Letters

Polyploidy in a 'living fossil' *Ginkgo biloba*

The 'living fossil' *Ginkgo biloba* L. is the only extant representative of Ginkgophyta, which is an ancient group of gymnosperms that constituted an important component of the Earth's forests in the Mesozoic and early to mid-Cenozoic (Tralau, 1967; Zhou, 1997, 2009; Royer *et al.*, 2003; Zhou & Zheng, 2003; Taylor *et al.*, 2009). The *Ginkgo* and its sister phylogenetic relatives, the cycads (Cycadophyta), are the last major lineages of green plants in which polyploidy (whole genome duplication) remains unknown. Moreover, current genomic evidence indicates that the Ginkgo + cycads and the Araucariaceae are the only two clades of gymnosperms and seed plants in which paleopolyploidy (ancient whole genome duplication) is completely absent in their evolutionary history (Li *et al.*, 2015) [Correction added after online publication 6 June 2016: citation has been corrected, and Li *et al.* (2015) was added to the reference list.], i.e. since their divergence from the common polyploid ancestor of seed plants *c.* 310 million years ago (Jiao *et al.*, 2011). Recent polyploidy is also rare in the remaining gymnosperm lineages, Gnetophyta and Pinophyta (conifers), in which it is known only in *Ephedra* (in about half of species) and four species of cupressoid conifers (Khoshoo, 1959; Husband *et al.*, 2013).

The rarity of polyploidy in gymnosperms strongly contrasts with that in related angiosperms (flowering plants), which have an evolutionary history full of various polyploid events (Leitch & Leitch, 2012; Husband *et al.*, 2013). Polyploidy is suggested to predate the origin of angiosperms, to have assisted in the survival of angiosperm lineages during the Cretaceous–Tertiary extinction and to precede the radiation of many angiosperm groups that currently form a dominant component of the Earth's vegetation (Fawcett *et al.*, 2009; Soltis *et al.*, 2009; Jiao *et al.*, 2011; Vanneste *et al.*, 2014). Polyploidy is an important mechanism providing new genetic substrates for evolution and can enable extensive genomic reorganizations and reprogramming, facilitating the adaptation of polyploid species to new environments (Levin, 2002; Otto, 2007; Leitch & Leitch, 2008; Van de Peer *et al.*, 2009). Therefore, the lack of polyploidy in gymnosperms could be one of the primary reasons for their evolutionary conservatism (Gorelick & Olson, 2011; Leitch & Leitch, 2012), leading to their consecutive replacement with the more adaptable polyploid-prone angiosperms in response to global climate and habitat changes during the Tertiary period (Lidgard & Crane, 1988; Royer *et al.*, 2003; Fawcett & Van de Peer, 2010; Fawcett *et al.*, 2013).

Surprisingly, however, *Ginkgo* has the potential to form spontaneous polyploid offspring. We found such a vital polyploid sapling of *Ginkgo* during a routine screening for genome size variation in plants used for cultivation experiments (Fig. 1a). This polyploid sapling (sex yet unknown) originated from the seeds collected from three female trees grown in the Botanical Garden of the Faculty of Science, Masaryk University in Brno (Czech Republic). Its genome size ($2C = 37.4 \pm 0.2$ Gbp) is approximately double that of the diploid *Ginkgo biloba* ($2C = 18.4 \pm 0.1$ Gbp, mean of all three possible mother trees), indicating that it is tetraploid (Fig. 1b). Compared with its diploid parental plants and same-age siblings, the leaves of the tetraploid had finely laciniate distal margins (Fig. 1c) and enlarged stomata (60 ± 6 µm in the tetraploid sapling vs 39 ± 5 µm in its same-age diploid siblings or 34 ± 6 µm in putatively parental trees; Fig. 1d). Such enlargement is an effect of polyploidy and a larger genome size in general (Masterson, 1994; Beaulieu *et al.*, 2008; Veselý *et al.*, 2012). The large stomatal size observed in the present tetraploid *Ginkgo* sapling has never been observed in any fossil *Ginkgo* species (17.5–34 µm; five species; Lomax *et al.*, 2014) or in other Ginkgoales (12.5–38 µm; seven species; Lomax *et al.*, 2014, their Supporting Information Table S1), suggesting its genome size is unusual from the paleo-historical perspective as well.

The discovery of this tetraploid *Ginkgo* sapling clearly indicates that there is no inherent intrinsic barrier for *Ginkgo*, and potentially cycads, to form spontaneous polyploids. However, why polyploidy does not appear to play a role in the *Ginkgo* + cycads clade and why it does not play a more significant role in the remaining gymnosperms still remain unclear (Khoshoo, 1959; Leitch & Leitch, 2012; Husband *et al.*, 2013). One remarkable difference between the *Ginkgo* with cycads and other gymnosperms is that they are completely dioecious (they form separate male and female individuals). When a polyploid plant newly arises in a parental diploid population, it will have difficulty finding an appropriate mating partner (of the same ploidy level). For monoecious or hermaphroditic species, the absence of a mating partner may be overcome by selfing (Levin, 1975). However, selfing is impossible in dioecious plants such as *Ginkgo* or cycads. Furthermore, the mate must be the other sex. This would greatly reduce the chances of the establishment of polyploids of dioecious species under natural conditions (Ashman *et al.*, 2013), especially in small populations and when polyploids are produced only rarely (a likely situation in gymnosperms; Khoshoo, 1959). This dioecy-determined barrier for polyploid establishment may add to other reasons limiting polyploidy in gymnosperms in general (discussed later) and explain the complete absence of polyploids in naturally occurring *Ginkgo* and cycads.

An analogy for the low polyploidy in gymnosperms may exist in the polyploid-rich angiosperms, where polyploidy is clearly less common in woody species (Müntzing, 1936; Stebbins, 1938) and in species with large diploid genome sizes (Grif, 2000). These two factors may act in concert in gymnosperms because they are exclusively woody and generally have very large monoploid genome sizes (Cx; genome size divided by ploidy level; Greilhuber *et al.*,

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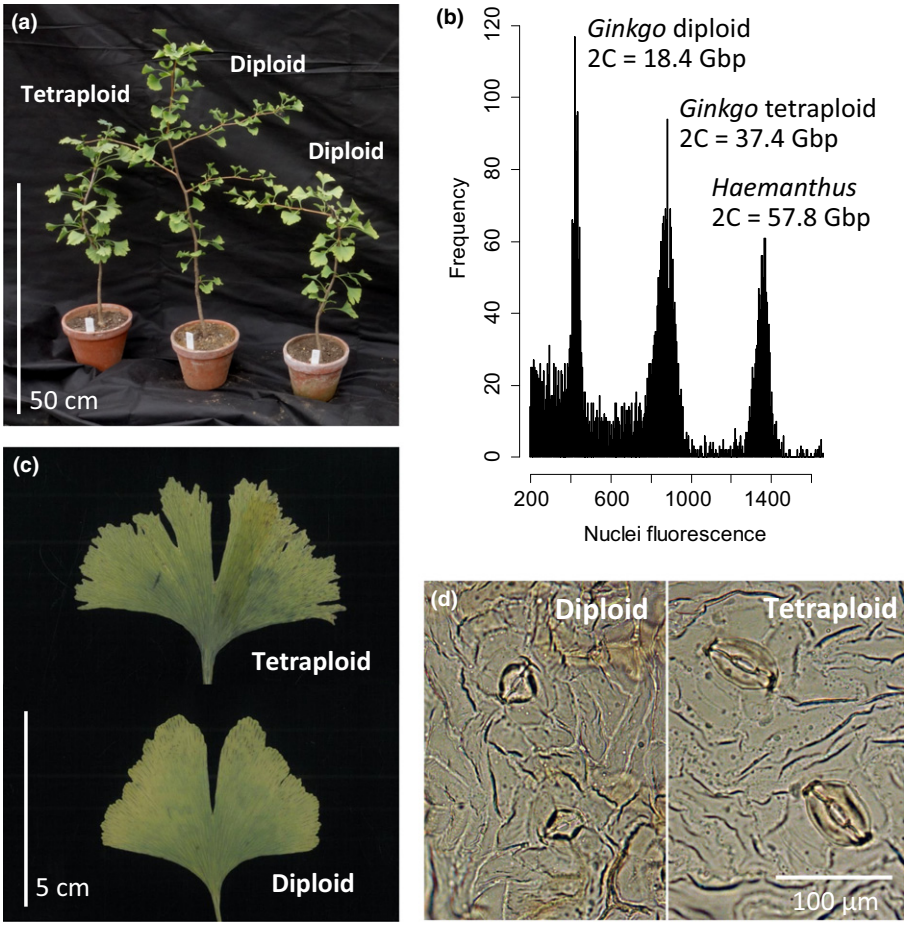

Fig. 1 Comparison of some properties of the tetraploid and diploid *Ginkgo* saplings (siblings). (a) General habit of plants cultivated together in one experimental treatment; (b) genome size in the flow cytometry histogram where both ploidy levels were chopped together with the internal standard; (c) mature leaves; (d) stomata in epidermal peels.

2005). For gymnosperms, the interquartile range (25^{th}–75^{th} percentile) of Cx is 11–24 Gbp; however, this range is only 1–5 Gbp for most angiosperms (based on the genome size and ploidy level data in the Plant C-value Database, data from flow cytometry or Feulgen densitometry only; Bennett & Leitch, 2012). Notably, all gymnosperm polyploids originated from groups in which the monoploid genome size is in the lower third of the known gymnosperm Cx values (*Juniperus* ~ 9–13 Gbp, *Fitzroya* ~ 9 Gbp, *Sequoia sempervirens* ~ 9 Gbp, *Ginkgo* ~ 9 Gbp). Of note, similar to gymnosperms, angiosperm trees or shrubs form polyploids only in circumstances in which their monoploid genome size is relatively small, with the absolute maximum representing shrubby succulent polyploid species of *Aloe* (Cx up to 18 Gbp) and the shrubby polyploid *Aucuba japonica* (Cx ~ 6 Gbp). Therefore, compared with angiosperms, the low polyploid frequency in gymnosperms may confirm a general trend that is potentially caused by the inability to evolve herbaceous forms and to downsize their very large genomes.

Why polyploidy is less common in woody plants with larger genomes remains debated. The most likely reason is that these newly arising polyploids will be counter-selected in maternal populations because of (1) disadvantages associated with having larger cells, leading to problems with (1a) the formation of woody fibres (Stebbins, 1938) and (1b) effective function of stomata needed to facilitate the movement of water and nutrients through the long xylem pathways (Beaulieu *et al.*, 2008) or because of (2) the increased nutrient demands associated with greater DNA replication in polyploid nuclei (Šmarda *et al.*, 2013). This is consistent with the developmental problems observed in artificially prepared or spontaneous conifer polyploids, which show reduced growth and premature mortality (Khoshoo, 1959; Ahuja, 2005). This, and the earlier evidence, indicate that although gymnosperms may occasionally form polyploid offspring, these offspring are unlikely to survive in natural populations and can likely only be established with targeted artificial selection (Khoshoo, 1959).

Although they are generally thought to be evolutionary dead ends, the rare gymnosperm polyploids warrant attention because they are a potential source of economically beneficial properties (Khoshoo, 1959). This may be particularly true for *Ginkgo* as a consequence of its exceptional ornamental and medicinal qualities (Hori *et al.*, 1997; Van Beek, 2002; Crane, 2013). *Ginkgo* is used to produce widely prescribed and sold herbal supplements to boost cognitive function and memory (including namely those containing the *Ginkgo* leaf extract, EGb 761) and several other pharmaceutically important compounds (Van Beek, 2000, 2002; Diamond & Bailey, 2013). The concentration of such compounds used to be increased in polyploids (Dhawan & Lavania, 1996), suggesting that polyploid *Ginkgo* may warrant breeding for commercial purposes. However, standard methods of inducing

polyploidy do not appear to be effective in *Ginkgo* (Sun *et al.*, 2015), and polyploid *Ginkgo* cells were obtained only from cell cultures (Tulecke, 1953). In light of the present discovery, it may be more reasonable to screen for the existence of polyploid plants that are already being cultivated in *Ginkgo* nurseries rather than producing such plants artificially. Nevertheless, depending on its vigour and chemical profile, this single *Gingko* sapling may encourage the production and wider cultivation of tetraploid *Ginkgo* plants over the next few years.

Methods

Stomatal sizes (guard cell lengths) were measured in several mature leaves of the tetraploid and two of its diploid siblings all cultivated in the field conditions in the experimental garden of the Department of Botany, Masaryk University in Brno-Bohunice, Czech Republic and in two mature diploid *Ginkgo biloba* trees grown in the Botanical Garden of the Faculty of Science, Masaryk University in Brno-Veveří, Czech Republic. Stomata were observed on epidermal peels prepared by boiling leaves in concentrated nitric acid, using an Olympus BX-51 microscope under ×200 magnification (Fig. 1d). Digitally documented slides were analysed manually with the Olympus CELL^F program.

Measurements of genome size were done by flow cytometry with propidium iodide dye and the internal genome size standard, *Haemanthus albiflos* (2*C* = 57.842 Gbp; Fig. 1b; Veselý *et al.*, 2012), using the same instruments and procedure as used in Šmarda *et al.* (2014). For improving the signal/background ratio the original OTTO I solution was mixed 1 : 1 with 0.1 M hydrochloric acid and supplied with two drops of Tween.

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Author contributions

P.Š. managed the research and wrote the paper, P.V. cultivated plants and observed the stomatal size, J.Š. carried out flow cytometry measurements, P.B. critically reviewed the draft of the manuscript, O.K. took part in cultivation and sampling, M.C. provided *Ginkgo* saplings and commented on an earlier draft of the manuscript.

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