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RESEARCH IN CONTEXT: PART OF A SPECIAL ISSUE ON SEXUAL PLANT REPRODUCTION

Cabomba as a model for studies of early angiosperm evolution

Aurelie C. M. Vialette-Guiraud[1,†], Michael Alaux[2,†], Fabrice Legeai[3], Cedric Finet[1,‡], Pierre Chambrier[1], Spencer C. Brown[4], Aurelie Chauvet[1], Carlos Magdalena[5], Paula J. Rudall[5,*] and Charles P. Scutt[1,*]

[1]*Laboratoire de Reproduction et Développement des Plantes, UMR 5667, CNRS-INRA-Université de Lyon, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France,* [2]*INRA-URGI Centre de Versailles, Bâtiment 18, Route de Saint-Cyr, 78026 Versailles Cedex, France,* [3]*UMR Bio3P, Campus de Beaulieu, 35042 Rennes Cedex, France,* [4]*Institut des Sciences du Végétal CNRS UPR2355, Bâtiment 23, Avenue de la Terrasse, F-91198 Gif-sur-Yvette Cedex, France and* [5]*Royal Botanic Gardens Kew, Richmond, Surrey TW9 3AB, UK*

[†]*These authors contributed equally to this work.*

[‡]*Present address: Howard Hughes Medical Institute, University of Wisconsin-Madison, 1525 Linden Drive, Madison, WI 53706, USA.*

* *Joint authors for correspondence. E-mail Charlie.Scutt@ens-lyon.fr, or P.Rudall@kew.org*

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• *Background* The angiosperms, or flowering plants, diversified in the Cretaceous to dominate almost all terrestrial environments. Molecular phylogenetic studies indicate that the orders Amborellales, Nymphaeales and Austrobaileyales, collectively termed the ANA grade, diverged as separate lineages from a remaining angiosperm clade at a very early stage in flowering plant evolution. By comparing these early diverging lineages, it is possible to infer the possible morphology and ecology of the last common ancestor of the extant angiosperms, and this analysis can now be extended to try to deduce the developmental mechanisms that were present in early flowering plants. However, not all species in the ANA grade form convenient molecular-genetic models.

• *Scope* The present study reviews the genus *Cabomba* (Nymphaeales), which shows a range of features that make it potentially useful as a genetic model. We focus on characters that have probably been conserved since the last common ancestor of the extant flowering plants. To facilitate the use of *Cabomba* as a molecular model, we describe methods for its cultivation to flowering in the laboratory, a novel *Cabomba* flower expressed sequence tag database, a well-adapted *in situ* hybridization protocol and a measurement of the nuclear genome size of *C. caroliniana*. We discuss the features required for species to become tractable models, and discuss the relative merits of *Cabomba* and other ANA-grade angiosperms in molecular-genetic studies aimed at understanding the origin of the flowering plants.

Key words: *Cabomba*, Cabombaceae, *Trithuria*, Hydatellaceae, Nymphaeales, ANA grade, angiosperm, flowering plant evolution, expressed sequence tags, EST, c-value, *in situ* hybridization, evo-devo.

INTRODUCTION

There are no widely accepted fossil angiosperms older than the Early Cretaceous, some 130 million years ago (Doyle, 2008). However, during the Cretaceous period, the angiosperms diversified to dominate almost all terrestrial environments. Press reports of studies currently nearing completion put the number of extant angiosperm species as high as 400 000 (Jowit, 2010), while the closest living relatives of the angiosperms, the remaining extant seed plants or gymnosperms, have dwindled to only approx. 1000 species. The angiosperm reproductive structure is termed the flower. Although diverse definitions of the flower exist (Bateman *et al.*, 2006), this structure is typically a bisexual reproductive axis of restricted growth that bears at its centre specialized female reproductive organs termed carpels. The angiosperm carpel encloses and protects the ovules: structures which in gymnosperms are usually naked, and borne in the axils of leaf-like organs such as the cone-scales of conifers. After fertilization, the carpel (or a group of fused carpels) develops into the fruit, which acts to protect and then disseminate the seeds. Some other features of the angiosperms are also rare or absent in gymnosperms (although several are present in the gymnosperm order Gnetales), including a double integument that covers their ovules and seeds, double fertilization that produces a zygote and a nutritive endosperm, specialized male reproductive organs termed stamens, and sterile perianth organs that are frequently differentiated into petals and sepals.

Phylogenetic studies performed over the last 15 years (Bremer *et al.*, 2009) have provided new insights into the relationships between extant angiosperm groups (Fig. 1). These studies indicate that three lineages, Amborellales, Nymphaeales and Austrobaileyales, diverged separately early in angiosperm evolution from a remaining common lineage from which all other living flowering plants are descended. These three lineages are collectively termed the ANA grade (or sometimes the ANITA grade). The order Amborellales contains a single species, *Amborella trichopoda*, which is a loosely branched shrub that is endemic to the southern Pacific island of New Caledonia. Nymphaeales, the most species-rich order, contains three families of herbaceous aquatic plants: Nymphaeaceae, Cabombaceae and

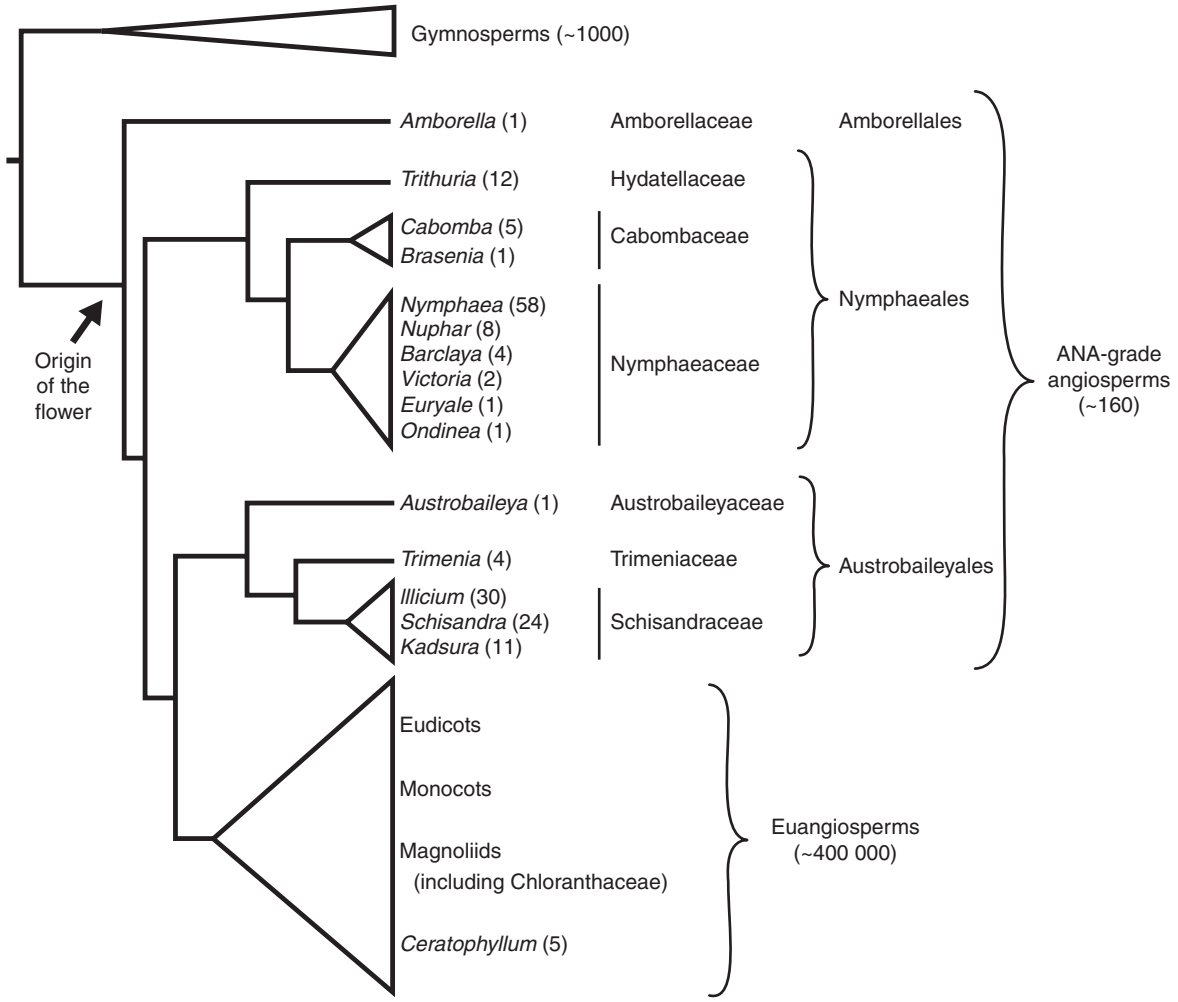

FIG. 1. Phylogenetic position of *Cabomba*. Schematic phylogeny based on Bremer *et al.* (2009) and related internet resources. Approximate species numbers are given in parentheses.

Hydatellaceae, which together constitute approx. 90 species in eight or nine genera (depending on whether *Ondinea* is grouped within *Nymphaea*: Löhne *et al.*, 2009). Finally, Austrobaileyales contains three families of woody plants: Austrobaileyaceae, Trimeniaceae and Schisandraceae, which include approx. 70 species in five genera. Most recent molecular phylogenetic analyses support the sequence of divergence of the three ANA-grade lineages as first Amborellales, then Nymphaeales and finally Austrobaileyales, although some studies have suggested an alternative topology in which Amborellales and Nymphaeales together form a first-diverging clade (Qiu *et al.*, 2001, 2006; Zanis *et al.*, 2002).

The relatively low number of extant ANA-grade species indicates a large number of extinctions in these ancient lineages, and/or the possibility that these groups have undergone fewer speciation events than the remaining angiosperm clade (Fig. 1). A relatively low speciation rate could have been a factor in the retention in the ANA grade of a high proportion of ancestral characters (Endress, 2004). Accordingly, comparisons of ANA-grade species have been used to speculate on the likely characteristics of the last common ancestor of the extant angiosperms, including both morphological/anatomical (Endress and Igersheim, 2000; Endress, 2001*a*, *b*; Rudall *et al.*, 2007, 2009*a*, *b*; Endress and Doyle, 2009; Friedman and Ryerson, 2009; Rudall and Bateman, 2010) and ecological (Feild *et al.*, 2003, 2004) studies. A major challenge is to extend these views of early angiosperms to include information on the mechanisms that controlled their development. Furthermore, by including gymnosperms in these comparisons as an outgroup, it may be possible to infer something of the molecular events that contributed to the evolution of the first flowers (Scutt *et al.*, 2006; Ferrandiz *et al.*, 2010; Rudall and Bateman, 2010; Rudall *et al.*, 2011). Thus, the aims of this article are to provide novel information and well-adapted protocols to facilitate the use of *Cabomba* as a potentially useful model within the ANA grade, and review the literature on *Cabomba* that is relevant to evo-devo studies.

CRITERIA FOR SELECTING MODEL ANA-GRADE ANGIOSPERMS

To facilitate molecular studies aimed at understanding the early evolution of angiosperms, it is useful to focus on some ANA-grade species, preferably covering all three orders.

Species used for in-depth investigation should be amenable for study using a wide variety of molecular biological and genetic methods (Bolker, 1995). Full genome sequencing of such models should be envisaged (see Soltis *et al.*, 2008), and manageable genome sizes are therefore an advantage. Ideally, model species should also contain a high number of likely plesiomorphic features. However, the plesiomorphic states are notoriously difficult to determine in angiosperms, because of the high morphological diversity among ANA-grade angiosperms and the absence of a closely related extant outgroup that would allow polarization of characters and reconstruction of the hypothetical ancestral states (Bateman *et al.*, 2006).

Recent molecular phylogenetic analyses (e.g. Löhne *et al.*, 2007; Borsch *et al.*, 2008) have resulted in the recognition of three families in Nymphaeales: Cabombaceae (two genera), Hydatellaceae (one) and Nymphaeaceae (six). In common with all other ANA-grade families, Nymphaeaceae show some derived traits, and the relatively large overall size and large flowers of most Nymphaeaceae could make these species problematic as model angiosperms. Despite these potential disadvantages, expressed sequence tag (EST) resources are available for *Nuphar* (Albert *et al.*, 2005), and this genus, together with *Nymphaea*, has already been incorporated into several evolutionary-developmental ('evo-devo') studies (Yamada *et al.*, 2003; Kim *et al.*, 2004, 2005; Yoo *et al.*, 2010).

Hydatellaceae, previously classified with the monocots, was recently shown to represent the earliest diverging extant lineage of Nymphaeales (Saarela *et al.*, 2007). This family contains a single genus, *Trithuria*, with ten species in Australia, one in New Zealand and one in southern India (Sokoloff *et al.*, 2008). Several species of *Trithuria* form bisexual reproductive axes, but these contain stamens at the centre, surrounded by carpels, and develop centripetally. These reproductive units, termed 'non-flowers' by Rudall *et al.* (2009*a*), could represent inside-out flowers, or cymose inflorescences in which each floral organ corresponds to a reduced flower. Set against their unusual reproductive arrangement, *Trithuria* plants are very small, and at least some species have a rapid generation time and can easily be grown to maturity in the laboratory (Rudall *et al.*, 2009*b*). Although relatively few molecular biological procedures have so far been tested on *Trithuria*, the important practical considerations of growth habit, size and life cycle make these plants potentially well adapted as molecular-genetic models.

The remaining family of Nymphaeales, Cabombaceae, consists of two genera: *Cabomba* (five species: Orgaard, 1991) and *Brasenia* (one: *B. schreberi*). All Cabombaceae possess trimerous flowers with a whorled perianth arrangement and non-fused ascidiate carpels. Although *Cabomba* and *Brasenia* share many morphological features, *Cabomba* seems better suited as a model given its smaller overall size. In addition, all submerged parts of *Brasenia* are covered in a thick, viscous slime that could cause serious problems for molecular biological procedures such as *in situ* hybridization.

CABOMBA: A BRIEF DESCRIPTION

Cabomba is a New World genus whose distribution extends from the north-eastern United States to northern Argentina, although most species are restricted to tropical or subtropical regions (Orgaard, 1991). All *Cabomba* species are aquatic and can grow in both stagnant and flowing fresh water. *Cabomba* plants form sparsely branched, erect stems and horizontal rhizomes that give rise to adventitious roots at leaf nodes. A marked foliar dimorphism is present in *Cabomba*, which forms submerged leaves that are highly dissected to reduce water drag, and floating leaves that have entire margins (Fig. 2). Both leaf types carry out photosynthesis, and the floating leaves also function to anchor and stabilize the plant to the water's surface and thus allow the flowers to be borne above the surface to enable pollination. As in many other aquatic plants, air canals ramify through the stems and petioles of *Cabomba* to facilitate gaseous exchange in the submerged parts of the plant. This and other aspects of the anatomy of *Cabomba* were described by Moseley *et al.* (1984), including a detailed treatment of the vascular arrangement.

A marked phase change to reproductive development is present in *Cabomba*, which can be manipulated by adjusting the light conditions (see below). Prior to phase change, the primordia arising from the shoot apical meristem develop into submerged leaves, whereas the primordia that arise after phase change develop into floating leaves that each subtend an axillary flower bud. Each shoot in its reproductive phase thus produces a succession of single flowers at the water's surface. *Cabomba* flowers are trimerous and contain two whorls of three petaloid tepals that form in alternating positions (Figs 2 and 3). Interestingly, a whorled, rather than a spiral, floral phyllotaxy is sometimes regarded as a derived condition in the angiosperms, but is present throughout both Nymphaeaceae and Cabombaceae, and may represent the basal condition in Nymphaeales. In *Cabomba*, the inner tepals are developmentally retarded with respect to the outer tepals and other floral organs (Tucker and Douglas, 1996; Endress, 2001*a*; Rudall *et al.*, 2009*a*), so that the reproductive organs are visible without dissection at early developmental stages (Fig. 3B): an unusual feature, also present in *Trithuria* (Rudall *et al.*, 2009*a*), that is potentially useful in a model organism. Three or six stamens are typically present in *Cabomba*, and one to four (most commonly three) separate carpels, which each contain one to five pendulous, anatropous ovules (Yamada *et al.*, 2001). The carpels of *Cabomba* are ascidiate and almost radially symmetric (Fig. 2D–F), resembling those of *Trithuria*, especially at early developmental stages (Endress, 2001; Schneider *et al.*, 2003; Rudall *et al.*, 2007). The carpels of *Cabomba* are not closed by cell fusion but by substances secreted into a narrow, open canal: a condition termed Type 1 angiospermy by Endress and Igersheim (2000), who concluded that this represents the likely ancestral condition in angiosperms because it occurs in many ANA-grade taxa (but not all; exceptions include *Nymphaea* and *Illicium*, in which carpels are postgenitally closed at anthesis; Endress and Igersheim, 2000).

The stigmatic surface in *Cabomba* is composed of multicellular protrusions, rather than unicellular papillae. Multicellular stigmatic hairs also occur in some other ANA-grade taxa, most notably *Trithuria* (Prychid *et al.*, 2011), so this could represent a plesiomorphic angiosperm feature (Endress and Igersheim, 2000). *Cabomba* is insect pollinated, and nectaries are

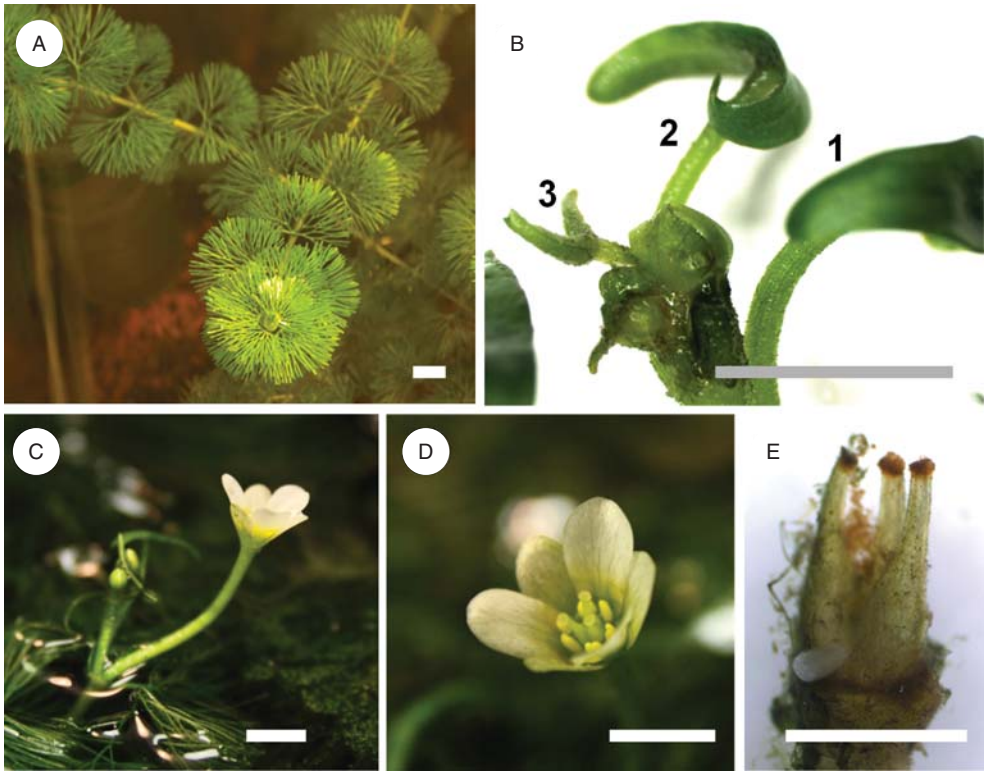

FIG. 2. Morphology of *Cabomba caroliniana*. (A) Erect stems carrying highly dissected, submerged leaves, seen from above. (B) Three successive floating leaves (1–3) emerging from the inflorescence apex, each subtending a flower bud present in the tissue at the centre. (C) Side view of a newly opened flower, supported above the water on its long pedicel by a floating leaf. (D) Open flower showing its trimerous, whorled arrangement. (E) A mature fruit, partially degraded under water to release the seeds. Scale bars = 1 cm.

present at the base of the inner tepals (Fig. 3F, G; Orgaard, 1991) to reward visiting pollinators, which include bees, wasps and flies (Schneider and Jeter, 1982). In *Cabomba*, as in many members of the ANA grade, protogyny acts as an outbreeding mechanism. Accordingly, each *Cabomba* flower opens on two successive days and closes during the intervening night. For fertilization to occur, the carpels of a first-day flower must be pollinated from the stamens of a second-day flower. Once pollination has occurred, the flower is rapidly pulled below the water by bending of the penduncle (Schneider and Jeter, 1982). The fruit then forms and, after approx. 1 month, releases its seeds directly into the water by decomposition of the fruit wall (Fig. 3). Vegetative propagation in the wild is also very common, and fragments of *Cabomba* stem can rapidly generate new plants under appropriate conditions: a capacity that has caused the clogging of ponds and waterways where *Cabomba* has been inadvertently introduced in Australia and elsewhere (Wilson *et al.*, 2007; Schooler, 2008).

HOW TO GROW *CABOMBA* IN THE LABORATORY

For a plant to be considered as a potential molecular-genetic model for the study of its flowers, it must be at least reasonably easy to grow to flowering in a laboratory or greenhouse environment. We have accordingly developed procedures to grow *Cabomba* through to flowering and seed-set in an aquarium of 200 L. Using such an aquarium, we have grown both *C. aquatica* and *C. caroliniana* plants rooted in a substrate composed of 50 % sand, 35 % ericaceous compost and 15 % kaolin (by volume). A 5-cm layer of this substrate was used, retained by a 2·5-cm layer of well-washed horticultural pumice. The aquarium was initially half-filled with deionized water, supplemented with 'Flourish' and 'Flourish Iron' nutrient solutions (Seachem, Madison, GA, USA), according to the manufacturer's recommendations. The use of peat-containing compost in the aquarium substrate was found to maintain the aquarium water at approx. pH 5·5–6·0, which proved suitable for growing both *Cabomba* species. The aquarium water was constantly filtered using an external pump through layers of both aquarium cell foam and active peat granules (JBL GmbH, Neuhofen, Germany). A UV lamp (Aqua Cristal; JBL GmbH) was also incorporated into the pump circuit to reduce algal growth. Once the initial turbulence of the water had subsided, after approx. 2 d of filtration, groups of *Cabomba* stems approx. 12 cm in length were planted into the substrate. *C. caroliniana* and *C. aquatica* were grown at water temperatures of 22 and 25 °C, respectively. The aquarium water was supplemented with carbon dioxide from a cylinder at approx. 120 bubbles per minute, these being retained for maximum dissolution in an inverted chamber attached to the return from the water pump. The aquarium water was also oxygenated by allowing part of the return

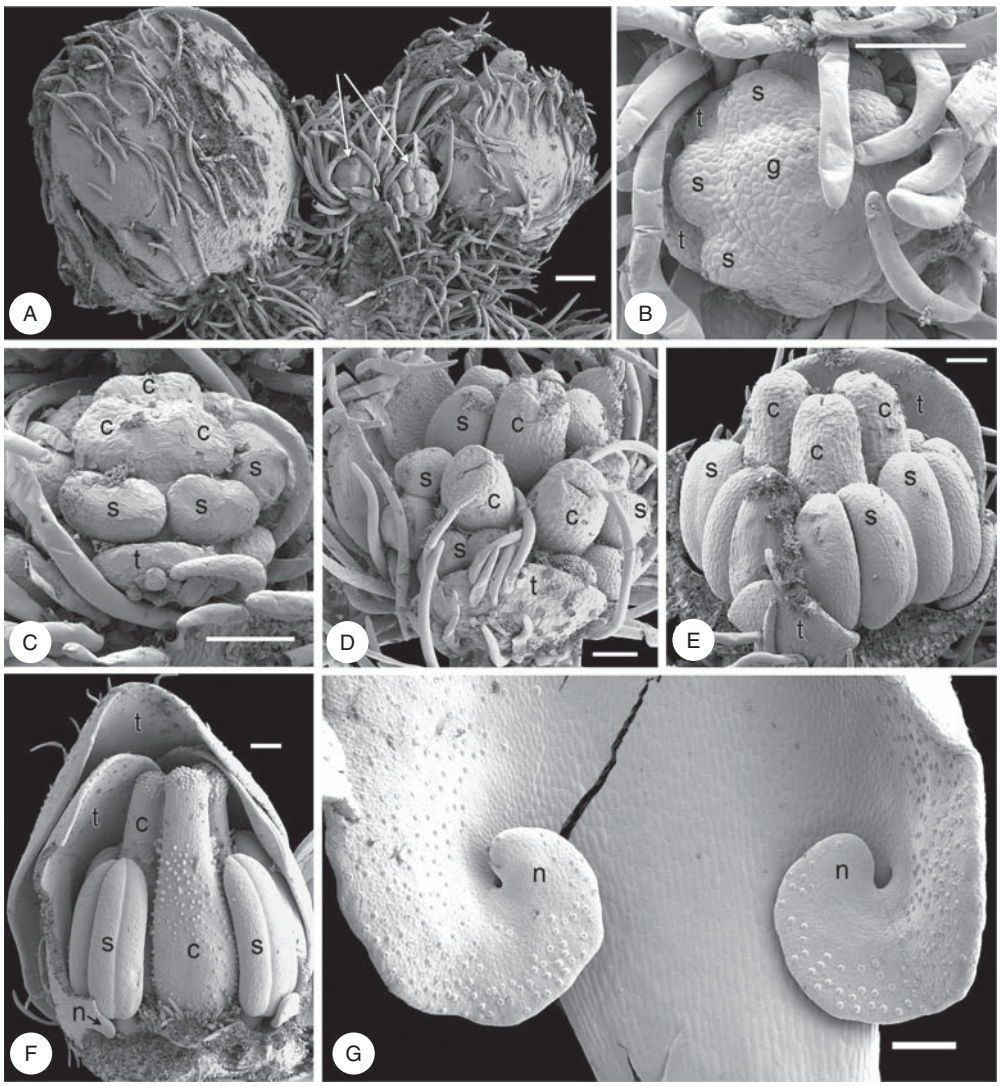

FIG. 3. Scanning electron micrographs of *Cabomba caroliniana* flowers growing at Kew. (A) Undissected flower buds, showing two preanthetic buds enclosed by tepals, and two early-stage buds (indicated by white arrows) not yet enclosed by tepals. (B) Early developmental stage showing early stamen, gynoecium and tepal primordia. (C) Later stage showing early carpel differentiation. (D–F) Successive developmental stages showing organ development and elongation; nectary developing on inner tepal in (F). (G) Inner tepal of anthetic flower showing nectaries. Abbreviations: c, carpel primordium or carpel; g, gynoecium primordium; n, nectary; s, stamen primordium or stamen; t, tepal. Scale bars: (A, F, G) = 200 μm, (B–E) = 100 μm.

from the water pump to fall from holes drilled into a pipe running along one side of the aquarium, approx. 5 cm above the water's surface. The water level was gradually increased, to keep up with plant growth.

To produce rapid vegetative growth of *Cabomba*, we used a purely artificial light source consisting of three specialized aquarium lamps (Floraset; Osram, Mississauga, Canada) fitted with 80-W discharge light bulbs (HDL-R; Osram) suspended at approx. 20 cm above the maximum water level. However, to induce flowering, it was necessary to add three domestic 60-W tungsten light bulbs, which had the effect of enriching the overall illumination in far-red wavelengths (700–750 nm). Under these conditions, *Cabomba* could be grown from short pieces of stem to flowering in approx. 6 weeks.

We have successfully pollinated *C. caroliniana* plants to generate fruits by taking dehisced anthers from flowers open on their second day and using these to manually pollinate flowers open on their first day. We have not, however, investigated seed viability, longevity or germination requirements.

AN EST RESOURCE FOR THE *CABOMBA* FLOWER

Publicly available EST resources of Nymphaeales have up to now been limited to *Nuphar* (Albert *et al.*, 2005). To facilitate molecular studies of *Cabomba*, we have generated a database of 3097 ESTs from a uni-directional *C. aquatica* flower cDNA bank constructed in the Bacteriophage λ Uni-Zap II vector (Stratagene, La Jolla, CA, USA). cDNAs for inclusion in this database were selected randomly from bacterial colonies harbouring recombinant pBluescriptII SK- plasmids which resulted from mass *in vivo* excisions of the λ-cloned bank, performed using ExAssist M13 helper phage (Stratagene) according to the manufacturer's protocols. The

selected cDNAs were sequenced from their 5′-ends and the resulting EST database has been made publicly available and can be interrogated by Blast and other procedures at http://www.ncbi.nlm.nih.gov/ and http://urgi.versailles.inra.fr/index.php/urgi/Data/Sequences/Run-GnpSeq.

We have characterized this database by performing a functional annotation using version 2·4·5 of the Blast2GO procedure described by Conesa *et al.* (2005). The NCBI non-redundant (nr) nucleotide database was searched by Blastx with *Cabomba* ESTs using the following parameters: number of hits, 100; expected value, $1{\cdot}0 \times 10^{-3}$; mode, QBlast-NCBI; HSP length cut-off, 33; filtering for low-complexity sequences, on. Following mapping onto GO databases, annotation was performed using the following parameters: E-value hit filter, $1{\cdot}0 \times 10^{-6}$; annotation cut-off, 55; GO weight, 5; Hsp hit coverage cut-off, 0. Sequences were categorized for biological processes, molecular functions and cellular components.

The results of these functional annotations are shown in Fig. 4, and demonstrate that the *Cabomba* EST database described contains numerous categories of sequences likely to be involved in developmental, regulatory and reproductive processes (Fig. 4A), of potential interest in the context of flower evo-devo research. High proportions of ESTs in the database encode domains of known binding or catalytic activity, while some 145 ESTs show homology to known transcription factors (Fig. 4B). Preliminary phylogenetic analyses of the ESTs encoding transcription factors indicate the presence of potential orthologues of several regulators of flower development, including members of the MADS-box, NAC and AUXIN RESPONSE FACTOR families. The majority of encoded proteins appear to be localized in the cytoplasm or nucleus, while the second largest group is addressed to organelles. Probable membrane-associated and extracellular proteins are also represented in significant numbers (Fig. 4C).

AN *IN SITU* HYBRIDIZATION PROTOCOL ADAPTED TO *CABOMBA*

In situ hybridization is an extremely valuable technique for the study of plant development. Conservation of gene expression patterns between species can be used to argue for the probable conservation of gene function. This type of evidence can be particularly useful for the molecular analysis of species that occupy key phylogenetic positions, but which are not amenable to the direct analysis of gene function, as is currently the case for the entire ANA grade of basal angiosperms.

We have tested several methods for non-radioactive mRNA *in situ* hybridization on *Cabomba* and found the method used by Nikovics *et al.* (2006) to give the best results, combining high signal strength and excellent tissue preservation. Using this technique, *in situ* hybridizations to the shoot apical meristem on a *HISTONE4* probe clearly show very high signal strength in actively dividing cells, with very little background elsewhere (Fig. 5). At high magnification, both individual cell contours and cell nuclei are clearly visible (Fig. 5C). As this method is described succinctly in its original publication, we reproduce it in the present work in the form of a user-friendly protocol (Supplementary Data).

COMPARISON OF NUCLEAR GENOME SIZES IN ANA-GRADE ANGIOSPERMS

Genome size is an important consideration in plant molecular biology, which must be taken into account when constructing and screening genomic libraries, performing Southern blots, or planning the sequencing of an entire genome. We measured the nuclear genome size of *C. caroliniana* material obtained from both Anthias SA (Les Chères, Rhône, France) and Bleu Aquarium (Caveriac, France). Leaves of each sample, and of the internal standard *Pisum sativum* 'Long Express' (2C = 8·37 pg; Marie and Brown, 1993), were chopped using a razor blade in 600 μL of cold Galbraith buffer (Galbraith *et al.*, 1983) supplemented with 1 % polyvinylpyrrolidone 10 000 and 50 μg mL^{-1} RNase A (Sigma Chemical Co., St. Louis, MO, USA). The suspension of nuclei thus produced was filtered through nylon mesh (pore size 48 μm), stained with 50 μg mL^{-1} propidium iodide (Sigma) and analysed on a flow cytometer (Elite, Beckman-Coulter, Villepinte, France). No significant difference was observed between the means of the two populations sampled, and the combined mean of the 23 measurements made was calculated as 2C = 6·73 ± 0·06 pg, giving a haploid genome size for *C. caroliniana* of 1C = 3290 ± 30 Mbp (taking 1 pg as equivalent to 978 Mbp, from Dolezel *et al.*, 2003).

The genome of *C. caroliniana* appears to be considerably larger than average for Nymphaeales, but slightly smaller than average for the ANA grade as a whole (Table 1). This genome, at approx. 30 % greater that of the monocot model *Zea mays*, remains sufficiently small to permit both genomic library construction and Southern blotting. In addition, with the current enormous increase in the speed of DNA sequencing technology, including recently published methods of up to 10^4 times faster than automated Sanger sequencing (Eid *et al.*, 2009), the genomes of *C. caroliniana* and many other ANA-grade angiosperms should rapidly fall within the reach of complete sequence analysis. Perhaps a greater challenge for evo-devo studies aimed at understanding the origin of the flower will be the sequencing and assembly of the much larger genomes typically present in gymnosperms. The 1C values of gymnosperms range from 9·62 Mbp in *Juniperus communis* subsp. *alpina* (Siljak-Yakovlev *et al.*, 2010) to 32·5 Mbp in *Pinus gerardiana* (Grotkopp *et al.*, 2004, recalculated with the reference value of Bogunic *et al.*, 2007): three to ten times bigger than that of *Cabomba.* Sequencing of gymnosperm genomes should provide an external reference to the angiosperms, which will surely be necessary if we are to identify the molecular events that generated the first flowers.

POTENTIAL OF *CABOMBA* AS A MODEL ANA-GRADE ANGIOSPERM

The present work examines the potential of *Cabomba* as a molecular-genetic model to represent Nymphaeales within the ANA grade. As explained above, all three families within Nymphaeales contain species of potential use as molecular models. Within Cabombaceae, it seems clear that *Cabomba* fulfils the requirements of a convenient model better than its sister genus *Brasenia*. However, within Nymphaeales as a whole, serious competition for super-model

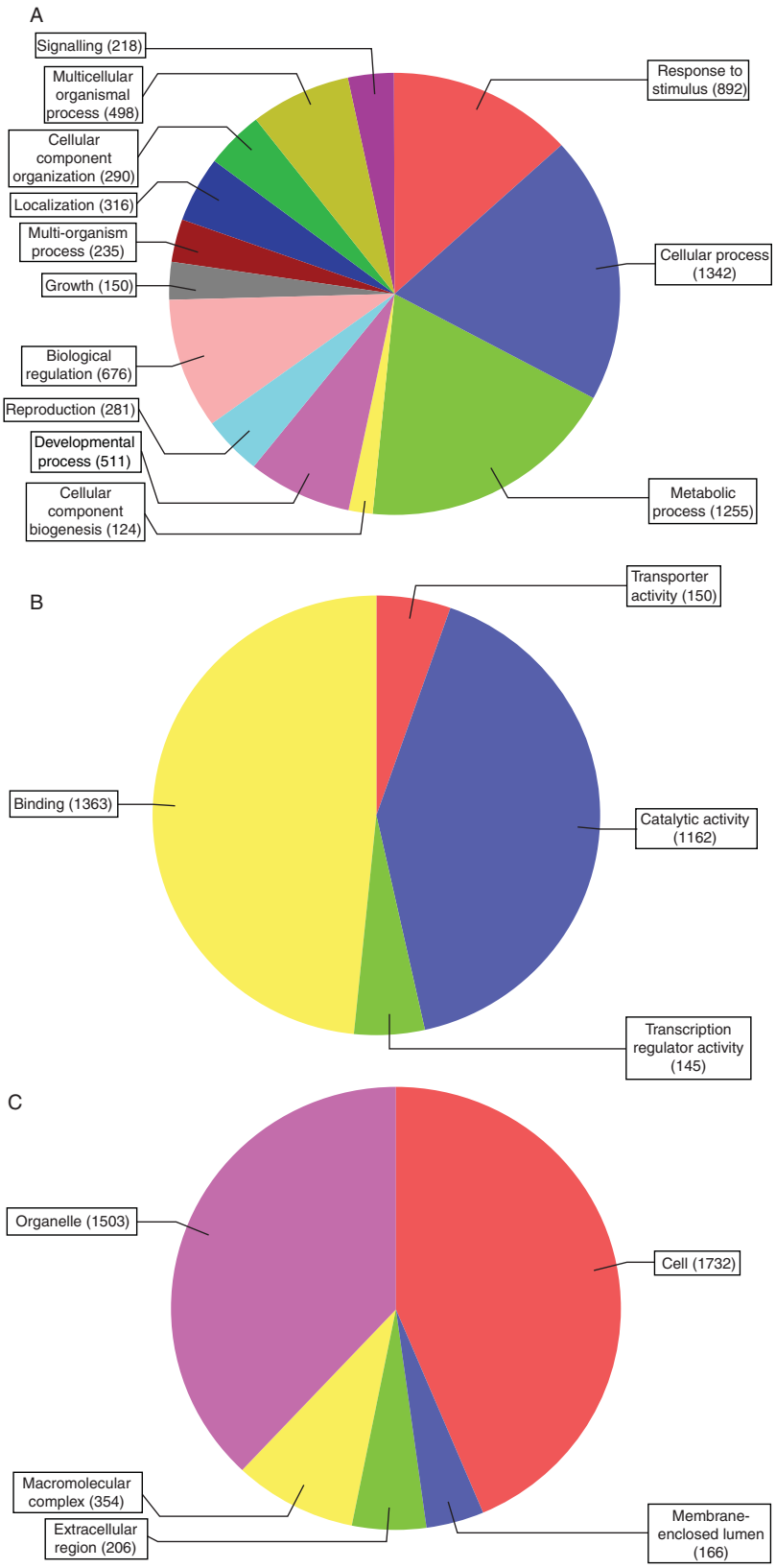

FIG. 4. Functional annotation of the *Cabomba* flower EST database. Sequences are annotated using Blast2GO to categorize 'Gene Ontology level 2 terms' matching with at least 100 sequences for: biological processes (A), molecular functions (B) and cellular components (C). Numbers of ESTs in each category are shown in parentheses.

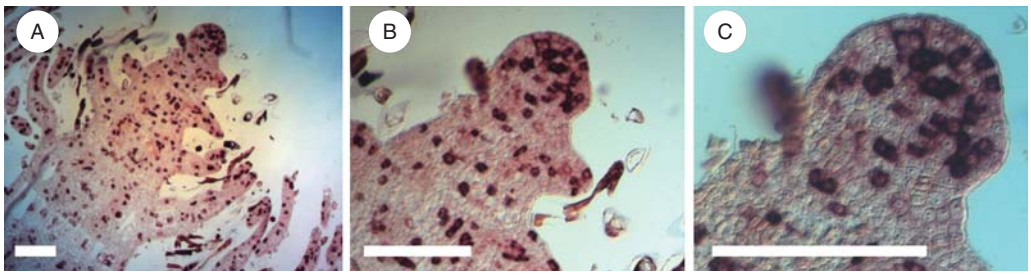

FIG. 5. *In situ* hybridization of a *HISTONE4* riboprobe to a *Cabomba aquatica* shoot apical meristem. Actively dividing cells that strongly express *HISTONE4* are shown by dark blue staining. The same specimen is shown at three magnifications, taken using brightfield illumination in conjunction with 4× (A) and 10× (B) objective lenses, and Nomarski contrast optics in conjunction with a 20× objective lens (C). Cell outlines and nuclei are clearly visible at high magnification. Scale bars = 400 μm. Full technical details are provided in Supplementary Data (available online).

TABLE 1. *Genome sizes of ANA-grade angiosperms*

ANA-grade species	Mass of 1C (pg)	Physical length (Mb)	Reference
Amborellaceae			
Amborella trichopoda	0·89	870	Leitch and Hanson (2002)
Mean for Amborellales	**0·89**	**870**	
Nymphaeaceae			
Nymphaea caerula	0·55	540	Bharathan *et al.* (1994)
Nymphaea lotus	1·08	1060	Bennett and Leitch (1995)
Nymphaea alba	1·99	1950	Zonneveld *et al.* (2005)
Nuphar luteum	2·53	2470	Zonneveld *et al.* (2005)
Cabombaceae			
Cabomba caroliniana	3·37	3290	Present work
Mean for Nymphaeales	**1·90**	**1860**	
Austrobaileyaceae			
Austrobaileya scandens	9·52	9310	Leitch and Hanson (2002)
Trimeniaceae			
Trimenia moorei	4·08	3990	Leitch and Hanson (2002)
Schisandraceae			
Illicium anisatum	3·35	3280	Nagl *et al.* (1977)
Kadsura coccinea	7·40	7240	Bennett *et al.* (2000)
Kadsura japonica	8·13	7950	Bennett *et al.* (2000)
Kadsura longespicata	8·85	8660	Bennett *et al.* (2000)
Schisandra rubriflora	9·12	8920	Leitch and Hanson (2002)
Mean for Austrobaileyales	**7·20**	**7050**	
Mean for ANA grade	**4·68**	**4580**	

status comes from *Trithuria*, which possesses the advantages of very small size, ease of cultivation and rapid generation time. Preliminary studies (R. Kynast and P. J. Rudall, unpubl. res.) also indicate the advantage of a relatively small genome size in *Trithuria*.

Despite the evident practical advantages of *Trithuria*, the need for a comparative approach is a compelling argument for the development of more than one model within each of the non-monotypic ANA-grade orders, Nymphaeales and Austrobaileyales. Furthermore, the unusual reproductive arrangement of *Trithuria* argues for the use of additional models within Nympheaeles. Indeed, for evo-devo studies of floral patterning and perianth development, which are highly derived features in *Trithuria*, it would seem essential to include additional models of Nymphaeales. *Cabomba*, as discussed above, combines simplicity of floral structure, numerous pleisiomorphic angiosperm characters, and practical features that make it amenable to study using a broad range of molecular biological techniques. We therefore argue that *Cabomba* can provide a useful contribution to evo-devo studies of angiosperm origin, alongside *Trithuria* in Nymphaeales, *Amborella* as the unique representative of Amborellales and suitable further models chosen from Austrobaileyales. Indeed, *Cabomba* has already made some contributions to evo-devo studies, with respect to both protein-coding genes (Fourquin *et al.*, 2005; Finet *et al.*, 2010; Yoo *et al.*, 2010; Vialette-Guiraud *et al.*, 2011) and small regulatory RNAs (Jasinski *et al.*, 2010).

Although *Cabomba* and several other ANA-grade taxa are clearly amenable to molecular biological analyses, a far greater challenge lies in the study of gene function in these plants. Conservation of gene expression between model and non-model species has provided a number of insights into probable gene functions in the ANA grade, although such data fall short of direct functional evidence and are relevant only to genes that show highly characteristic expression patterns, which is not the case for many important developmental regulators. In addition, we should not be content to study only what has been conserved during evolution: differences in gene function should also be investigated if we are to understand the genetic basis for morphological biodiversity. For evo-devo studies to engage fully with the question of the origin and early evolution of the angiosperms, we should develop methods of functional analysis for use in the ANA grade. As yet, almost no work has been performed in this direction, but it is worth considering at this stage the techniques and ANA-grade taxa that might be best adapted to the needs of evo-devo research.

Traditionally, plant functional genetics has been performed through the production of mutants, which can be generated by pseudo-random T-DNA insertion, transposon mutagenesis, or exposure to mutagenic chemicals or radiation. The resulting mutant collections can be screened phenotypically by 'forward genetic' approaches, or for mutations in genes of interest through 'reverse genetic' approaches, including the TILLING technique, which does not require

genetic transformation (Wang *et al.*, 2010). Both forward and reverse genetic screens require the growth of large numbers of plants and the long-term storage of mutant collections, usually in the form of seed. Unfortunately, *Cabomba* is not sufficiently easy to grow in large numbers to permit forward or reverse genetic screening, and seed production and storage issues have yet to be fully addressed in this genus. *Trithuria*, by contrast, is small and flowers rapidly, and its seed can be stored conveniently (Tuckett *et al.*, 2010). *Trithuria* is thus potentially amenable to forward and reverse genetic screening, and indeed appears to be the only ANA-grade angiosperm that might readily lend itself to the use of these techniques.

Happily for the future of reverse genetics in the ANA grade, further techniques are available that do not depend on the generation of large mutant collections. In these methods, much smaller groups of plants can either be transformed to integrate a chosen transgene or, in an approach termed virus-induced gene silencing (VIGS), infected with a transgenic virus to reduce the expression of one or more endogenous genes (Burch-Smith *et al.*, 2004). Through the transformation approach, gene expression may be either down-regulated by RNA interference (RNAi; Helliwell and Waterhouse, 2003), or misdirected through the use of a non-native or modified gene promoter. Further, more precise techniques for the analysis of gene function are also being developed, including the use of zinc-finger nucleases (Osakabe *et al.*, 2010). These recombinant enzymes are engineered to generate double-stranded DNA breaks at pre-defined loci, whose subsequent repair by endogenous mechanisms can be exploited to perform precise gene replacements. Needless to say, the success of these approaches depends on the availability of efficient transformation and regeneration methods, or on a VIGS vector that is capable of infecting the species of interest. Almost no work has been performed to date on the development of any of these techniques in the ANA grade, and such studies must now be a high priority. Although several members of the ANA grade have been shown to be amenable to methods for molecular analysis, it is the successful development of stable transformation and/or VIGS methods that will define the long-term value of these species as experimental models. As discussed above, *Trithuria* may prove to be the only ANA-grade angiosperm that readily lends itself to large-scale forward and reverse genetic screening. However, the requirements of evo-devo research mean that smaller-scale methods must be developed to provide functional data in other ANA-grade taxa. In this context, *Cabomba*, which can be grown relatively easily and rapidly in small numbers in the laboratory, certainly seems worthy of the investment of time and effort necessary to attempt the development of such techniques as RNAi and VIGS.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of a protocol for mRNA *in situ* hybridization for tissue sections suitable for use with *Cabomba*.

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LITERATURE CITED

Albert VA, Soltis DE, Carlson JE, *et al.* 2005. Floral gene resources from basal angiosperms for comparative genomics research. *BMC Plant Biology* **5**: 5. doi:10.1186/1471-2229-5-5.

Bateman RM, Hilton J, Rudall PJ. 2006. Morphological and molecular phylogenetic context of the angiosperms: contrasting the 'top-down' and 'bottom-up' approaches used to infer the likely characteristics of the first flowers. *Journal of Experimental Botany* **57**: 3471–3503.

Bennett MD, Leitch IJ. 1995. Nuclear-DNA amounts in Angiosperms. *Annals of Botany* **76**: 113–176.

Bennett MD, Bhandol P, Leitch IJ. 2000. Nuclear DNA amounts in angiosperms and their modern uses – 807 new estimates. *Annals of Botany* **86**: 859–909.

Bharathan G, Lambert G, Galbraith DW. 1994. Nuclear-DNA content of monocotyledons and related taxa. *American Journal of Botany* **81**: 381–386.

Bogunic F, Muratovic E, Ballian D, Siljak-Yakovlev S, Brown S. 2007. Genome size stability among five subspecies of *Pinus nigra* Arnold s.l. *Environmental & Experimental Botany* **59**: 354–360.

Bolker JA. 1995. Model systems in developmental biology. *BioEssays* **17**: 451–455.

Borsch T, Löhne C, Wiersema J. 2008. Phylogeny and evolutionary patterns in Nymphaeales: integrating genes, genomes and morphology. *Taxon* **57**: 1052–1081.

Bremer B, Bremer K, Chase MW, *et al.* 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society* **161**: 105–121.

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant Journal* **39**: 734–746.

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**: 3674–3676.

Dolezel J, Bartos J, Voglmayr H, Greilhuber J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry Part A* **51A**: 127–128.

Doyle JA. 2008. Integrating molecular phylogenetic and paleobotanical evidence on the origin of the flower. *International Journal of Plant Sciences* **169**: 816–843.

Eid J, Fehr A, Gray J, *et al.* 2009. Real-time DNA sequencing from single polymerase molecules. *Science* **323**: 133–138.

Endress PK. 2001a. Origins of flower morphology. *Journal of Experimental Zoology* **291**: 105–115.

Endress PK. 2001b. The flowers in extant basal angiosperms and inferences on ancestral flowers. *International Journal of Plant Sciences* **162**: 1111–1140.

Endress PK. 2004. Structure and relationships of basal relictual angiosperms. *Australian Systematic Botany* **17**: 343–366.

Endress PK, Doyle JA. 2009. Reconstructing the ancestral angiosperm flower and its initial specializations. *American Journal of Botany* **96**: 22–66.

Endress PK, Igersheim A. 2000. Gynoecium structure and evolution in basal angiosperms. *International Journal of Plant Sciences* **161**: S211–S223.

Feild TS, Arens NC, Dawson TE. 2003. The ancestral ecology of angiosperms: emerging perspectives from extant basal lineages. *International Journal of Plant Sciences* **164**: S129–S142.

Feild TS, Arens NC, Doyle JA, Dawson TE, Donoghue MJ. 2004. Dark and disturbed: a new image of early angiosperm ecology. *Paleobiology* **30**: 82–107.

Ferrandiz C, Fourquin C, Prunet N, *et al.* 2010. Carpel development. *Advances in Botanical Research* **55**: 1–73.

Finet C, Fourquin C, Vinauger M, *et al*. 2010. Parallel structural evolution of auxin response factors in the angiosperms. *Plant Journal* **63**: 952–959.

Fourquin C, Vinauger-Douard M, Fogliani B, Dumas C, Scutt CP. 2005. Evidence that CRABS CLAW and TOUSLED have conserved their roles in carpel development since the ancestor of the extant angiosperms. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 4649–4654.

Friedman WE, Ryerson KC. 2009. Reconstructing the ancestral female gametophyte of angiosperms: insights from *Amborella* and other ancient lineages of flowering plants. *American Journal of Botany* **96**: 129–143.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell-cycle in intact plant-tissues. *Science* **220**: 1049–1051.

Grotkopp E, Rejmanek M, Sanderson MJ, Rost TL. 2004. Evolution of genome size in pines (*Pinus*) and its life-history correlates: supertree analyses. *Evolution* **58**: 1705–1729.

Helliwell C, Waterhouse P. 2003. Constructs and methods for high-throughput gene silencing in plants. *Methods* **30**: 289–295.

Jasinski S, Vialette-Guiraud ACM, Scutt CP. 2010. The evolutionary-developmental analysis of plant microRNAs. *Philosophical Transactions of the Royal Society B-Biological Sciences* **365**: 469–476.

Jowit J. 2010. Scientists prune list of world's plants. *The Guardian* (London). http://www.guardian.co.uk/science/2010/sep/19/scientists-prune-world-plant-list.

Kim ST, Yoo MJ, Albert VA, Farris JS, Soltis PS, Soltis DE. 2004. Phylogeny and diversification of B-function MADS-box genes in angiosperms: evolutionary and functional implications of a 260-million-year-old duplication. *American Journal of Botany* **91**: 2102–2118.

Kim S, Koh J, Yoo MJ, *et al*. 2005. Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. *Plant Journal* **43**: 724–744.

Leitch IJ, Hanson L. 2002. DNA C-values in seven families fill phylogenetic gaps in the basal angiosperms. *Botanical Journal of the Linnean Society* **140**: 175–179.

Löhne C, Borsch T, Wiersema JH. 2007. Phylogenetic analysis of Nymphaeales using fast-evolving and noncoding chloroplast markers. *Botanical Journal of the Linnean Society* **154**: 141–163.

Löhne C, Wiersema JH, Borsch T. 2009. The unusual *Ondinea*, actually just another Australian water-lily of *Nymphaea* subg. *Anecphya* (Nymphaeaceae). *Willdenowia* **39**: 55–58.

Marie D, Brown SC. 1993. A cytometric exercise in plant DNA histograms, with 2c-values for 70 species. *Biology of the Cell* **78**: 41–51.

Moseley MF, Mehta IJ, Williamson PS, Kosakai H. 1984. Morphological-studies of the Nymphaeaceae (Sensu Lato). 13. Contributions to the vegetative and floral structure of *Cabomba*. *American Journal of Botany* **71**: 902–924.

Nagl W, Habermann T, Fusenig HP. 1977. Nuclear-DNA contents in 4 primitive Angiosperms. *Plant Systematics and Evolution* **127**: 103–105.

Nikovics K, Blein T, Peaucelle A, *et al*. 2006. The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. *Plant Cell* **18**: 2929–2945.

Orgaard M. 1991. The genus *Cabomba* (Cabombaceae) – a taxonomic study. *Nordic Journal of Botany* **11**: 179–203.

Osakabe K, Osakabe Y, Toki S. 2010. Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 12034–12039.

Prychid CJ, Sokoloff DD, Remizowa MV, Tuckett RE, Ramsay MM, Rudall PJ. 2011. Unique stigmatic hairs and pollen-tube growth within the stigmatic cell wall in the early-divergent angiosperm family Hydatellaceae. *Annals of Botany* **108**: 599–608.

Qiu YL, Lee J, Whitlock BA, Bernasconi-Quadroni F, Dombrovska O. 2001. Was the ANITA rooting of the angiosperm phylogeny affected by long-branch attraction? *Molecular Biology and Evolution* **18**: 1745–1753.

Qiu YL, Li LB, Hendry TA, *et al*. 2006. Reconstructing the basal angiosperm phylogeny: evaluating information content of mitochondrial genes. *Taxon* **55**: 837–856.

Rudall PJ, Bateman RM. 2010. Defining the limits of flowers: the challenge of distinguishing between the evolutionary products of simple versus compound strobili. *Philosophical Transactions of the Royal Society B* **365**: 397–409.

Rudall PJ, Sokoloff DD, Remizowa MV, *et al*. 2007. Morphology of Hydatellaceae, an anomalous aquatic family recently recognized as an early-divergent angiosperm lineage. *American Journal of Botany* **94**: 1073–1092.

Rudall PJ, Remizowa MV, Prenner G, Prychid CJ, Tuckett RE, Sokoloff DD. 2009*a*. Nonflowers near the base of extant angiosperms? Spatiotemporal arrangement of organs in reproductive units of Hydatellaceae and its bearing on the origin of the flower. *American Journal of Botany* **96**: 67–82.

Rudall PJ, Eldridge T, Tratt J, *et al*. 2009*b*. Seed fertilization, development and germination in Hydatellaceae (Nymphaeales): implications for endosperm evolution in early angiosperms. *American Journal of Botany* **96**: 1581–1593.

Rudall PJ, Vergara-Silva F, Hilton J, Bateman RM. 2011. Recurrent abnormalities in conifer cones and the evolutionary origins of flower-like structures. *Trends in Plant Science* **16**: 151–159.

Saarela JM, Rai HS, Doyle JA, *et al*. 2007. Hydatellaceae identified as a new branch near the base of the angiosperm phylogenetic tree. *Nature* **446**: 312–315.

Schneider EL, Jeter JM. 1982. Morphological studies of the Nymphaeaceae. 12. The floral biology of *Cabomba caroliniana*. *American Journal of Botany* **69**: 1410–1419.

Schneider EL, Tucker SC, Williamson PS. 2003. Floral development in the Nymphaeales. *International Journal of Plant Sciences* **164**: S279–S292.

Schooler SS. 2008. Shade as a management tool for the invasive submerged macrophyte, *Cabomba caroliniana*. *Journal of Aquatic Plant Management* **46**: 168–171.

Scutt CP, Vinauger-Douard M, Fourquin C, Finet C, Dumas C. 2006. An evolutionary perspective on the regulation of carpel development. *Journal of Experimental Botany* **57**: 2143–2152.

Siljak-Yakovlev S, Pustahija F, Solic EM, *et al*. 2010. Towards a genome size and chromosome number database of Balkan flora: C-values in 343 taxa with novel values for 242. *Advanced Science Letters* **3**: 190–213.

Sokoloff DD, Remizowa MV, Macfarlane TD, Rudall PJ. 2008. Classification of the early-divergent angiosperm family Hydatellaceae: one genus instead of two, four new species and sexual dimorphism in dioecious taxa. *Taxon* **57**: 179–200.

Soltis DE, Albert VA, Leebens-Mack J, *et al*. 2008. The *Amborella* genome: an evolutionary reference for plant biology. *Genome Biology* **9**: 402. doi:10.1186/gb-2008-9-3-402.

Tucker SC, Douglas AW. 1996. Floral structure, development, and relationships of paleoherbs: *Saruma*, *Cabomba*, *Lactoris*, and selected Piperales. In Taylor DW, Hickey LJ, eds. *Flowering plant origin, evolution and phylogeny*. New York: Chapman and Hall, 141–175.

Tuckett RE, Merritt DJ, Rudall PJ, *et al*. 2010. A new type of specialized morphophysiological dormancy and seed storage behaviour in Hydatellaceae, an early-divergent angiosperm family. *Annals of Botany* **105**: 1053–1061.

Vialette-Guiraud ACM, Adam H, Finet C, Jasinski S, Jouannic S, Scutt CP. 2011. Insights from ANA-grade angiosperms into the early evolution of *CUP-SHAPED COTYLEDON* genes. *Annals of Botany* **107**: 1511–1519.

Wang T, Uauy C, Till B, Liu CM. 2010. TILLING and associated technologies. *Journal of Integrative Plant Biology* **52**: 1027–1030.

Wilson CE, Darbyshire SJ, Jones R. 2007. The biology of invasive alien plants in Canada. 7. *Cabomba caroliniana* A. Gray. *Canadian Journal of Plant Science* **87**: 615–638.

Yamada T, Imaichi R, Kato M. 2001. Developmental morphology of ovules and seeds of Nymphaeales. *American Journal of Botany* **88**: 963–974.

Yamada T, Ito M, Kato M. 2003. Expression pattern of INNER NOOUTER homologue in *Nymphaea* (water lily family, Nymphaeaceae). *Development Genes and Evolution* **213**: 510–513.

Yoo MJ, Soltis PS, Soltis DE. 2010. Expression of floral Mads-box genes in two divergent water lilies: Nymphaeales and Nelumbo. *International Journal of Plant Sciences* **171**: 121–146.

Zanis MJ, Soltis DE, Soltis PS, Mathews S, Donoghue MJ. 2002. The root of the angiosperms revisited. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 6848–6853.

Zonneveld BJM, Leitch IJ, Bennett MD. 2005. First nuclear DNA amounts in more than 300 angiosperms. *Annals of Botany* **96**: 229–244.