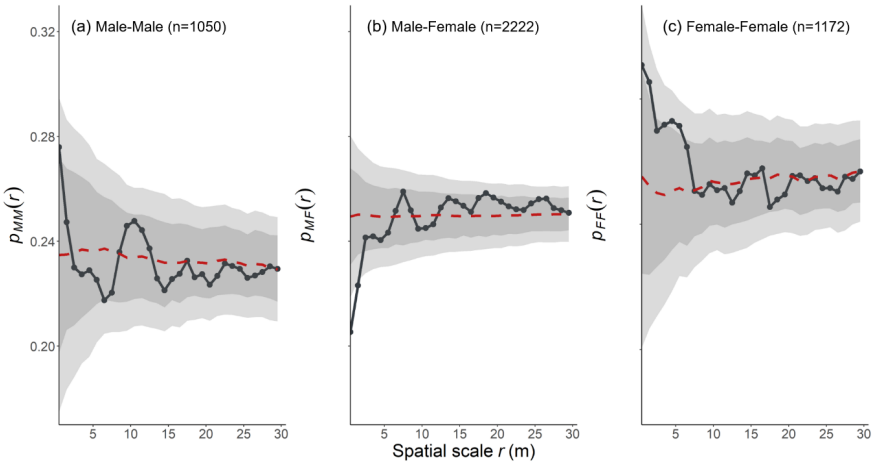


Figure 4 Spatial association within and between sexes in*Diospyros morrisiana* based on mark connection function$p$ (r). pMM (r) and pFF (r) detect the strength of association within males (a) and females (c), whilepMF (r) detects spatial segregation between sexes (b). Local random labelling was used as the null model, which switches the sexes of points closer than 30m. Observed and expected mark connection function p (r) under the null model are represented by solid (black) and dashed (red) lines respectively. Shaded areas represent 95% pointwise simulation envelopes (dark grey boundary) and global envelopes (light grey boundary) constructed using the 25th highest and lowest values of 999 Monte Carlo simulations of the null model. Values outside the envelopes indicate significant departure from the randomness.