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Valerie Poncet, Philippe Birnbaum, Valérie Burtet-Sarramegna, Alexandre de Kochko, Bruno Fogliani, et al.. Amborella – Bearing witness to the past ?. Annual Plant Reviews Online, 2019, 3, pp.1 - 41. ⟨10.1002/9781119312994.apr0689⟩. ⟨hal-02291318⟩

AMBORELLA – BEARING WITNESS TO THE PAST?

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Abstract: *Amborella trichopoda* (Amborellaceae) is a shrub endemic to New Caledonia in the Southwest Pacific region. This plant suddenly became famous when molecular phylogenetic studies revealed that this sole species is likely the sister taxon to all other angiosperms. It has thus been a prime research model for reconstructing plant evolution and gaining insight into what the earliest angiosperms looked like. A wealth of studies on *Amborella* have now shed considerable light on its genome, morphology, anatomy, physiology, development and architecture—this research is reviewed here. While *Amborella* likely retained some ancestral traits, critical character reconstructions have also highlighted some derived and sometimes unique characters in this species. The history of *Amborella* is also tied to the South Pacific archipelago of New Caledonia, its homeland. It was part of the New Caledonian biogeography puzzle and its genetic history shed light on the dynamics of its ecosystem, the rainforest understorey. *Amborella* is now more broadly cultivated and has been the focus of some conservation measures that will benefit other species in this biodiversity hotspot.

Keywords: *Amborella trichopoda*, New Caledonia, angiosperms

1 Introduction

Amborella trichopoda (Amborellaceae), referred below as *Amborella* is a shrub endemic to New Caledonia in the Southwest Pacific region that rose to fame almost 20 years ago because of its phylogenetic position as a likely sister taxon to all other living angiosperms. First only mentioned in a footnote by Baillon in 1869 (1869), *Amborella* was then described in more detail by Baillon himself (1873) based on a study of herbarium specimens. At that time, Baillon only had male flower and fruit material. It was first considered to be close to *Hedycarya* (Monimiaceae, Laurales), but later placed in its own family, i.e. Amborellaceae, by Pichon (1948) but still in the Laurales order. Finally, with the advent of the Angiosperm Phylogeny Group (APG) system and the use of molecular tools, *Amborella* was firmly classified in its own monotypic family (Amborellaceae), within its own order Amborellales (APGII, 2003).

Amborella was early recognized as being an exceptional plant because of its unique morphological features, mainly its flowers. But what raised more questions among botanists in the mid-20th century is the absence of xylem vessels in this plant (Bailey and Swamy, 1948). Finally, it was only recently determined—with the advent of molecular tools—that its taxonomic status became the one that is now widely accepted (APGIV, 2016). *Amborella* had mainly been studied by botanists since the 19th century, but in the late 1990s the species started to interest a wider range of plant and evolutionary scientists. A wealth of studies on *Amborella* have now brought considerable insight into the ancestral angiosperm genome, seed, flower and plant architecture, as well as into the unique ecology, biogeography and conservation biology of its home island, New Caledonia—we review these aspects here.

2 Amborella's Position on the Tree of Life

Amborella was included in the first broad-scale phylogenetic study of angiosperms (Chase et al, 1993) and was recovered in a clade (named 'Paleoherb II') containing taxa that are all now thought to represent relatively early-diverging angiosperm lineages, but not Monimiaceae or Laurales. The first phylogenetic study, published in 1997, that suggested *Amborella* may be sister to the rest of living angiosperms was based on nuclear 18S ribosomal sequences (Soltis et al, 1997) (Figure 1). Several parallel studies published in late 1999, based on nuclear, plastid and/or mitochondrial genes, confirmed this phylogenetic position of *Amborella* with increased confidence (Mathews and Donoghue, 1999; Parkinson et al, 1999; Qiu et al, 1999; Soltis et al, 1999).

In the following few years, sequencing of the complete plastid genome was used as a tool to reconstruct phylogenies, and the earliest studies suggested that monocots were the actual sister group to the rest of the angiosperms (Goremykin et al, 2003), and that *Amborella* and Nymphaeales may be sisters (Goremykin et al, 2004). However, these studies included a limited number of species, and grasses were used as sole representatives of monocots. Moreover, the fast molecular evolutionary rates observed in the latter probably had long-branch attraction effects on phylogenetic reconstruction (Soltis et al, 2004; Soltis and Soltis, 2004; Stefanovic et al, 2004). Subsequent phylogenetic studies based on plastid genomic data with broader sampling (including non-grass monocots) revealed *Amborella* to be a sister to all other angiosperms (Drew et al, 2014; Moore et al, 2007). Over the past decade there has been a dramatic increase in data available based on transcriptome and complete genome sequencing. With access to high numbers of unlinked nuclear genes, conflicts emerged regarding the two ways to analyze them, i.e. concatenated and coaelescent methods, where the first one placed

Amborella as a sister to the rest of the angiosperms, while the second one was considered as sister to Nymphaeales (Xi et al, 2014). The latter topology may have resulted from artifacts associated with misalignment, mis-rooted gene trees, and character subsampling (Simmons, 2017; Zhong and Betancur-R, 2017). Although some debate remains, the current scientific consensus indicates that *Amborella* alone is likely sister to the rest of the angiosperms (APGIV, 2016), and it will be considered as such in the present review. In this consensus phylogeny, the Amborellales, Nymphaeales and Austrobaileyales orders, collectively referred to as the ANA grade, diverged successively from a lineage that led to a clade containing all other living angiosperms, which are referred to as mesangiosperms. This very large group comprises monocots, eudicots, magnoliids, Chloranthales, and Ceratophyllales (Figure 1).

The age of the divergence between Amborellales and all other living angiosperms (i.e. the most recent common ancestor, or MRCA, of living angiosperms) remains a topic of substantial debate. There is no known fossil record of *Amborella,* or any fossil that could be attributed to the Amborellales lineage. The earliest unambiguous crown group angiosperm fossil is *Montsechia,* a probable relative of the living genus *Ceratophyllum* that dates back to 130-125 million years ago (Mya) (Gomez et al, 2015), so the MRCA of living angiosperms can be considered to have lived some time before that approximate date. Molecular clock studies can provide estimates to date the initial divergence between extant angiosperms, but the ages obtained vary widely between studies, from 256 to 140 Mya (Barba-Montoya et al, 2018; Bell et al, 2010; Magallón et al, 2015), see Soltis et al. (2018) for a more thorough review of the many age estimates available. A recently discovered fossil flower (Fu et al, 2018) provides further physical evidence of angiosperms as early as 174 Mya, but the precise taxonomic affinities of that species are not currently known, so it may predate the MRCA of living angiosperms.

The position of *Amborella* among angiosperms somewhat resembles that of platypus among mammals. It also displays some features that may be regarded as gymnosperm-like, though these must be interpreted with caution as many of them (e.g. spiral phyllotaxy in reproductive organs, dioecy, orthotropic ovule symmetry) may represent secondarily derived characters that were not present in the MRCA of living angiosperms. Though *Amborella* may be derived (i.e. contain characteristics that have diverged from those of the MRCA of extant angiosperms) in many respects, its likely phylogenetic position gives it considerable weight in reconstruction analyses, and *Amborella* provides a major contribution to understanding the origin and evolution of flowering plants, and hence to unravel what Charles Darwin in 1879 (1903) considered to be an "abominable mystery". Indeed, since the moment *Amborella* was shown to be the likely sister group of all other angiosperms, it became the focus of studies in a broad range of disciplines (Figure 2).

3 *Amborella* and the Evolution of the Angiosperm Genome

When most phylogenetic studies confirmed the unique position of *Amborella*, the full sequencing of its genome soon became a priority (Soltis et al, 2008). *Amborella* has a relatively small genome (1C= 0.89 pg, 870 Mbp, Leitch and Hanson, 2002; 2n=26, Oginuma et al, 2000), a feature that is suspected to be ancestral in angiosperms (Soltis et al, 2003). The sequencing of the *Amborella* genome *via* the The *Amborella* Genome Project was expected to provide clues as to the genetic adaptations that helped flowers emerge and dominate the world 256-140 Mya. Just as the sequencing of the genome of the duck-billed platypus in mammals yielded insight into the emergence of mammals (Warren et al, 2008), *Amborella*'s genome provides a glimpse at changes that helped angiosperms diversify from a common ancestor with gymnosperms. The goal of this project was to generate a high quality *Amborella* genome sequence, which was released in December 2013 (Amborella Genome Project et al, 2013), using a new assembly and validation approach (Chamala et al, 2013).

3.1 Whole Genome Duplication to Gain New Functions

Amborella genome comparisons with three eudicot genomes (grape, peach, and cacao) allowed inference of the hypothetical structure of pre-hexaploidization chromosomes in the ancestor of the core eudicots (Amborella Genome Project et al, 2013). Comparisons with other seed plant genomes provided clear evidence that an early ancestor of flowering plants gained a duplicate copy of its genome, a feature known as whole genome duplication (WGD) or polyploidy, and previously suggested on the basis of other data (Jiao et al, 2011). The *Amborella* genome appears to be relatively simple as there is no evidence of WGD since its divergence from the rest of the angiosperms, contrary to e.g. tomato (Tomato Genome Consortium et al, 2012). Indeed, the vast majority of living angiosperms have undergone one or more genome multiplications since the epsilon WGD, and many show traces of ancient WGD events that occurred in shared ancestors at different points in the angiosperm phylogeny. Analyses of paralogous gene copies have indicated that the epsilon WGD event occurred shortly before the diversification of all living angiosperms. This duplication may have spurred the diversification and expansion of flowering plants by providing an extra copy of each gene that could subsequently evolve towards new functions (Wendel et al, 2016). Some duplicated genes were lost over time but others took on new functions (neofunctionalization), such as genes involved in flower development that have homologs in other seed plants.

At least 14,000 protein-coding genes existed in the last common ancestor of all flowering plants (Amborella Genome Project et al, 2013). Among these genes, 1179 gene lineages (orthogroups) are unique to flowering plants (or have diverged sufficiently since no gymnosperm homologs have been detected) and many are known to be important for producing flowers as well as other structures and processes specific to flowering plants. Among the genes related to reproduction, the data revealed the existence of 36 MADS-box genes of 21 major clades, which implies that at least 21 such genes were present in the MRCA of living angiosperms. Some of these genes were previously demonstrated to have expression patterns in flower tissues (Kim et al, 2005), consistent with putative roles in floral organ specification that had been conserved from early angiosperms. The diversity of MADS-box transcription factors, and their interaction through protein-protein interactions, was probably important in the diversification of floral forms.

The *Amborella* genome is also unique in that there is no evidence of recent transposon insertions while ancient and divergent transposons have been retained (Amborella Genome Project et al, 2013). Its transposable elements seem to have stabilized in the genome in the past several million years. As an additional feature, the genome harbors an abundance of atypical lineage-specific 24-nucleotide microRNAs, with at least 27 regulatory microRNA families inferred as having been present in the ancestral angiosperm.

3.2 Reconstruction of the Ancestral Genome as a Central Reference Genome

The *Amborella* genome was the first nuclear genome sequence released, and it is still the only one available, among ANA grade species, so it has considerable importance in the reconstruction of trait evolutions associated with flowering plants. The information released was considered as pivotal for all genome evolution, gene family evolution, and phylogenetic studies (Adams, 2013). Since its publication and release in 2013, the *Amborella* genome has been referred to nearly 300 times (Scopus) in the literature and included as a reference genome in public resources such as Ensembl Genomes (Kersey et al, 2016). As the *Amborella* genome shows no evidence of WGD since it diverged from the rest of the angiosperms, it turned out to be a good reference to assist in the analysis of recently sequenced genomes of angiosperms such as the ash tree (genus *Fraxinus*) (Sollars et al, 2017) or the obligate mycoheterotrophic *Gastrodia elata* (Yuan et al, 2018), but also of conifers such as *Pinus taeda* (Neale et al, 2014) or of a liverwort, *Marchantia polymorpha* (Bowman et al, 2017).

Because of the absence of additional whole-genome duplication specific to the *Amborella* lineage, the *Amborella* genome has enabled reconstruction of the genome of the MRCA of extant angiosperms. Likewise, it helped gain insight into how genome evolution has proceeded in different lineages after genome doubling (the gamma hexaploidy event, Figure 3) occurred early in eudicot evolution (Chanderbali et al, 2017).

Researchers studying other plants considered the *Amborella* genome useful as a reference point to identify and study families of genes in other plants, including crops. The genome sequence provides insight into the evolution of complex seeds, floral scents and other flowering plant features. For example, in striving to identify proteome signatures that could be associated with the origin and early diversification of angiosperms, the *Amborella* vacuolar processing enzyme (VPE) family was characterized (Poncet et al, 2015). This family is part of a major process involved in seed storage protein synthesis and as such governs the establishment of vigorous seedlings. Phylogenetic analyses showed that *Amborella* sequences grouped within two major clades of angiosperm VPEs, indicating that the duplication that generated the ancestors of these clades occurred before the most recent common ancestor of living angiosperms (Poncet et al, 2015) (see the seed chapter below for further detail).

In conclusion, the availability of the *Amborella* genome has paved the way for a multitude of analyses and interpretations of evolutionary traits associated with the domination of flowering plant species worldwide. It has broadened the horizons and enhanced the data interpretation potential for any analysis of genomics, transcriptomics or proteomics data, thus helping to gain a better overall understanding of plant evolution.

4 *Amborella* Mitochondrial Genome, a Sponge for Foreign Genes

The struggle to resolve the exact phylogenetic position of *Amborella* has led to the sequencing of a wide range of markers from all three genomic compartments while revealing some interesting cases of horizontal transfer in the mitochondrial genome. Vertical gene transfer involves the transfer of genes from parents to their progeny (i.e. sexual reproduction), and occurs within species or very closely related taxa (hybridization). In contrast, horizontal (i.e. lateral) gene transfer (HGT), refers to nonsexual transmission of genetic material between unrelated organisms. Hence, HGT involves gene transfer across species limits (Crisp et al, 2015; Soltis et al, 2011). HGT is common in bacterial evolution (Boucher et al, 2003), and the advent of eukaryotic mitochondria and plastids—derived from endosymbiosis of bacterial progenitors (Archibald, 2011; Keeling and Palmer, 2008)—is a key HGT in plant evolution. Besides these endosymbiosis events, little is currently known about the evolution of mitochondrial and plastid genomes with regard to HGT, except in angiosperms, where many cases of HGT in mitochondrial genomes have been reported (Bergthorsson et al, 2003; Xi et al, 2013; Yang et al, 2016), while this process remains apparently rare or absent in plastid genomes (Park et al, 2007; Rice and Palmer, 2006).

HGT is probably one evolutionary driving force playing an important role in adaptation to parasitism, pathogenesis (Soucy et al, 2015) and other specific environmental conditions (Keeling and Palmer, 2008), and even in adaptive radiation (Cooper, 2014). Studies with fungi suggest that mitochondrial HGT may play an important role in genome evolution in a much broader spectrum of eukaryotes, and some authors have pointed out that there is "a critical need to systematically study the frequency, extent, and importance of mitochondrial HGT across eukaryotes" (Wu et al, 2015).

Most horizontal transfers identified in mitochondrial genomes were single events, and *Amborella* was also known to have one case of HGT (Bergthorsson et al, 2003), which was previously misinterpreted as being a 'divergent sequence' (Qiu et al, 1999) or a 'paralogue' gene (Barkman et al, 2000). But another study of the *Amborella* mitochondrial genome revealed no fewer than 28 foreign genes, from other diverse land plants (Bergthorsson et al, 2004). Other angiosperms (especially eudicots) were the donors of these genes, whereas other genes are derived from non-angiosperms, including at least three different moss donors (Figure 4). *Amborella* mtDNA has undergone the most numerous HGT events than any other eukaryotic genome examined (Bergthorsson et al, 2004), thus strangely serving as a kind of "sponge" for foreign genes. Some plants are known to have several mitochondrial HGTs (Mower et al, 2010; Xi et al, 2013), but *Amborella*—with so many cases of HGT involving so many different foreign donors—appeared to be the best model for studying this phenomenon.

A more in-depth study was thus carried out involving sequencing of the complete mitochondrial genome of *Amborella*, revealing a huge 3.9-megabase genome (Rice et al, 2013). This study confirmed the presence of many previously identified foreign genes, but also six apparently complete mitochondrial genomes acquired from non-angiosperm plants (three green algae and one moss) and other angiosperms. Most of these transfers probably date back millions of years. The foreign genomes are remarkably well preserved, with very little deletion and rearrangement. The authors suggested a fusion-compatibility model was involved, where the whole eukaryotic mitochondrion was captured, followed by mitochondrial fusion and genome recombination.

One might consider that these incredible horizontal gene and genome transfers where detected in *Amborella* because it was studied first based on its exceptional phylogenetic position. However, recent whole mitochondrial sequencing studies of other basal angiosperms such as *Nymphaea colorata* (Nymphaeaceae) did not reveal any horizontal gene transfer event (Dong et al, 2018), while the mitochondiral genome of *Liriodendron tulipifera* (Magnoliaceae) was qualified as "fossilized" (Richardson et al, 2013). In eudicots, *Hibiscus cannabinus* (Malvaceae) was recently sequenced and only a single protein-coding gene having a characteristic chimeric suspected to be of HGT origin was identified (Liao et al, 2018). *Amborella* nevertheless still remains unique because of its huge sponge-like mitochondrial genome, which highlights the potential scale of neutral evolution.

Finally, several known mitochondrial HGTs occurred between parasitic plants and their hosts (Mower et al, 2010; Xi et al, 2013; Yang et al, 2016), where the intimate physical association of these plants probably facilitated DNA material transfer. In *Amborella* (Rice et al, 2013), the presence of several genes from a donor in the parasite-rich Santalales order (Rubiales and Heide-Jørgensen, 2011) might have involved the same transfer mechanism. The presence of genes and genomes from mosses and green algae could possibly be explained by the frequent presence of many epiphylls and epiphytes on the host plant (Rice et al, 2013), but this phenomenon is relatively common in many tropical rainforests. However, the presence of genes with exceptionally high and specific similarity to *Ricinus* and *Bambusa* in the *Amborella* mitochondrial genome is enigmatic, as both genera are not native to New Caledonia. So the mitochondrial genome of *Amborella* is still a treasure trove of mysteries.

5 Insight into Early Flowers

5.1 Flower Morphology

Amborella trichopoda is a dioecious species possessing unisexual flowers borne on separate male and female individuals (Figure 5a-e). Its floral anatomy has been extensively described by Endress and Igersheim (Endress and Igersheim, 2000b), and Buzgo et al. (Buzgo et al, 2004). In both sexes, floral organs arise on a continuous spiral, and organs of intermediate form can arise in transition zones between floral organ types. Mature male flowers typically contain 9-11 tepals and 12-21 stamens, while female flowers contain 7-8 tepals, 1-2 staminodes (sterile, stamen-like organs) and 5-6 carpels. The anthers of male flowers are triangular, 4-loculate, introrse, and borne on short, laterally flattened filaments. The carpels of female flowers are free and ascidiate (bottle-shaped; Figure 5c-f) and each contains one pendant, nearly orthotropous ovule, which is covered by two integuments (Figure 5g). The embryo sac within the *Amborella* ovule has a unique arrangement of eight cells, containing a total of nine haploid nuclei. Double fertilization of the egg and central cells of the *Amborella* embryo sac results in the production of a diploid zygote and a triploid endosperm, respectively (Friedman, 2006). The mature stigmatic surfaces of the carpels can come into contact (Figure 5f), providing a compitum to allow pollen tubes to pass between carpels and thereby improve the fertilization efficiency (Endress and Igersheim, 2000a). A secretion-filled canal in each carpel guides pollen tubes towards the downwards-facing micropyle of the ovule (Figure 5f-g). Pollen dispersion is both wind- and insect-mediated, with the nectarless flowers attracting a wide range of insects, including beetles (Doyle and Endress, 2000).

Amborella's unique position in angiosperm phylogeny gives this species considerable importance in studies aimed at reconstructing, from living species, the characteristics of the MRCA of living angiosperms. No living angiosperm appears to have conserved all the floral traits of their common ancestor, and *Amborella* appears to possess a mixture of potentially pleisiomorphic and derived traits. Reconstruction studies indicate that the flowers of the MRCA of living angiosperms, like those of *Amborella*, were probably small (Endress, 2001), actinomorphic, and contained an undifferentiated perianth of tepals (Figure 6) (Endress and Doyle, 2009; Endress and Doyle, 2015; Sauquet et al, 2017). However, unlike *Amborella*, the ancestral angiosperm almost certainly bore bisexual flowers (Sauquet et al, 2017). Protogyny is also a likely ancestral trait in angiosperms (Endress and Doyle, 2015), logically absent in *Amborella* due to its dioecious reproductive system. Sauquet et al. (2017) conclude that the perianth and androecium of the MRCA of living angiosperms was probably whorled, while its gynoecium was probably of spiral phyllotaxy, in contrast to the entirely spiral form of *Amborella* flowers. However, this interpretation was questioned by Sokoloff et al. (2018), who point out that developmental constraints may prevent such a switch in phyllotaxy within the flower, and by De-Paula et al. (2018), who suggest that reconstructed models should be constrained to ensure that these contain internally consistent lists of features including the type of phyllotaxy present in each set of floral organs. The floral phyllotaxy of the MRCA of living angiosperms therefore remains unclear.

The gynoecium of the MCRA of living angiosperms, as with *Amborella*, was almost certainly superior and contained several free ascidiate carpels that were incompletely fused at the apex by post-genital cell division, but were closed instead by the secretion of substances into a stigmatic aperture or canal (Endress, 2001). The ovary of each carpel in the MRCA of living angiosperms probably contained a single pendant ovule (Figure 6b) (Endress and Doyle, 2015) (Endress and Doyle, 2015)—these characteristics are conserved in *Amborella*. As in *Amborella*, each ovule in the MRCA of living angiosperms almost certainly had two integuments and an extensive nucellus, or maternal nutritive tissue. Interestingly, the eight-celled *Amborella* embryo sac is similar to the seven-celled *Polygonum*-type embryo sac prevalent in extant angiosperms, which contains two synergid cells at its micropyle end, whereas there are three such cells in *Amborella*. However, Friedman and Ryerson (2009) consider that both the *Amborella* and *Polygonum* embryo sac arrangements represent a doubled form of an ancestral four-celled embryo sac, which is still present in the ANA-grade Nymphaeales and Austrobaileyales orders. In this putatively ancestral-type embryo sac, double fertilization leads to the production of a diploid zygote and a diploid, rather than triploid, endosperm.

5.2 Flower Development

Numerous mechanisms controlling flower development in eudicot models such as *Arabidopsis thaliana* have probably been conserved since the MRCA of extant angiosperms. In the (A)BC model of flower development (Causier et al, 2010), the partially overlapping expression domains in the floral meristem of several classes of transcription factors, almost all of which belong to the MADS-box gene family, control the identities of sepals, petals, stamens and carpels in the first to fourth floral whorls, respectively, of the typical eudicot flower. These expression domains appear to be largely conserved in ANA-grade angiosperms such as *Amborella* (Kim et al, 2005), suggesting that much of the (A)BC model has been conserved since the MRCA of living angiosperms (Figure 6c). Notably, the MADS-box C-function appears to be specifically expressed in both male and female reproductive organs of the flower in *Amborella*, as in eudicots, while B-function expression is excluded from female reproductive organs but present in both the stamens and perianth organs. The expression boundaries of (A)BC floral MADS-box genes tend, however, to be less sharply delineated in ANA-grade angiosperms than in typical eudicot models, which may explain the presence of intermediate organ types in ANA-grade species such as *Amborella*. Interestingly, certain mechanisms acting upstream of MADS-box floral homeotic genes, notably involving the master transcriptional regulator LEAFY (LFY), appear to be conserved since the common ancestor of all living seed plants (Moyroud et al, 2017), which may have lived some 300 Mya. Numerous other classes of transcriptional and post-transcriptional regulators, known to control flower and/or ovule development in one or more model angiosperms, show conserved expression patterns in *Amborella*, suggesting that the functions of these regulators are also at least partially conserved in the lineages studied since the MRCA of living angiosperms (Figure 6a,b) (Arnault et al, 2018; Fourquin et al, 2005; Jasinski et al, 2010; Vialette-Guiraud et al, 2011).

5.3 *Amborella*'s sexual system

In the vast majority of dioecious angiosperms, which make up around 6% of angiosperm species (Renner, 2014), the sex determination mechanism is unknown, although it was reported to be controlled by sex chromosomes in some 50 cases (Fruchard and Marais, 2017). A young, seed-grown population of ~300 *Amborella* plants showed no significant deviation from a 1:1 sex-ratio, and remained ~99% stable between consecutive flowering seasons (Anger et al, 2017).

These data are compatible with the presence of chromosomal sex determination mechanism in *Amborella*. Previous studies on the *Amborella* 2n=26 karyotype (Chamala et al, 2013; Oginuma et al, 2000) did not reveal the presence of morphologically distinguishable sex chromosomes, so any sex-determining genes present may be confined to a relatively short chromosomal region. Interestingly, natural *Amborella* populations have been reported with male to female ratios between 1.4:1 (Anger et al, 2017) and 1.8:1 (Thien et al, 2003), an effect which in dioecious angiosperms was found to be generally correlated with long-lived growth habits, fleshy fruits and biotic seed distribution (Field et al, 2013). Two studies have provided evidence of relatively rare complete sex-change events in *Amborella*, either among young seed-grown plants (Anger et al, 2017), or following propagation by cuttings (Buzgo et al, 2004), which could indicate the involvement of epigenetic factors that can modify sex-expression. Anger et al. (2017) also provided evidence of sexual instability through the production of a proportion of morphologically bisexual flowers on otherwise male individuals, which may indicate the evolution of dioecy via a gynodioecious intermediate (Fruchard and Marais, 2017). As pointed out by Anger et al. (2017), transcriptomic analyses could be used to identify the hypothesized non-recombinant sex-determining region of the *Amborella* genome.

6 *Amborella* and the Seed of the First Angiosperms

The *Amborella* unit of dispersal is characterized by a drupe (with the fruit being constituted by one or several drupes) composed of a soft red mesocarp (Figure 7a) and a hard endocarp surrounding a large seed, as revealed by the dissection of a dry drupe without pulp (Figure 7b). The size and bright color of the drupe suggest bird dispersal, but the long persistence of fruits on the plant (Fourcade et al, 2015) and the genetic differentiation observed between populations (Poncet et al, 2013) indicate that the dispersal mechanism is not very efficient. The seed *stricto sensu* has a thin seed coat (Figure 7c,d) and a small embryo surrounded by a copious endosperm (Figure 7d). This embryo is underdeveloped and arrested at the heart-shaped stage (Figure 7d). This is a typical feature of ANA-grade ancestral flowering plant species (Fogliani et al, 2017; Villegente et al, 2017). Indeed, the ratio (E:S) between the length of the embryo (E) and that of the seed (S) has increased during evolution (Forbis et al, 2002). Seeds with an underdeveloped embryo have morphological dormancy, which is considered to be the most primitive type of dormancy (Fogliani et al, 2017).

6.1 Seed Storage Proteins

Contrary to gymnosperms, which have a diploid maternally-derived nutritive tissue in the seed, most angiosperms, including *Amborella*, display a triploid, biparental endosperm (Friedman and Ryerson, 2009). Furthermore, the timing of resource accumulation in the seed varies in different seed-plant taxa (Cailleau et al, 2010). Presumably, these differences have an impact on the specialization of the biochemical machinery necessary for seed storage-protein accumulation. Of the three genes encoding 11S globulins (the major seed storage proteins in angiosperms) in *Amborella*, two of them encode globulins that exhibit strong amino acid sequence similarities with the corresponding proteins in modern angiosperms (Amborella Genome Project et al, 2013; Villegente et al, 2017). However, a third one deviates notably from the two others as it exhibits an amino acid sequence involved in globulin processing/packaging during seed maturation, i.e. analogous to that present in 11S globulins from the oldest living species of tree, *Gingko Ginkgo* biloba (Amborella Genome Project et al, 2013), and the gymnosperm

Metasequoia glyptostroboides (Amborella Genome Project et al, 2013; Hager and Wind, 1997). Because the three 11S *Amborella* globulins are highly abundant in both the endosperm and embryo (Villegente et al, 2017), angiosperm- and gymnosperm-type 11S globulins coexist in *Amborella* seeds.

This feature prompted Poncet et al. (2015) to characterize the vacuolar processing enzyme (VPE) system in *Amborella* seeds. In particular, a seed-specific β-VPE isoform was characterized, whose role is to process 11S globulins at the above-mentioned amino acid sequence, so they are correctly assembled and stored within protein storage vacuoles during seed maturation on mother plants. Logically, this role is only necessary in seed plants, and in this regard, the absence of a direct ortholog of β-VPEs in non-seed plants, such as bryophytes and lycophytes, is not incongruent. Furthermore, the findings showed that the β-VPE lineage is specific to angiosperms, so the remaining seed plants, i.e. gymnosperms, also lack direct orthologs of the angiosperm β-VPE lineage (Poncet et al, 2015).

6.2 Physiological Maturity of *Amborella* Embryos during Seed Dispersal

When mature *Amborella* seeds are dispersed, they contain small underdeveloped embryos that must develop before germination, which is an ancient strategy within seed plants (Willis et al, 2014) to extend germination over time. This has raised the question of the physiological stage reached by the *Amborella* embryo by the end of seed maturation. One intriguing aspect concerns the acquisition of desiccation tolerance, as mature *Amborella* seeds are able to survive for several months in the dry state, whereas numerous studies (Dickie and Pritchard, 2002) have found that this tolerance only occurs very late during maturation of modern angiosperm seeds capable of withstanding drying and/or freezing (i.e. orthodox seeds).

Despite the underdeveloped nature of the embryo (Figure 7 c,d), proteomics data are in agreement with the acquisition of desiccation tolerance by the *Amborella* embryo (Villegente et al, 2017). The embryo was shown to contain an arsenal of chaperone proteins, whose function is to assist other proteins in their maturation, while ensuring proper three-dimensional folding, notably under seed desiccation stress. These include so-called late embryogenesis abundant (LEA) and heat shock proteins (HSP) that protect macromolecular complexes from stress induced by desiccation, dry storage and imbibition, etc. (Villegente et al, 2017). A recent study on desiccation sensitivity in 45 New Caledonian plant species, including *Amborella*, confirmed its tolerance (Toublanc-Lambault et al, 2019). These data are important since the origin of tolerance to desiccation during evolution is controversial. For example, a study of 45 species concluded that recalcitrant seeds (non-tolerant to desiccation) were associated with ancestral-type ovaries and orthodoxy was the more evolved character (von Teichman and van Wyk, 1994). However, more recent studies of a larger number of species came to the opposite conclusion (Dickie and Pritchard, 2002).

Thus, despite its rudimentary appearance, the *Amborella* embryo has characteristics generally associated with late stages of development in orthodox seeds, including: (i) the ability to accumulate stored reserves, and (ii) the ability to tolerate desiccation. Because this occurs while the *Amborella* embryo is still immature, it strongly suggests that *Amborella* seeds and seeds of other angiosperms with large embryos differ markedly with regard to the timing of the expression of genes associated with such processes.

7 Wood Anatomy and Architecture of *Amborella*

The vegetative anatomy of *Amborella* has long attracted attention because of the lack of vessels in the xylem (Bailey, 1957; Bailey and Swamy, 1948; Young, 1981). The vesselless condition is of considerable interest in angiosperm evolution as it is generally recognized that angiosperms originally emerged with tracheids or tracheid-like vessel elements, as observed in extant gymnosperms. The first observation of the absence of xylem vessels in *Amborella* was detected in a specimen collected by Vieillard on loan to the Arnold Arboretum (Bailey and Swamy, 1948), and the exclusive presence of tracheids was further confirmed by Carlquist and Schneider (2001). Apart from *Amborella*, the vesselless condition is known in Nymphaeales, Winteraceae and Trochodendrales, but is likely to have resulted from an evolutionary reversal for Winteraceae and Trochodendrales (Doyle and Endress, 2000; Feild et al, 2002).

In *Amborella*, tracheids occur in relatively uniform files, with little differentiation in cell diameter and wall thickness (Figure 8a), in contrast to secondary xylem in typical vessel-bearing plants (Feild et al, 2000). A major distinction between tracheids and vessels is better observed with scanning electron microscopy (SEM) and concerns the pronounced differences between end wall and lateral wall pitting area characterizing vessels (Figure. 8b). Moreover, tracheids have intact pit membranes derived from the primary cell walls (Carlquist and Schneider, 2002). The transition from tracheids to vessels involved various degrees of pit membrane remnants in the form of scalariform end walls (Carlquist, 2012; Carlquist and Schneider, 2002). More hydraulically efficient vessel elements are shorter, wider, their end walls lack pit membranes, and their perforation plates vary from scalariform to simple (Zimmermann, 1983). Detailed anatomical studies of *Amborella* revealed that tracheids have porose pit membranes at the end wall, and larger pits at the end walls than on the lateral walls were observed (Carlquist and Schneider, 2001). Thus, from an anatomical perspective, the tracheary elements of *Amborella* do not strictly conform to true tracheids. Despite the presence of pit membranes, scalariform pitting on end walls of tracheids is observed in *Amborella*, as well as in Nymphaeaceae, suggesting that this character state is a plesiomorphic condition in angiosperms. These observations concern the development of porosity, while tracheids in *Amborella* have been considered as transitional. The anatomical structure of conductive cells in *Amborella* consequently suggests that the evolution of vessels took place early in angiosperm history. Indeed, several species belonging to the first diverging angiosperm lineages (*Illicium*, *Trimenia*, *Austrobaileya*) have vessels with scalariform perforation plates and various degrees of pit membrane remnants (occasional retention of intact pit membranes) in the perforation plates of their vessels (Carlquist, 1996; Carlquist and Schneider, 2001). These vessels were even called "semi-tracheids" due to the prominence of pit membrane remnants (Carlquist, 2012). A combination of simple and scalariform perforation plates has been observed in some species (Carlquist, 2012). These observations suggest that membrane porosity, a result of a relatively pronounced lysis of the membrane, likely yielded to simple perforation. Recent SEM observations of pit membranes in basal angiosperms (ANA grade) have revealed that their microstructures might be more diverse than previously thought (Carlquist et al, 2009). This diversity makes the phylogenetic inferences more complex. However, the anatomical structure of *Amborella*, together with the remaining ANA grade species, suggests that vessels evolved from tracheary elements at an intermediate state between tracheids and vessels.

The transition from tracheids or tracheid-like vessels to more efficient vessels has been of major importance in angiosperm diversification (Carlquist, 2012; Sperry et al, 2007). Natural selection favored vessels with a greater end wall conductive area, which might have enabled vessel-bearing plants to occupy drier and more seasonal habitats. Anatomists however now recognize that vessel transition is not a linear evolutionary trend and the presence or absence of vessels should not be considered as a binary character state (Carlquist and Schneider, 2002; Olson, 2012). Several intermediate states between vessels and tracheids have been observed, given that xylem structure is the result of selective pressures confronted under different environmental conditions (Carlquist and Schneider, 2002; Spicer, 2017). A major advantage of vessel evolution is the development of wood structural complexity (i.e. heteroxyly). The division of labor into distinct support and conductive tissues (e.g. vessels, fibers and parenchyma), which characterizes heteroxylous wood, is believed to represent a major advance in angiosperm diversification, and is correlated with increased functional and ecological diversity (Bond, 1989; Carlquist, 2009; Feild et al, 2012; Isnard and Feild, 2015). The mechanical organization of *Amborella* is that of a self-supporting plant with material stiffness increasing with diameter (Trueba et al, 2016). However, mechanical reinforcement does not fully compensate for the increased plant stature in *Amborella*. As such, the so called “mechanical laxness” (Feild et al, 2009) of stems reflects a limited mechanical adjustment as increases in diameter are not offset by an increase in mechanical stiffness. The development of tracheid porosity in *Amborella* could have favored hydraulic performance to the detriment of mechanical stiffness (Feild et al, 2009). This indicates that selection for mechanical stability has probably been of major importance during the evolution of heteroxylous wood fibers.

Xylem evolution has been key in the diversification of angiosperms, notably in opening developmental opportunities in response to environmental cues (Rowe and Speck, 2005; Spicer, 2017). Given that *Amborella* xylem has transitional features, its growth habit is also potentially of evolutionary significance to reconstruct the architecture of early woody angiosperms. *Amborella* is often described as a scrambling shrub (Jérémie, 1982) or a small tree that grows to 7–12 m height in the understory of rainforests, and whose habit exhibits some plasticity in response to light exposure (Trueba et al, 2016). The architecture of *Amborella*, built up by the stacking of axes that are initially orthotropic and bend secondarily under gravity, gives the seemingly scandent habit (Figure 9a,b). A single meristem consequently determines both the main trunk and branches, and there is no hierarchical architectural organization, with all axes being morphologically similar (Figure 9a,b). Other morphological features of *Amborella* are the presence of axillary surnumerary buds (Figure 9c) and a strong ability to reiterate by mesotonic and basitonic branching from dormant buds in response to light or mechanical damage (Trueba et al, 2016). An abundant resprouting ability was also described for Austrobaileyales and is in line with the “dark and disturbed” hypothesis, according to which the earliest angiosperms were woody plants that grew in dimly lit, disturbed forest understory habitats (Feild and Arens, 2005; Feild et al, 2004).

Species with hydraulically inefficient xylem conduits are restricted to rainforest habitats, while species with more efficient vessels (like those presenting simple perforation plates) occupy more seasonal habitats (Pouteau et al, 2015). More recently, *Amborella* xylem has been shown to be highly vulnerable to drought-induced cavitation, a feature probably associated with its growth in humid forests (Trueba et al, 2019; Trueba et al, 2017). *Amborella* therefore has the anatomical and physiological features of a shade-adapted and drought-intolerant plant. The observation of these features in *Amborella*, along with other basal angiosperms, has fueled evolutionary arguments on the possible plesiomorphic condition of these features, suggesting that they reflect the first stages of angiosperm evolution.

8 *Amborella* and the Biogeography of New Caledonia

The restricted distribution of *Amborella* to a single island in the South Pacific has long been considered as a biogeographic enigma (Figure 10). Its homeland, New Caledonia, has attracted the interest of botanists and biogeographers because of its rich and unique flora, with over 3,300 species of vascular plants and an endemism rate of ≈ 75% (Morat et al, 2012), including a large number of unique lineages: 62 to 91 endemic genera (Pillon et al, 2017) and three endemic families (Amborellaceae, Oncothecaceae, Phellinaceae). The origin of the richness and uniqueness of this island flora has been the focus of major scientific research in recent decades. Since the wood of *Amborella* was found to lack vessels (Bailey and Swamy, 1948) and its placement in its own family (Pichon, 1948), this species has been qualified as a "museum-piece plant", "primitive" (Carlquist, 1965), "archaic", "peculiar relict" (Thorne, 1965) and "relictual endemic" (Lowry, 1998). In this regard, *Amborella* has often been cited alongside two other plant groups. First, Winteraceae (Canellales), which is well-represented in New Caledonia with the genus *Zygogynum*, is another family of vesselless angiosperms, whose flowers were once considered amongst the most primitive (Carlquist, 1965). Second, conifer diversity in New Caledonia is among the highest worldwide, including the charimastic *Aracauria* (Cook's pines and relatives) and *Parasitaxus* (the sole parasitic gymnosperm).

Unlike most Pacific islands, New Caledonia is not volcanic but instead has a continental base which is a legacy of its past inclusion in the Gondwanan super-continent, until it separated from Australia in the Late Cretaceous and drifted eastward (Aitchison et al, 1995). The co-existence of "ancient" elements such as *Amborella*, Winteraceae and conifers, with recent radiations (*Phyllanthus*, *Psychotria*, etc.), has often been explained by several waves of migration to New Caledonia (Carlquist, 1965; Lowry, 1998). "Old" lineages with supposedly limited dispersal capacity would have first colonized the island while it was connected or in close proximity to a continent, while "younger" lineages would have reached the island through long-distance dispersal when the island became more isolated. This scenario overlooks two facts: the geological evidence of the subsidence and immersion of New Caledonia during the Early Cenozoic (Paris, 1981; Pelletier, 2006), and the disharmony of its fauna, particularly the absence of native land mammals (apart from bats), land snakes and amphibians (Thorne, 1965). This suggests that the entire biota of New Caledonia probably reached the island through long distance dispersal across the sea, and indeed, most molecular dating evidence (Cruaud et al, 2012; Grandcolas et al, 2008; Nattier et al, 2017; Pillon, 2012) indicates a recent origin of the biota that postdates its emersion ≈37 Mya (Cluzel et al, 1998). This is also true for the endemic radiations of Winteraceae and conifers that occurred during a more recent period than previously expected (Leslie et al, 2018; Setoguchi et al, 1998; Thomas et al, 2014). New Caledonia should no longer be considered as a continental island in its biogeographical sense, as defined by Wallace (Wallace, 1880) that has retained ancient groups since its separation from Australia, and would be better qualified as a "Darwinian" island *sensu* Gillespie and Roderick (2002) *i. e.* an island that has never been in contact with the source of colonists.

The occurrence of *Amborella*, Winteraceae and many conifers in New Caledonia has long been considered as evidence for an ancient origin of its biota, but this hypothesis is no longer tenable. Island endemics should not be assumed to be the same age as island biotas (Pillon and Buerki, 2017). It seems most likely that the *Amborella* lineage was once more widespread, colonized New Caledonia long after its divergence from other angiosperms, and later disappeared from elsewhere. It then secondarily became restricted to New Caledonia, as was the case for some other endemic genera that have a fossil record in Australia or New Zealand (Pillon et al, 2017; Pole, 2010). No plant fossil similar to *Amborella* has been found to date, so its exact history is unclear, and it is not known what happened to this lineage between its divergence 140-256 Mya and the oldest genetic split within extant populations <100,000 years before present (BP) in New Caledonia (Tournebize et al, 2017). Groups such as *Amborella* should be considered as relicts, i.e. remnants of lineages that were once more widespread and/or diverse but were generally wiped out by extinction (Grandcolas et al, 2014). If the flora of New Caledonia is not so ancient, then how can the richness in relict lineages be explained (Pillon et al, 2017)? It has been suggested that the relative climatic stability in Southeast Asia and Australasia compared to other areas of the world could have favored the survival of "primitive" angiosperms in areas such as New Caledonia (Morley, 2001). Indeed, most of the ANA-grade and magnoliid species of this island have drought-vulnerable wood and thus tend to grow in rainforest habitats, particularly in areas that were putative refugia during the Late Glacial Maximum (Pouteau et al, 2015). Climate stability rather than geological history could thus explain the occurrence of *Amborella* and some other relicts in New Caledonia, which could have served as a refugium while other parts of the world were getting drier or colder, and losing these unique lineages. It is often presumed that the unusual ultramafic substrates of New Caledonia have slowed down the immigration and invasion of New Caledonian flora by novel species (Isnard et al, 2016), therefore safeguarding relict lineages that are strongly biased in favour of these substrates (Pillon et al, 2017). *Amborella* is unlike most other endemic genera given that it is never found on ultramafic rock environments and, interestingly *Austrotaxus* (Taxaceae), the next oldest relict and only Southern Hemisphere yew, is rarely found on this substrate. Further clues into the biogeographic history of New Caledonia could emerge via studies on the ecology of its endemic plants.

8 *Amborella*, a Forest Dweller

Amborella is found in scattered populations in mid-elevation rainforests (200-1000 m) on old metamorphic rocks across the main island of New Caledonia, with an estimated density of 433 trees/ha (Col d'Amieu, Thien et al, 2003). In a network of forest plots covering New Caledonia (Ibanez et al, 2013; Ibanez et al, 2014), *Amborella* is actually a quite abundant species of the rainforest understory (mean diameter at breast height, DBH, = 8 cm; DBH max = 14 cm), accounting for up to 5% of trees with a DBH >5 cm and ranked among the 5-10 most abundant species per plot. This species is part of communities that include Lauraceae (e.g. *Cryptocarya*), Winteraceae (e.g. *Zygogynum*) or Monimiaceae (e.g. *Hedycarya*). Furthermore, plots where the species is the most abundant also host communities where palms are abundant, especially *Burretiokentia vieillardii*. More generally, *Amborella*, along with half of the 109 species of the ANA grade and magnoliids endemic to New Caledonia, occur in the buffered climatic conditions where rainfall is greater than 2000 mm/year, with diurnal temperature variations lower than 7°C (Pouteau et al, 2015).

New Caledonian rainforests feature a contrasting mosaic that includes an exceptional tree density (average of 1250 trees DBH > 10 cm/ha), low alpha diversity (average of 95 species/ha) and very high beta diversity (45-81% of species dissimilarities between plots, Bray-Curtis index) (Ibanez et al, 2018; Ibanez et al, 2014). The tree density increases significantly with elevation, while the species richness peaks at medium elevation (400-600 m) (Birnbaum et al, 2015a; Ibanez et al, 2014). The low canopy height is another unique feature of New Caledonian rainforests. Trees are smaller than expected on the basis of allometric relationships applied in other biogeographical areas (Blanchard et al, 2016).

The high tree density and low canopy height are both unique structural features of New Caledonian forests (Figure 11) that are directly related to the cyclone frequency and intensity (Ibanez et al, 2019). Because of this disturbance cycle, the sylvigenetic dynamics are based on the probability of occurrence of a disturbance rather than on the lifespan of largest trees. The recurrence of cyclones and storms is also a key factor involved in establishing and maintaining long-lived gymnosperms (e.g. Araucariaceae). Without periodic disturbances, gymnosperms would not be able to coexist within angiosperm forests (Enright et al, 1999). The rainforest dynamics would thus be partly driven by the regime of disturbances impacting the whole forest, including the understory, as observed on a 1-ha 'wekori' plot that the Cook cyclone crossed in April 2017 (P. Birnbaum pers. obs.). Consequently, canopy gaps might not be closed by fast-growing heliophilous tree species but rather by a mixture of tree species able to grow under different light conditions (Birnbaum et al, 2015b). Finally, the recurrence of these climatic events maintains these habitats in an early-succession phase, so they could be defined as ever-young forests.

According to the forest architecture and dynamics, New Caledonian forest understories are clearly in line with the putative dark and disturbed understory habitat of early angiosperms (Feild et al, 2004). Furthermore, while only 70% of the families composing the native vascular flora grow in rainforests, all the indigenous families and nearly all of the genera of the ANA-grade and magnoliids occur in rainforests (Morat et al, 2012; Pouteau et al, 2015). This co-occurrence suggests that wet, shaded and frequently disturbed rainforest understories provided an ideal refugium for ANA-grade and magnoliid woody angiosperms in New Caledonia.

9 *Amborella* through the Ice Age

The recent history of *Amborella* is tightly linked to its favoured rainforest ecosystem. While the effects of the Ice Age on temperate ecosystems is well known, the palaeoenvironmental context of most tropical Pacific islands is still poorly understood (Keppel et al, 2012). Yet, some studies based on terrestrial pollen diagrams have indicated that tropical rainforests, including those of New Caledonia, also experienced episodes of range contraction during the Pleistocene, while the humid vegetation later expanded at the Pleistocene–Holocene transition (Hope et al, 2004), until the arrival of humans ≈3,000 years BP and the expansion of secondary vegetation (Stevenson et al, 2001).

Amborella was among the first relatively widespread species endemic to New Caledonia to have been the focus of an in-depth population genetics study. It was pivotal to demonstrate the impact of past climate change on the New Caledonian flora and vegetation. The last glacial cycles entailed climatic variations and an alternation of rainforest contraction and fragmentation. Their effects in New Caledonia were first highlighted by Pintaud et al. (2001) who revealed a small number of palm micro-endemism hotspots in the wettest part of the island, which may have served as rainforest refugia during the last glacial maximum, 23,400 years BP. A nuclear microsatellite population genetics study revealed that genetic diversity was clearly structured between populations within *Amborella* (Poncet et al, 2012; Poncet et al, 2013). Differentiation among *Amborella* genetic groups apparently does not result from current ecological gradients and gene flow barriers, but rather reflects the survival and expansion of lineages from past refugia, as predicted by niche modelling. The dramatic decrease in suitable *Amborella* habitat areas during the last glacial maximum (96.5% reduction) confirmed the palaeoenvironmental evidence that tropical forests regressed in southern New Caledonia during drier periods (Stevenson et al, 2001; Stevenson and Hope, 2005).

Amborella demographic dynamics has thus been influenced by: (i) a reduction in past population size compared to present, with a recent expansion and (ii) a reduction of gene flow among populations during the unfavourable period (associated with fragmentation of refugial areas). The dynamics can be inferred from genetic diversity patterns in current populations, which depends on genetic drift (random fluctuation in allele frequency) and migration. Tournebize (2017) used two genetic datasets to infer the *Amborella* demographic history: a whole-genome resequencing dataset and a microsatellite dataset. Genetic analyses based on the resequencing dataset (100,000 SNPs) supported two main latitudinally distinct groups. Their genetic structure could reflect the divergence of two ancestral populations during the Last Glacial Maximum, 23,400 years BP. The Northern population was estimated to be located in the Aoupinié region and the Southern one close to Dogny Plateau (Figure 12b), which both represent major centres of relict angiosperm diversity and micro-endemism (Pouteau et al, 2015; Wulff et al, 2013). The inferred demographic changes were related to variations in $\delta^{13}C$ isotopic ratio reflecting productivity changes of lowland forest in New Zealand, ~2,000 km south of New Caledonia (Figure 12a) (Hellstrom et al, 1998). *Amborella* plausibly re-expanded in New Caledonia from c. ~12,800 years BP, shortly after a rapid forest re-expansion in northern New Zealand (c. ~14,000 years BP).

10 *Amborella* ex situ Cultivation, an Emerging Plant Model

Before advances in molecular phylogenetics, *Amborella* was almost unknown outside its natural range and scarcely cultivated. Besides their importance for conservation and educational purposes in botanical gardens, living *Amborella* plant collections are of great value for research teams seeking to understand the evolutionary history of angiosperms, including their development, physiology, cell biology and genetics. However, the ease of cultivation (including sexual and asexual propagation, crossing experiments, fecundity, generation time, etc.) is a key issue for any potential model plant (Chang et al, 2016). The first successful *ex situ* cultivation of *Amborella* was presumably performed by Todd and Virginia Keeler-Wolf, two former University of California Santa Cruz (USA) students, who back in 1974-1975 (Anonymous, 1976; Stephens, 1999) sent *Amborella* cuttings by air from New Caledonia to their professor, Ray Collett, who was also the founder of the Santa Cruz Arboretum. The resulting plants were grown outdoors at Santa Cruz, and even survived some periods of frost (Perlman, 1999). Shortly after the true phylogenetic position of *Amborella* was revealed, *Amborella* became a “must have” for all botanical gardens worldwide (Husby et al, 2010) and cuttings were mainly distributed in the US and European institutions from the Santa Cruz mother rootstock plant (Buzgo et al, 2004), but also from plant material directly obtained from New Caledonian forests (Gâteblé, 2015; Große-Veldmann et al, 2011).

From a horticultural perspective, *Amborella* is a fairly easy plant to cultivate compared to many New Caledonian endemic species that can be hard to propagate and are slow growing (Gâteblé, 2015) (Figure 13). Over the last 10 years, *Amborella* has been successfully propagated through sexual and asexual propagation and grown in New Caledonia in quite a large quantity. The purpose of this extensive work was to set up an *ex situ* collection that could be used for further research, including functional genetics, e.g. genetic manipulation through *Agrobacterium*-mediated plant transformation and virus-induced gene silencing (Scutt et al, 2015) or gene expression studies (Arnault et al, 2018).

Vegetative propagation through cuttings with the help of indole-3-butyric acid (IBA) application is an effective way to produce new plants in 6-24 weeks (Gâteblé, 2015; Große-Veldmann et al, 2011; Scutt et al, 2015). Seed germination is also an easy way to propagate this species, which undergoes simple non-deep morphophysiological dormancy (see above) (Fogliani et al, 2017). Depending on the growing conditions, germination (radicle emergence) can occur in less than a month, but under less favourable conditions it can also take over 2 years (Gâteblé, 2015; Scutt et al, 2015). It has also been shown that *Amborella* seeds can be stored for a long time for conservation purposes because they are desiccation-tolerant (Toublanc-Lambault et al, 2019). In trials performed in Nouméa (New Caledonia) and Lyon (France), *in vitro* vegetative propagation and seed germination also proved to be valuable methods for generating plant material (Gâteblé, 2015; Scutt et al, 2015). When grown from cuttings or seeds, or acclimatised after *in vitro* culture, *Amborella* plants were found to flourish in pots containing peat-based substrates and regular slow-release fertilizer under simple shade houses with scheduled overhead watering in New Caledonia, or in temperate glasshouses in Europe and USA (Gâteblé, 2015; Große-Veldmann et al, 2011). However, because of *Amborella*'s architecture and adaptations to the rainforest understory habitat (Trueba et al, 2016), *ex-situ* cultivated plants are quite sensitive to the light intensity, relative humidity and watering changes. Large branches can easily break when manipulating such *ex-situ* plants (Gâteblé, 2015). Flowering can start as soon as cuttings are rooted, depending on the season, while seed-grown plants flower as early as 15 months after sowing (Anger et al, 2017).

11 General Conclusion

Amborella features include a mixture of plesiomorphic characters that were probably present in the first angiosperms (e.g. incompletely closed carpels, an undifferentiated perianth, tracheid-like elements), and unique derived characters, such as massive foreign gene transfer into its mitochondrial genome. *Amborella* represents a good reference to understand plant evolution. *Amborella* soon became famous after it was widely accepted that it was the sister group of the rest of the angiosperms (Pillon and Munzinger, 2005) (Figure 2). It was one of the first wild species to have its genome fully sequenced, and novel studies on this plant are continuously being published. Since 2005, more studies have been carried on wild populations with an increasing contribution of institutions based in New Caledonia, including the University of New Caledonia (UNC), the New Caledonian Agronomic Institute (IAC) and the French National Research Institute for Sustainable Development (IRD). *Amborella* was given a "Least Concerned" protection status by the New Caledonia Red List Authority (unpublished) and until recently, did not benefit from any protection. However, since 2009 the populations of Mount Aoupinié (a putative glacial refugium with high genetic diversity) have been included for protection in a nature reserve covering this mountain. In addition, wild *Amborella* populations have also been documented in the vicinity of the recently (2008) created nature reserve *Parc des Grandes Fougères*, where a few plants have been outplanted, and which could be expanded in the future.

There has been considerable discussion in the New Caledonian and international media about the uniqueness of *Amborella*. On the island, this plant is often proudly (but with some exaggeration) qualified as the "oldest flowering plant" (Pantz et al, 2006) or the "most primitive flowering plant" (Suprin, 2011), and it is now a well-known plant in New Caledonia. Ships, streets, businesses, etc., have also been named after it (Figure 14). *Amborella* fully exemplifies the scientific and popular interest for relicts and it is hoped that it could also serve as an umbrella species to preserve the unique biodiversity of New Caledonia.

Acknowledgments

This work is devoted to the memory of our colleagues and friends Jean-Christophe Pintaud and Charly Zongo.

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FIG 1. Summary phylogenetic tree of the major clades within angiosperms showing the position of *Amborella* as the sister group to the rest of flowering plants. Most angiosperm species belong to eudicots, monocots and to a lesser extent magnoliids. The three small Amborellales, Nymphaeales, and Austrobaileyales orders are often designated together as ANA-grade.

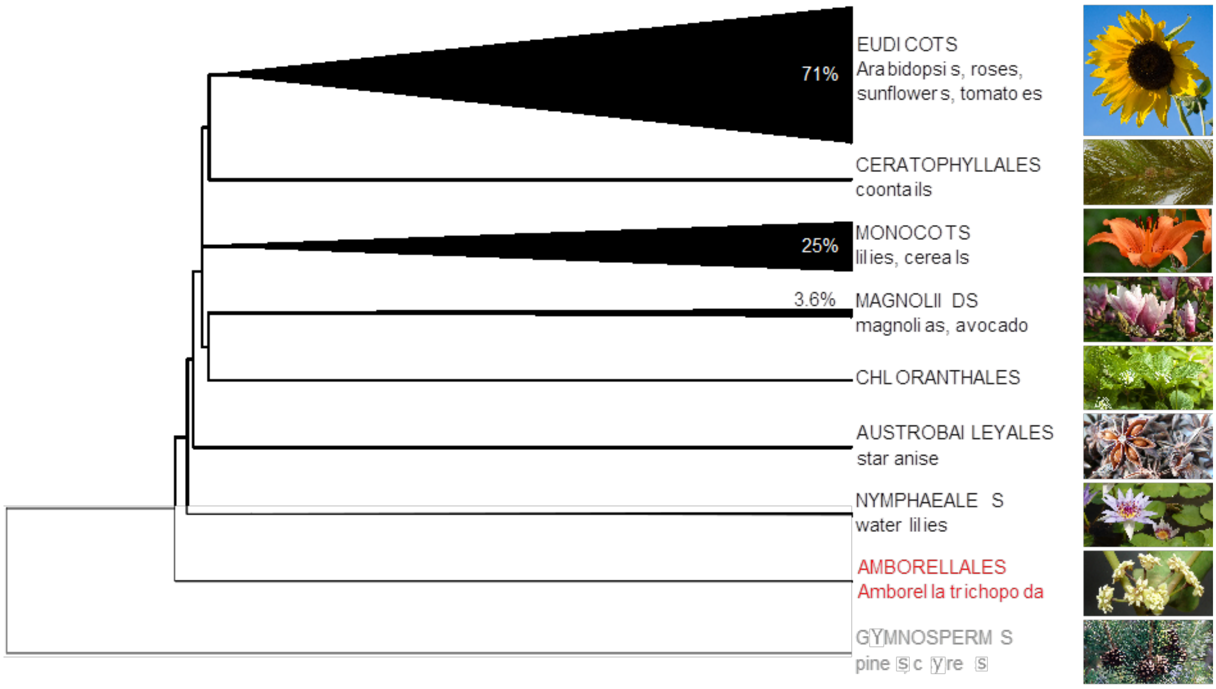

FIG 2. The number of scientific articles published since 1975 with the words "*Amborella*" or "Amborellaceae" in their title, according to Web of Science. For comparison, here we show the results of the same search for Phellinaceae and Oncothecaceae, the two other plant families endemic to New Caledonia. The Hydatellaceae family was considered as a member of monocots until a phylogenetic study (Saarela et al, 2007) indicated that it belonged to ANA-grade angiosperms.

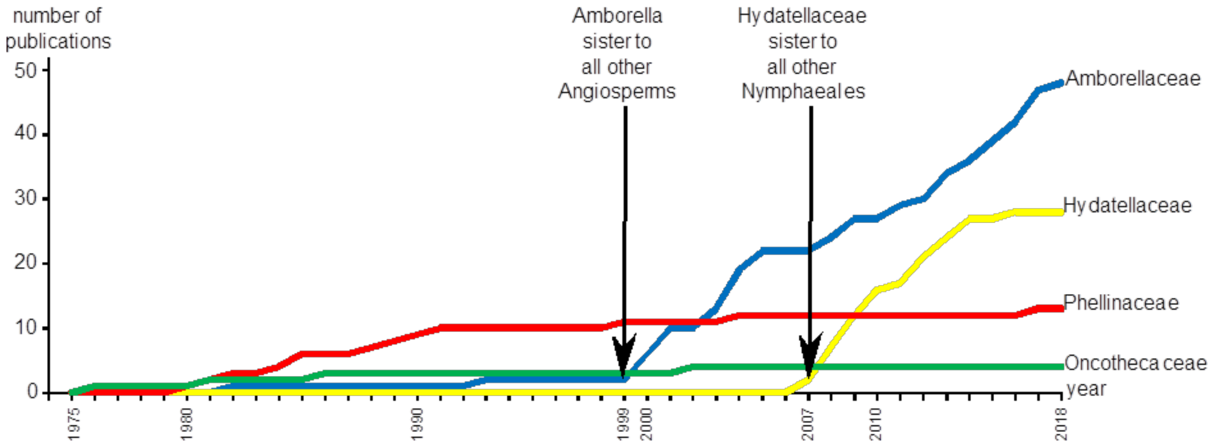

FIG 3. The *Amborella* genome provides an exceptional reference for inferring features of the first flowering plants and identifies an ancient angiosperm-wide whole-genome duplication (red star). An overview of land plant phylogeny, including relationships among major angiosperm lineages. Hypothesized polyploidy events in land plant evolution are overlaid on the phylogeny with symbols. The red star indicates the common ancestor of angiosperms and the evolutionary timing of epsilon WGD. The evolutionary timing of zeta and gamma polyploidy events are shown with empty and purple stars, respectively. Additional polyploidy events are indicated with ellipses (Amborella Genome Project et al, 2013).

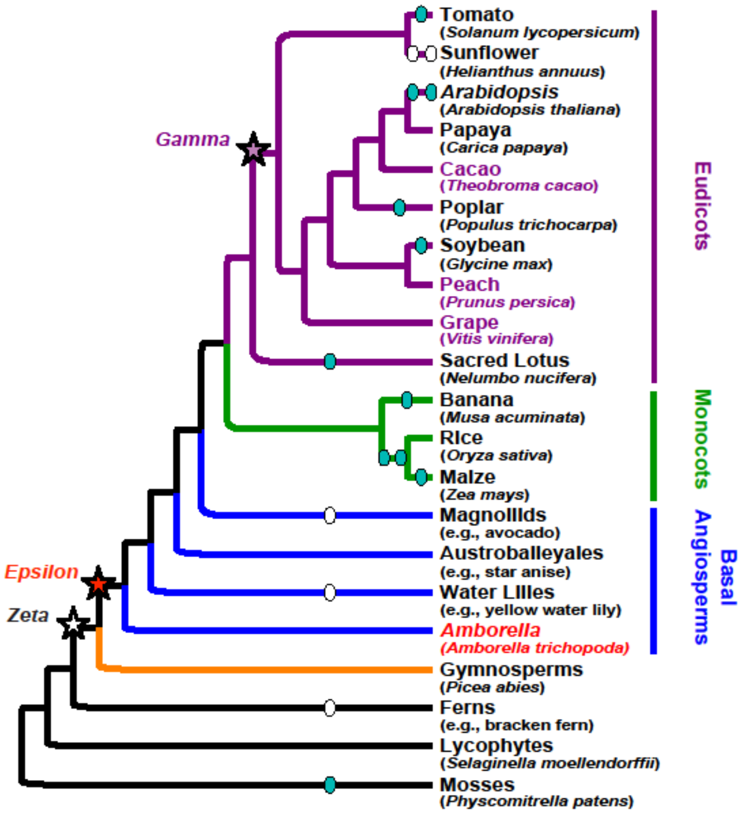

FIG 4. Horizontal gene transfer **(a)** Direct contact of epiphylls (liverworts) on *Amborella* leaves that explain the horizontal transfer of genetic material (Photo: J. Munzinger). **(b)** Maximum likelihood evidence for HGT in *Amborella* mtDNA, with the original *Amborella* copy of the *atp1* sequence, and its multiple copies gained via horizontal transfer (modified from (Rice et al, 2013))

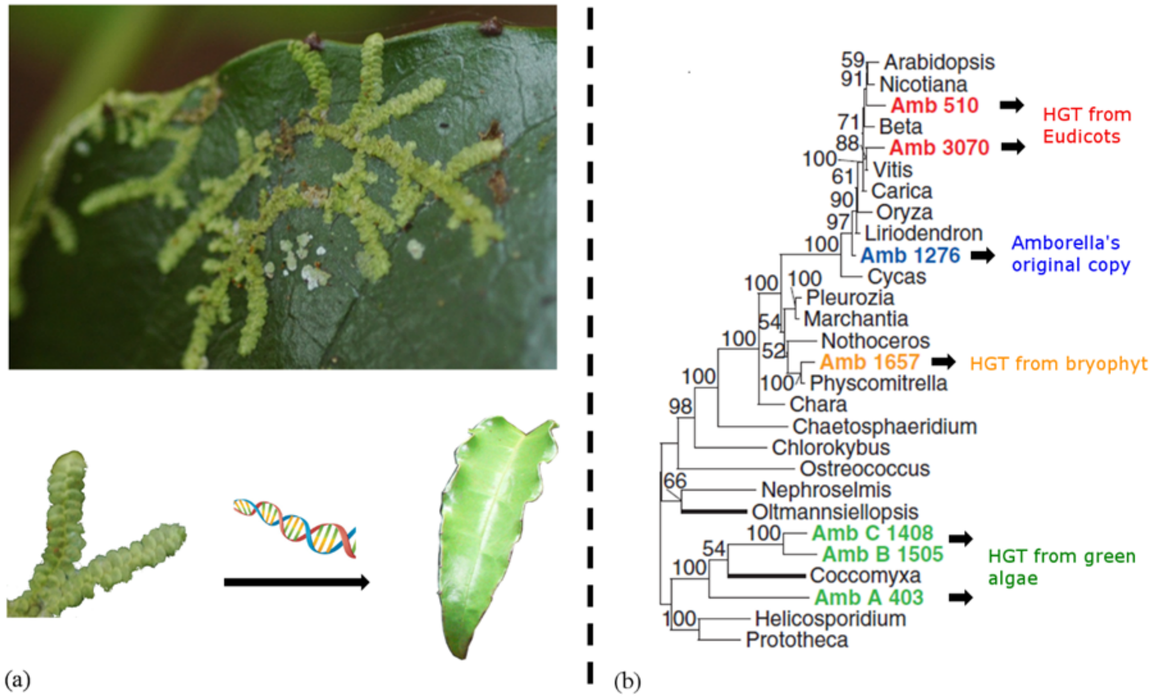

FIG 5. *Amborella* reproductive structures. **(a-b).** Male *Amborella* flowers. **(c-e).** Female *Amborella* flowers. **F.** Diagram of two adjacent *Amborella* carpels, based on the study of Endress and Igersheim (2000b), showing their communicating stigmatic surfaces (light blue) that form a compitum, and secretion-filled stigmatic canals (darker blue) that guide pollen tube growth. **(g)** Diagram of the near orthotropic *Amborella* ovule (corresponding to the boxed section of Fig. 5F) with its unique eight-celled embryo sac, based on the findings of Buzgo et al. (2004) and Friedman (2006). ac = antipodal cells, cc = central cell (containing two polar nuclei), ch = chalaza, co = compitum, ec = egg cell, f = funiculus, ii = inner integument, mp = micropyle, n = nucellus, oi = outer integument, sc = stigmatic canal, st = staminodium, sy = synergids.

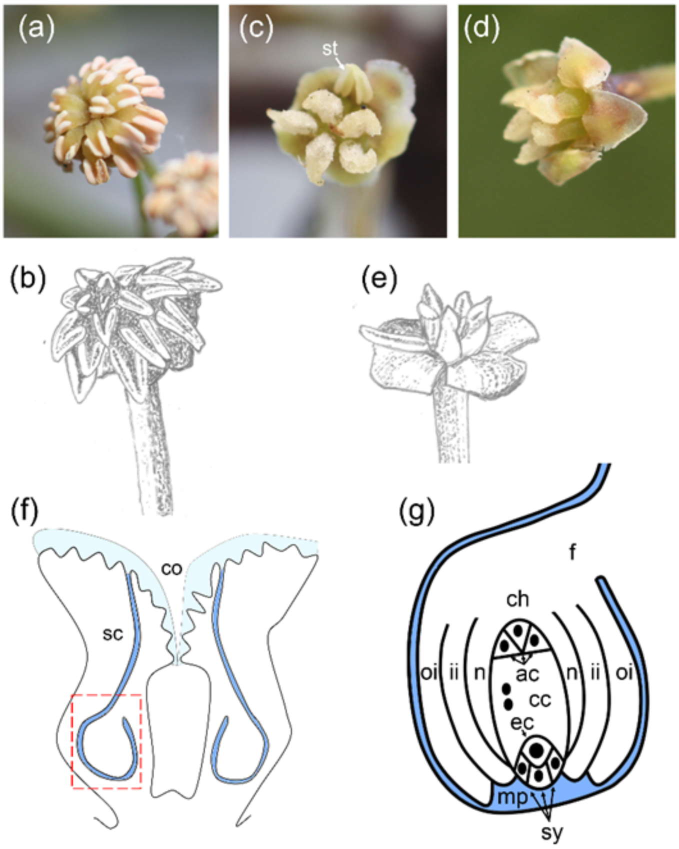

FIG 6. Schematic morphological and molecular reconstructions of the flower of the MRCA of living angiosperms based in part on *Amborella* studies.

(a). The flower of the MRCA of living angiosperms was probably actinomorphic, bisexual and protogynous. Reconstructed floral organ characters and selected gene expression patterns are indicated. Note: no attempt has been made to show accurate numbers, shapes or relative sizes of floral organs.

(b). A single bitegmic ovule was probably present in each carpel, possessing either anatropous or orthotropous symmetry, and containing a 4-celled embryo sac and a large nucellus. The expression of regulators controlling boundary formation in the ovule axis and abaxial/adaxial polarity in the integuments is indicated.

(c) Floral organ identity in the ancestral flower was determined by the combinatorial expression of MADS-box transcription factors of the ABC model, whose expression boundaries (indicated by fading colours) may have been less distinct than those of typical model eudicots, producing intermediate organs in boundary regions (asterisks). MADS-box floral regulators were themselves controlled by master regulators of floral patterning, including LEAFY.

Gene abbreviations refer to the ancestors, in the MRCA of living angiosperms, of the present-day *Arabidopsis thaliana* genes listed below. MADS-box family: AP3 = APETALA3; PI = PISTILLATA; SEP1/2/4 = SEPALLATA1, 2 & 4; SEP3 = SEPALLATA3. YABBY family: CRC = CRABS CLAW; INO = INNER NO OUTER; NAC family: CUC1/2 = CUP-SHAPED COTYLEDON1 & 2. AUXIN RESPONSE FACTOR family: ETT = ETTIN/AUXIN RESPONSE FACTOR3; ARF4 = AUXIN RESPONSE FACTOR4. KANADI family: ATS = ABBERANT TESTA SHAPE/KANADI4. HOMEODOMAIN-LEUCINE ZIPPER CLASS III family: PHB/PHV = PHABOLOSA & PHAVOLUTA; REV = REVOLUTA. Other abbreviations: cc = central cell, ch = chalaza, ec = egg cell, ii = inner integument, mp = micropyle, oi = outer integument, pn = polar nucleus, n = nucellus, sy = synergids. References are given in the text.

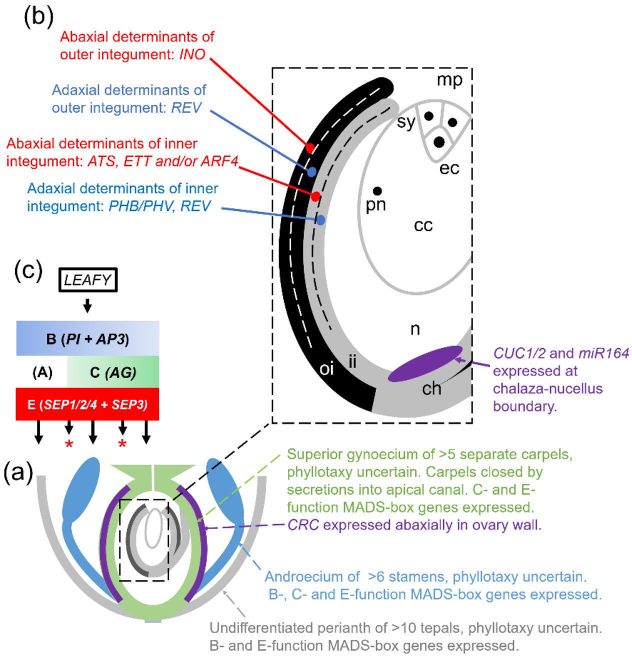

FIG 7. *Amborella* fruits and seeds. **(a).** fresh fruits **(b).** drupe without fleshly part. **(c).** longitudinally sectioned. **(d).** longitudinal section of a seed showing the heart-shaped underdeveloped embryo

(emb, embryo; endo: endosperm; sc, seed coat). Photos: (a) B. Fogliani, (b-d) adapted from Fogliani et al. (2017)

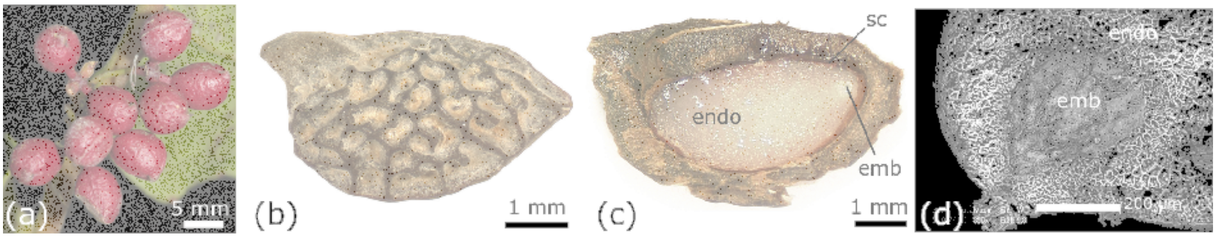

FIG 8. Anatomical features of *Amborella trichopoda*. **(a).** Transverse section showing the parenchymatous pith (p) and the vesselless xylem (x) of *Amborella trichopoda*. Scale bars = 500 µm, and 125 µm for the inset; **(b).** SEM image of the lateral walls of two adjacent tracheids showing different pitting patterns (arrows). Scale bar = 10 µm.

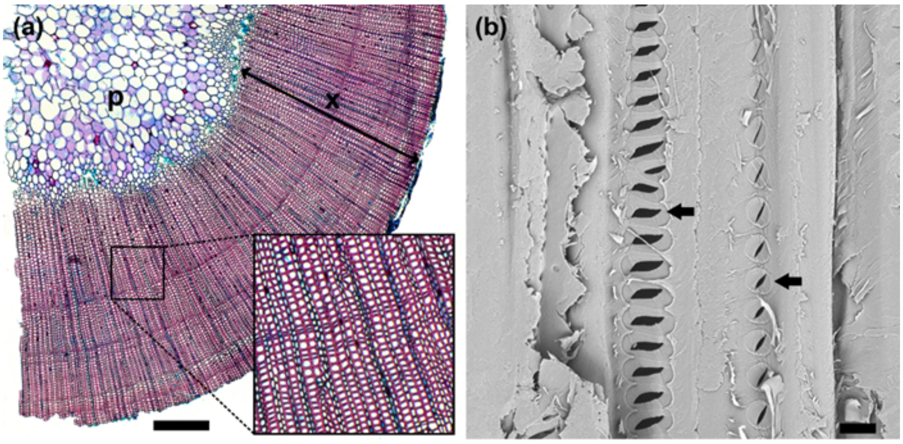

FIG 9. Illustration of architectural and morphological features of *Amborella trichopoda*. **(a)** A young individual (c. 6-year-old plant) presenting a sympodial branching pattern. Axes are morphologically similar without a hierarchical architectural construction with distinctive axis categories; **(b)** individual growing in the understory showing sympodiality by stacking of modules. Changes in the diameter of the parent axes (A1, A2) are observed after branching; **(c)** *Amborella trichopoda* presents a supernumerary bud, which is located below a first axillary bud. Under high lighting conditions both axillary buds can activate, producing small axes. Abbreviations: A1, A2, A3: axis orders; b1, axis deriving from an axillary bud; b2, axis deriving from a supernumerary bud; L, axillary leaf; P, parent axis. Modified from Trueba et al. (2016).

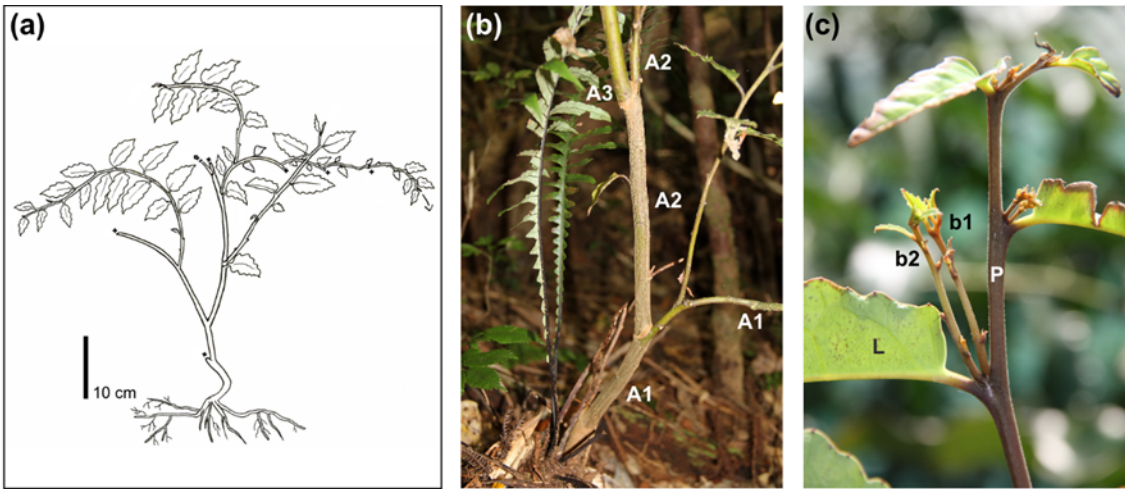

FIG 10. **(a)** Distribution of ANA-grade and magnoliids families endemic to Oceania. **(b)** *Amborella* distribution in New Caledonia.

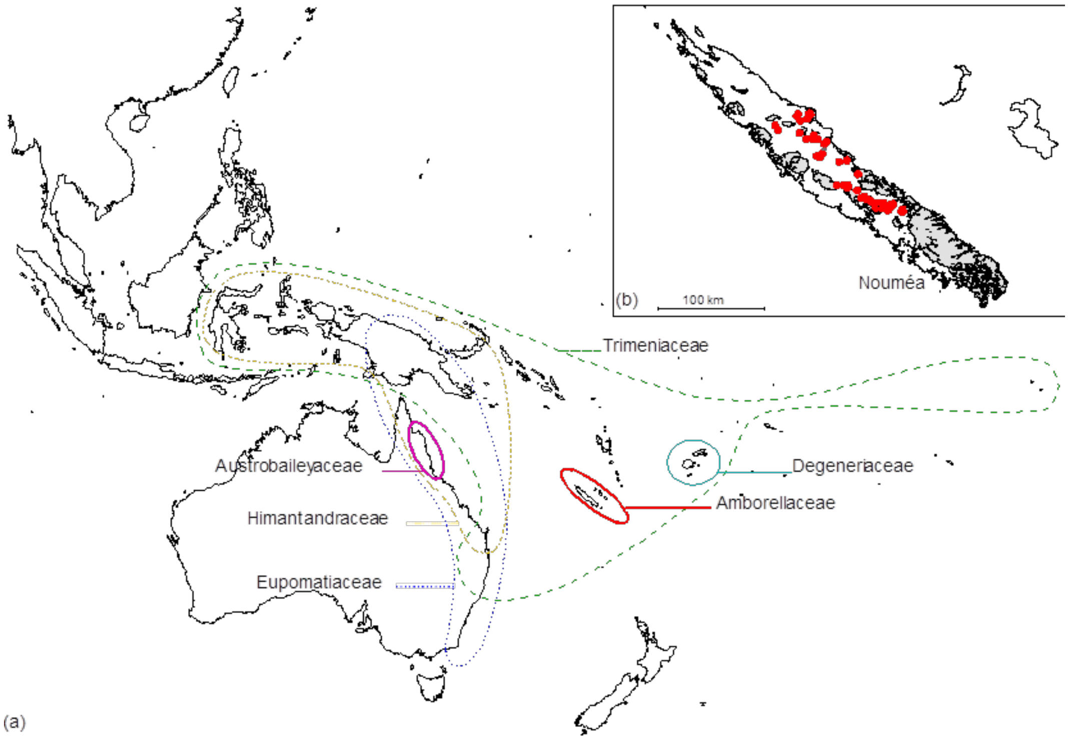

FIG 11. A New Caledonian rainforest understorey—a favorite habitat of *Amborella*. Photo: P. Birnbaum.

FIG 12. *Amborella* demography on a spatiotemporal scale**. (a).** Variations in the δ^{13}C isotopic ratio reflecting changes in lowland forest productivity in northern New Zealand (Hellstrom et al, 1998) and, on the right, the best estimated model describing the demographic history of *Amborella trichopoda*. The size of the rectangles are proportional to the effective population sizes and it is a time-course diagram. **(b).** Location of the putative ancestral *Amborella* populations around 12,800 years BP, as inferred from spatially explicit modelling of demogenetic dynamics. Solid/dotted curves define the 50/95% posterior density envelope for each ancestral population. Diamonds indicate the estimated location of populations with maximum probability: 21.70°S–165.88°E, 12 km southwards to Dogny; 21.13°S–165.30°E, 5 km northwards to Aoupinié.

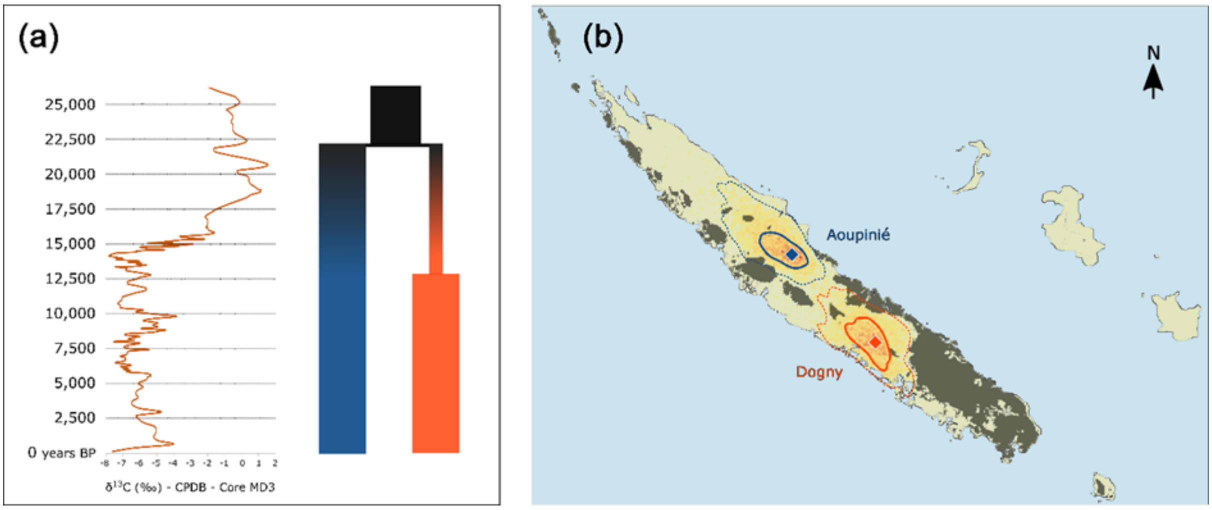

FIG 13. *Amborella* in cultivation. **(a) & (b).** a potted *Amborella* plant showing variability in architecture. **(c).** *in vitro* culture of *Amborella* (Photos: G. Gâteblé).

FIG 14. Objects illustrating or named after *Amborella*. **(a).** New Caledonian postage stamp drawn by M. Morat, © OPT Nouvelle-Calédonie, **(b).** ship of the New Caledonian government, **(c).** street plate in Dumbéa (New Caledonia) (photos: B. Fogliani), **(d).** lollipop named after *Amborella* (Photos: Amborella Organics, Heather Marie Collins)