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 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 basal 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 gynoecium (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, monocious 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 *Austrobaileyia 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 (Remer, 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 *Onodinea* as a polytomy as, according to the phylogeny of Lohne et al. (2007). *Nymphaea* is polyphyletic, one section being sister to a clade composed of *Victoria* plus *Euryale*, while another includes *Onodinea*. 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 Aoupinie, Northern Province, 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 1:0.1 (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.
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 *Amborella 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).

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

<table>
<thead>
<tr>
<th>Plants surveyed</th>
<th>Males (including partially bisexual males)</th>
<th>Females</th>
<th>Not in flower</th>
<th>M:F ratio</th>
<th>Sign test two-tailed P-value for divergence from expected 1:1 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex situ population, 2014</td>
<td>310</td>
<td>155 (33)</td>
<td>153</td>
<td>2</td>
<td>1-01:1</td>
</tr>
<tr>
<td>Ex situ population, 2015</td>
<td>305</td>
<td>150 (11)</td>
<td>154</td>
<td>1</td>
<td>0-98:1</td>
</tr>
<tr>
<td>In situ population, 2014</td>
<td>162</td>
<td>95 (2)</td>
<td>Not determined</td>
<td>1-42:1</td>
<td>0-034</td>
</tr>
</tbody>
</table>

**DISCUSSION**

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

In this study, the sex ratio of a sizeable seed-grown ex situ population of *Amborella 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 *Amborella 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 *Amborella trichopoda* of genetic sex determination.

In contrast, a male:female ratio of 1-42:1 was observed in a natural population of *Amborella 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 *Amborella 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 *Amborella 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 *Amborella 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 *Amborella 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 Aoupinie. (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

<table>
<thead>
<tr>
<th>Phenotype in 2014</th>
<th>Phenotype in 2015</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely male</td>
<td>Completely male</td>
<td>111</td>
</tr>
<tr>
<td>Female</td>
<td>Female</td>
<td>151</td>
</tr>
<tr>
<td>Partially bisexual male</td>
<td>Partially bisexual male</td>
<td>6</td>
</tr>
<tr>
<td>Completely male</td>
<td>Female</td>
<td>1</td>
</tr>
<tr>
<td>Completely male</td>
<td>Partially bisexual male</td>
<td>5</td>
</tr>
<tr>
<td>Completely male</td>
<td>Dead</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>Completely male</td>
<td>2</td>
</tr>
<tr>
<td>Partially bisexual male</td>
<td>Completely male</td>
<td>26</td>
</tr>
<tr>
<td>Partially bisexual male</td>
<td>Did not flower</td>
<td>1</td>
</tr>
<tr>
<td>Did not flower</td>
<td>Female</td>
<td>2</td>
</tr>
</tbody>
</table>

*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 (hypoth- esized) 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.

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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LITERATURE CITED


