

Dioecy in *Amborella trichopoda*: evidence for genetically based sex determination and its consequences for inferences of the breeding system in early angiosperms

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- **Background and Aims** This work aimed to gain insight into the breeding system at the base of living angiosperms through both character state reconstructions and the study of sex ratios and phenotypes in the likely sister to all other living angiosperms, *Amborella trichopoda*.
- **Methods** Sex phenotypes were mapped onto a phylogeny of basally diverging angiosperms using maximum parsimony. In parallel, sex ratios and phenotypes were studied over two consecutive flowering seasons in an *ex situ* population of *A. trichopoda*, while the sex ratio of an *in situ* population was also assessed.
- **Key Results** Parsimony analyses failed to resolve the breeding system present at the base of living angiosperms, but indicated the importance of *A. trichopoda* for the future elucidation of this question. The *ex situ* *A. trichopoda* population studied showed a primary sex ratio close to 1:1, though sex ratio bias was found in the *in situ* population studied. Instances of sexual instability were quantified in both populations.
- **Conclusions** Sex ratio data support the presence of genetic sex determination in *A. trichopoda*, whose further elucidation may guide inferences on the breeding system at the base of living angiosperms. Sexual instability in *A. trichopoda* suggests the operation of epigenetic mechanisms, and the evolution of dioecy via a gynodioecious intermediate.

Key words: Dioecy, sex determination, sex ratio, *Amborella trichopoda*, ANA grade, angiosperms, flowering plants.

INTRODUCTION

An important question of plant evolutionary biology concerns the characteristics of the earliest angiosperms. Among the character states of interest, the breeding system present in early angiosperms, which may have made a vital contribution to the rapid expansion and diversification of this group, is of particular importance. Any attempt at ancestral reconstruction requires a reliable phylogenetic framework, and this is provided in the case of early angiosperms by a consensus of recent molecular studies which clearly indicate the orders Amborellales, Nymphaeales and Austrobaileyales, collectively termed the ANA grade, to have diverged at an early evolutionary stage from all remaining angiosperms, termed euangiosperms. Within this framework, two slightly different alternative topologies emerge from the current literature: the majority of recent studies, summarized by Byng *et al.* (2016), place Amborellales as the earliest diverging ANA grade order, followed sequentially by Nymphaeales and Austrobaileyales. The alternative view emerges from studies by Qiu *et al.* (2001, 2010) and Xi *et al.* (2015), who analysed matrices consisting of five genes from all three genomic compartments, four mitochondrial genes and 310 nuclear genes, respectively. These phylogenies all conclude that the earliest bifurcation in the living angiosperm tree divides a clade composed

of Amborellales and Nymphaeales from one composed of Austrobaileyales and euangiosperms.

Amborellales contains the single species, *Amborella trichopoda*, a dioecious understorey shrub, endemic to New Caledonia. Flowers of male *A. trichopoda* plants typically contain 9–12 tepals and 12–20 stamens, whereas those of females typically contain 7–8 tepals and five carpels, in addition to 1–2 sterile stamen-like organs termed staminodes (Endress, 2001). These staminodes have been interpreted as possible vestiges of a bisexual ancestor, as has a protrusion in the centre of male flowers which may represent a degenerated gynoeceum (Endress and Doyle, 2015). No morphologically distinct sex chromosomes have been identified in *A. trichopoda*, the karyotype being $2n = 26$ in both males and females (Oginuma *et al.*, 2000; Chamala *et al.*, 2013).

Of the remaining early-diverging ANA-grade angiosperms, Nymphaeales contains three families, of which the most basally diverging, Hydatellaceae, is represented by the single genus *Trithuria*. *Trithuria* contains approx. 12 species possessing a mixture of dioecious, monoecious and bisexual breeding systems (Iles *et al.*, 2012). All remaining Nymphaeales, comprising six genera of Nymphaeaceae and two genera of Cabombaceae, possess bisexual flowers (Thien *et al.*, 2009). Within Austrobaileyales, the most basally diverging family,

Austrobaileyaceae, contains the single species *Austrobaileya scandens*, which has bisexual flowers, while Trimeniaceae contains the single genus *Trimenia*, containing both bisexual and andromonoecious species (Endress and Sampson, 1983). The remaining family of Austrobaileyales, Schisandraceae, contains three genera, of which *Illicium* is fully bisexual, *Schisandra* contains a mixture of monoecious and dioecious species (Saunders, 2000), and *Kadsura* is predominantly monoecious, with a tendency towards dioecy (Saunders, 1998).

Within the flowering plants as a whole, around 6 % of species are dioecious, and between 871 and 5000 transitions from bisexuality to dioecy have been inferred (Renner, 2014). Such transitions have been hypothesized to pass via two main pathways involving intermediate gynodioecious and monoecious states, respectively (reviewed by Barrett, 2002).

Here, we use a maximum parsimony analysis to show that the base of living angiosperms is formally ambiguous between dioecious and bisexual breeding systems. We furthermore establish sex ratios in both *ex situ* and *in situ* populations of *A. trichopoda*, the former of which provides strong support for the presence of a genetic sex determination system. We discuss the potential importance of this observation for future inferences on the breeding system at the base of living angiosperms. We also observe sex ratio bias in an *in situ* population of *A. trichopoda*, and instability of sex phenotypes, which provide further clues on the mechanism and evolution of dioecy in this species.

MATERIALS AND METHODS

Character reconstruction by parsimony

Cladograms representing two alternative topologies for the base of angiosperm phylogeny were manually created in Newick format, corresponding to the topologies given by the Angiosperm Phylogeny Group Website (Byng et al., 2016) and by Xi et al. (2014), respectively, with additional data from Saarela et al. (2007), Lohne et al. (2007) and Iles et al. (2012). The internal phylogeny of Nymphaeaceae was simplified by representing *Nymphaea*, *Victoria*, *Euryale* and *Ondinea* as a polytomy as, according to the phylogeny of Loehne et al. (2007), *Nymphaea* is polyphyletic, one section being sister to a clade composed of *Victoria* plus *Euryale*, while another includes *Ondinea*. Character states relating to sex phenotypes (dioecy, monoecy, andromonoecy and bisexuality) were mapped onto both cladograms in Mesquite (Maddison and Maddison, 2015) using parsimony reconstruction. Character states were also mapped without data from *A. trichopoda* to test the importance of this species to the inferences made using the full data set.

Ex situ assessments

Mature fruits were harvested on 5 September 2012 from a single female individual growing at an altitude of 658 m on the eastern slopes of Mont Aoupinié, Northern Province, New Caledonia (S21°9'56.70", E165°18'53.70"). The outer fleshy tissues were removed and seeds were dried for 7 d at room temperature before scarification in concentrated sulphuric acid for 35 min, followed by rinsing with tap water (Fogliani et al., 2017). Scarified seeds were sown in seed trays in a 1:1 (v/v)

mixture of perlite/peat compost and covered with a thin layer of vermiculite. The trays were then placed in a greenhouse at the IAC, Mont Dore in the Southern Province of New Caledonia (S22°13'52.56", E166°32'19.43") at 85 ± 10 % relative humidity and 25 ± 3 °C. Germinated seedlings were re-potted at the 3–6 leaf stage in a 1:1 (v/v) perlite/peat mixture to which a slow-release fertilizer had been added. Plants were transferred to larger pots when necessary and maintained in a greenhouse under 80 % shade conditions.

Sex ratios were assessed during the first and second flowering seasons in observations carried out at least once a month from January to June (inclusive), in 2014 and 2015. The difference between observed and expected sex ratios, using the null hypothesis of an expected 1:1 ratio of males to females, was made using the Sign test.

In situ assessments

The sex of flowers was assessed on the 16 and 17 April 2014 in 162 individuals growing at an altitude of 515–70 m on the slope and central ridge of Mont Aoupinié. To detect the presence of any deviations from strict dioecy, numerous flowers were closely inspected on each of 127 of these individuals, which were located next to a track and thus easily accessible. The sex of flowers in the remaining 35 individuals, which were located further from the track, was assessed using binoculars. The difference between observed and expected sex ratios was analysed statistically as described for *ex situ* assessments.

RESULTS

Character mapping by parsimony fails to resolve the breeding system present at the base of living angiosperms.

We mapped an extensive data set of floral phenotypes in ANA-grade angiosperms, covering monoecious, dioecious, andromonoecious and bisexual arrangements, onto two alternative topologies that emerge from the current literature for the base of angiosperm phylogeny. In both topologies (Fig. 1A, B), the base of extant angiosperms emerged as unresolved between bisexual and dioecious breeding systems. Both phylogenies suggest a transition to monoecy within *Trithuria*.

To test the importance of data from *A. trichopoda* to the reconstructions performed, we removed these from analyses made using both topologies considered. In both cases (Fig. 1C, D), the base of living angiosperms resolved as bisexual, confirming the importance of further studies of sex determination in *A. trichopoda* for the resolution of the breeding system present at the base of extant angiosperms, as discussed by Endress and Doyle (2009, 2015),

A large, seed-grown ex situ population of *Amborella trichopoda* showed a 1:1 sex ratio over two consecutive flowering seasons.

A group of 310 *A. trichopoda* plants, grown under greenhouse conditions, were found in their first flowering season to show a male:female ratio of 1.01:1 (Table 1), thus failing by a wide margin to reject the null hypothesis of a 1:1 sex ratio,

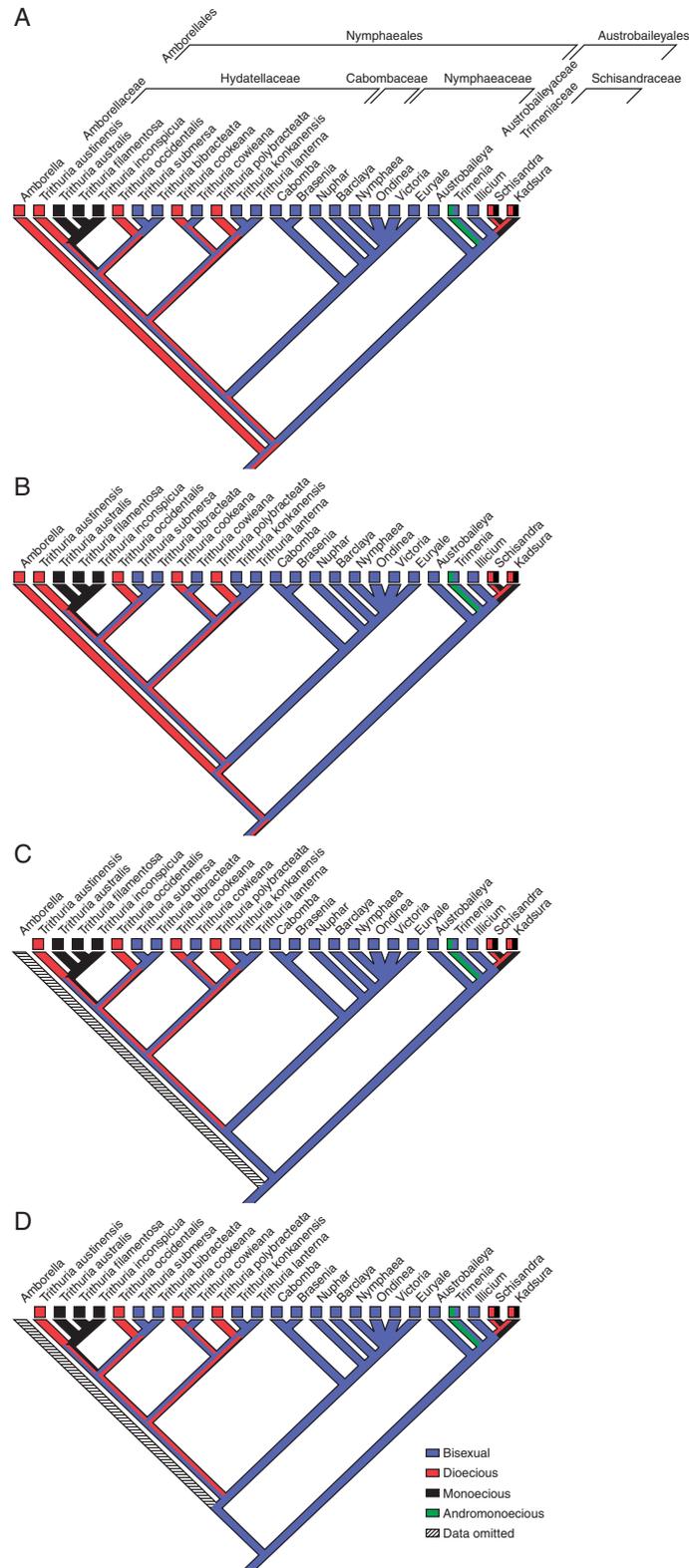


FIG. 1. Character state mapping of breeding system data onto two alternative topologies for the base of angiosperm phylogeny. (A) A phylogenetic reconstruction based on the consensus phylogeny of Byng *et al.* (2016), in which Amborellales is sister to all other angiosperms. (B) An alternative phylogeny, based on Xi *et al.* (2014), in which Amborellales + Nymphaeales is sister to all other angiosperms. In both topologies, the base of the living angiosperms emerges as ambiguous between dioecious and bisexual types. (C and D) Analyses as in (A) and (B), respectively, but excluding data from *A. trichopoda*, showing that, without data from this species, the base of angiosperms resolves as bisexual. The classification of ANA-grade taxa into families and orders is shown.

TABLE 1. Sex ratios for *ex situ* and *in situ* populations of *Amborella trichopoda*

	<i>Ex situ</i> population, 2014	<i>Ex situ</i> population, 2015	<i>In situ</i> population, 2014
Plants surveyed	310	305	162
Males (including partially bisexual males)	155 (33)	150 (11)	95 (2)
Females	153	154	67
Not in flower	2	1	Not determined
M:F ratio	1.01:1	0.98:1	1.42:1
Sign test two-tailed <i>P</i> -value for divergence from expected 1:1 ratio	0.95	0.86	0.034

characteristic of genetic sex determination. Almost all plants flowered during the period of observation. With the exception of five plants that died in the latter half of the first year of observations, all of which had been male, the same group of plants was reassessed in its second flowering season. The overall morphology of typical male and female plants during the second flowering season is shown in [Supplementary Data Fig. S1](#), together with a timeline of cultivation procedures and observations. Again, almost all plants flowered, and only three complete sex change events were noted (one from male to female and two from female to male) between the two seasons. Sex ratio data therefore support a primary sex ratio in *A. trichopoda* of 1:1, with high stability of sex phenotypes (approx. 99 %) between successive flowering seasons. Interestingly, male plants were observed to flower, on average, earlier than females in both 2014 and 2015 ([Supplementary Data Fig. S2](#)).

An in situ Amborella trichopoda population shows a male-biased sex ratio

A sample of 162 plants from an *in situ* population showed a male:female ratio of 1.42:1, indicating a statistically significant deviation from the primary sex ratio of 1:1 observed in an *ex situ* population (Table 1). These data appear to reveal the phenomenon of a sex ratio bias, in favour of males, in a natural population of *A. trichopoda*.

A proportion of male Amborella trichopoda plants produce some bisexual flowers

A small number of male plants in the *ex situ* population studied produced some bisexual flowers in one or both flowering seasons (Table 2). This partial bisexuality varied from the presence of a single carpel (in addition to multiple stamens) in a few flowers, to the production of up to 50 % of fully bisexual flowers, together with male flowers (Fig. 2C–G). The carpels in bisexual flowers appeared to replace stamens. However, male and partially bisexual male phenotypes of *A. trichopoda* appeared readily interconvertible between flowering seasons (Table 2). Fruits were observed on partially bisexual males (Fig. 2F, H) and these were able to grow to maturity (Fig. 2I). Two male plants in the *in situ* sample of 162 plants were observed to produce a small proportion of bisexual flowers (Fig. 2J–L), and fruits were also seen on one of these (Fig. 2M).

DISCUSSION

Sex ratio data support the presence of genetic sex determination in A. trichopoda, with male-biased sex ratios in natural populations.

In this study, the sex ratio of a sizeable seed-grown *ex situ* population of *A. trichopoda*, in which almost all plants flowered, proved very close to 1:1 when plants bearing male and some bisexual flowers were considered as male. No transitions between partially bisexual male and completely female phenotypes were observed, supporting this classification. The sex of *A. trichopoda* individuals was very stable (approx. 99 %) between two consecutive flowering seasons. Both the 1:1 primary sex ratio and the high stability of sex phenotypes clearly support the presence in *A. trichopoda* of genetic sex determination.

In contrast, a male:female ratio of 1.42:1 was observed in a natural population of *A. trichopoda*, representing a statistically significant deviation from the expected 1:1 ratio. Interestingly, Thien *et al.* (2003) performed a similar survey, and found an even greater male bias of approx. 1.8:1. Work by Field *et al.* (2013b) indicates that sex ratio bias is commonly encountered in dioecious species, with male bias being more common than female bias. These authors find instances of male bias to correlate with the long-lived growth habit, fleshy fruits and biotic seed distribution. Both the long-lived growth habit and fleshy fruits are present in *A. trichopoda*, while biotic seed distribution may be present in this species (Endress and Igersheim, 2000), or may have been present recently in its evolutionary past (Fourcade *et al.*, 2015). Observed sex ratios may also be biased through sampling effort (Field *et al.*, 2013a). The tendency for male *A. trichopoda* plants to flower earlier than females (Fig. S2), coupled to the limited time scale over which *in situ* observations were carried out in the present work, may also therefore have contributed to the male bias observed.

Bioinformatics analyses of Amborella trichopoda may help to elucidate the breeding system present at the base of living angiosperms

Maximum parsimony analyses performed in this study (Fig. 1A, B) failed to resolve the breeding system at the base of living angiosperms between bisexual and dioecious types. However, the exclusion of data from *A. trichopoda* led to the resolution of the basal node in this phylogeny as bisexual (Fig. 1C, D). Clearly, a date for the origin of dioecy in *A. trichopoda* could help to elucidate this question further: if that origin were more recent than the initial radiation of living angiosperms, we could conclude that the most recent common

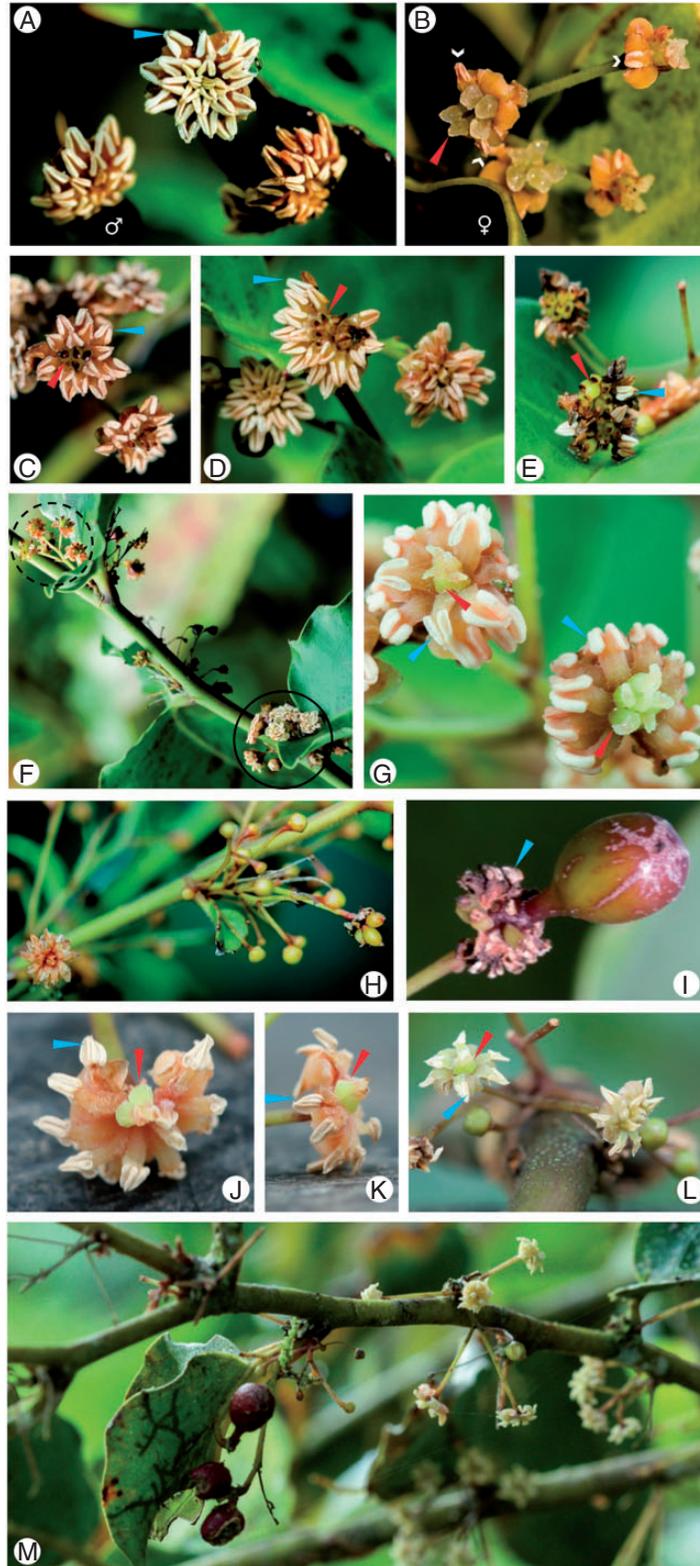


FIG. 2. Sex phenotypes in *Amborella trichopoda*. (A–I) Plants from an *ex situ* population. (A) Completely male flowers. (B) Female flowers. (C–E) Increasing levels of sexual ambiguity among male flowers. (F) Inflorescences borne on the same branch harbouring both male (solid line) and ambiguous (dashed line) flowers at early fruit set. (G) Ambiguous male flowers after (left) and before (right) anther dehiscence. (H) Late fruit set and male flower observed on the same axis. (I) Maturing fruit among degenerating stamens. (J–M) Plants from an *in situ* population growing on Mount Aoupinié. (J and K). An ambiguous male flower with two carpels. (L) An ambiguous male flower with three carpels (left) next to a completely male flower (right). (M) Male flowers and mature fruits on the same axis. Stamens, blue arrows; carpels, red arrows; staminodes, white arrows.

TABLE 2. *Floral phenotypes in an ex situ population of 310 Amborella trichopoda plants, 2014–2015, indicating instances of sexual instability*

Phenotype in 2014	Phenotype in 2015	Number of plants
Completely male	Completely male	111
Female	Female	151
Partially bisexual male	Partially bisexual male	6
Completely male	Female	1*
Completely male	Partially bisexual male	5
Completely male	Dead	5
Female	Completely male	2*
Partially bisexual male	Completely male	26
Partially bisexual male	Did not flower	1
Did not flower	Female	2

*Complete sex change event.

ancestor (MRCA) of living angiosperms was probably bisexual. In contrast, an origin of dioecy in *A. trichopoda* that pre-dated the radiation of living angiosperms would support the presence of dioecy in the MRCA of that group. It should be emphasized that, whatever the breeding system in the MRCA of living angiosperms, other breeding systems may have been present in contemporaneous early angiosperms (whose lineages later became extinct).

It can be expected that genes physically linked to the (hypothesized) sex-determining chromosomal region in *A. trichopoda* will have ceased to recombine. As no morphologically distinct sex chromosomes have been observed in cytological examinations (Oginuma *et al.*, 2000; Chamala *et al.*, 2013), this sex-determining region is likely to be of limited size, though might still cover some tens of mega-bases. Non-recombining polymorphic genes within this region could be detected from RNA sequencing data of families or populations using a number of recently developed bioinformatics methods (Bergero and Charlesworth, 2011; Chibalina and Filatov, 2011; Muyle *et al.*, 2012). Such genes could then be localized within the (male) reference genome of *A. trichopoda* and the several available re-sequenced genomes of both sexes (*Amborella* Genome Project, 2013), potentially providing an efficient means to characterize the entire sex-determining region. Molecular sequence divergence within this region could then be used (Charlesworth, 2008) to date the initial arrest of sex chromosome recombination in the *A. trichopoda* lineage, thereby informing further efforts to reconstruct the breeding system at the base of living angiosperms.

Sexual instability provides clues on the mechanism and evolution of dioecy in Amborella trichopoda

In the present work, we identified two types of sexual instability in *A. trichopoda*. First, we noted a small proportion (approx. 1 %) of complete sex change events in a population of young plants grown from seed. These findings confirm observations by Buzgo *et al.* (2004), who noted similar sex changes in *A. trichopoda* grown from cuttings. Secondly, we found a variable proportion of bisexual flowers on some predominantly male individuals in both *ex situ* and *in situ* populations. Sexual instability is a common phenomenon in dioecious plant species, and many exogenous, environmental and demographic factors

are known to affect sex ratios, including plant hormones (Durand and Durand, 1991; Papadopoulou and Grumet, 2005), non-methylable nucleotide analogues (Janousek *et al.*, 1996), temperature (Manzano *et al.*, 2014), pathogens (Scutt *et al.*, 1997), timing of seed set (Freeman *et al.*, 1994) and population structure (Stehlik *et al.*, 2008). Some of these factors strongly suggest the involvement of epigenetic mechanisms operating in sex determination, and sexual instability in *A. trichopoda* may therefore involve such factors, operating either at, or downstream of, sex-determining loci.

Partially bisexual males have previously been observed in multiple species in which dioecy is thought to have evolved through the gynodioecious pathway (Charlesworth and Charlesworth, 1978, Lloyd, 1980), which suggests that dioecy in *A. trichopoda* may also have evolved via this route. Such ‘inconstant males’ are predicted by mathematical modelling to be stably maintained in populations, causing a tendency to subdioecy (Ehlers and Bataillon, 2007). The presence of partially bisexual males is not, therefore, necessarily informative on the timing of the origin of dioecy in *A. trichopoda*. Fruits generated from bisexual flowers on otherwise male *A. trichopoda* plants have been observed to grow to maturity (Fig. 2). However, we have not yet tested the viability of the seed generated within these, or the sex ratio and fertility of the following generation. It is, therefore, not yet certain that the carpels that develop in a proportion of otherwise male *A. trichopoda* flowers represent a functional deviation from complete dioecy.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: 32-month-old *A. trichopoda* plants in May 2015. Figure S2: flowering time in *ex situ* male and female plants.

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