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Small genomes and large seeds: chromosome numbers, genome size and seed mass in diploid *Aesculus* species (Sapindaceae)

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• **Background and Aims** *Aesculus* L. (horse chestnut, buckeye) is a genus of 12–19 extant woody species native to the temperate Northern Hemisphere. This genus is known for unusually large seeds among angiosperms. While chromosome counts are available for many *Aesculus* species, only one has had its genome size measured. The aim of this study is to provide more genome size data and analyse the relationship between genome size and seed mass in this genus.
• **Methods** Chromosome numbers in root tip cuttings were confirmed for four species and reported for the first time for three additional species. Flow cytometric measurements of 2C nuclear DNA values were conducted on eight species, and mean seed mass values were estimated for the same taxa.
• **Key Results** The same chromosome number, $2n = 40$, was determined in all investigated taxa. Original measurements of 2C values for seven *Aesculus* species (eight taxa), added to just one reliable datum for *A. hippocastanum*, confirmed the notion that the genome size in this genus with relatively large seeds is surprisingly low, ranging from 0·955 pg $2C^{-1}$ in *A. parviflora* to 1·275 pg $2C^{-1}$ in *A. glabra* var. *glabra*.
• **Conclusions** The chromosome number of $2n = 40$ seems to be conclusively the universal $2n$ number for non-hybrid species in this genus. *Aesculus* genome sizes are relatively small, not only within its own family, Sapindaceae, but also within woody angiosperms. The genome sizes seem to be distinct and non-overlapping among the four major *Aesculus* clades. These results provide an extra support for the most recent reconstruction of *Aesculus* phylogeny. The correlation between the 2C values and seed masses in examined *Aesculus* species is slightly negative and not significant. However, when the four major clades are treated separately, there is consistent positive association between larger genome size and larger seed mass within individual lineages.

Key words: *Aesculus*, chromosome number, genome size, phylogeny, seed mass.

INTRODUCTION

The genus *Aesculus* L. comprises woody plants, all native to the temperate Northern Hemisphere (Harris *et al*., 2009). *Aesculus* is known to be widespread in Tertiary forests that linked Europe, Asia and North America, and contemporary species are considered to be relics of these ancestral populations (Hardin, 1960; Danoghue and Smith, 2004). The number of recognized species ranges from 12 to 19, depending on acceptance of seven eastern Asian species, all putative constituents of section *Calothyrsus* (Xiang *et al*., 1998; Turland and Xia, 2005; Harris *et al*., 2009). Traditional assignment of species to five sections was exclusively based on morphology (Hardin, 1957a, *b*, 1960). Later, proposed *Aesculus* phylogenies were based either on both molecular and morphological data (Xiang *et al*., 1998) or exclusively on morphology (Forest *et al*., 2001). Recently, a robust phylogeny of *Aesculus* constructed by Harris *et al*. (2009) on the basis of extensive data on morphology, DNA sequences and fossils revealed four major clades, namely (1) an Asian clade, i.e. sect. *Calothyrsus* excluding *A. californica* (Spach) Nutt.; (2) sect. *Macrothyrsus* + *A. californica*; (3) sect. *Pavia* + sect. *Parryana*; and (4) sect. *Aesculus* (Table 1).

The chromosome number of $2n = 40$, published so far for nine non-hybrid *Aesculus* species, corresponds to the diploid level (Fedorov, 1969; IPCN Chromosome Reports Database, 2015; Table 2). The triploid ($2n = 60$) and tetraploid ($2n = 80$) chromosome numbers (Fedorov, 1969) refer exclusively to taxa which arose from interspecific hybridization, namely to *A.* ×*carnea*, *A.* ×*plantierensis* and *A.* ×*rubicunda*. Chromosomes in *Aesculus* are small sized, as inferred from depicted metaphase chromosomes in *A. hippocastanum* L. (Pogan *et al*., 1980); the chromosome length in this species ranges approximately from 1 µm to 2 µm. Nevertheless, certain differences in chromosome size among species may exist, as, for example, between reportedly larger chromosomes of *A. pavia* L. compared with chromosomes of *A. hippocastanum* (Skovsted, 1929). Accordingly, differently sized parental chromosomes of *A. pavia* and *A. hippocastanum* were allegedly also detectable in their hybrid *A.* ×*carnea* (Skovsted, 1929); however, this finding was later questioned (Upcott, 1936). So far, the nuclear genome size has only been reported for *A. hippocastanum* (Bennett *et al*., 1982; Hanson *et al*., 2002 in Bennett and Leitch, 2005; Table 3). Using Feulgen densitometry, the first analysis of nuclear DNA content measured 0·250 pg $2C^{-1}$ in this species (Bennett *et al*., 1982). Such an extremely low C-value would

assign *A. hippocastanum* to angiosperms with the smallest genomes, which are represented, for example, by *Arabidopsis thaliana* (Bennett and Leitch, 2005). Later on, using flow cytometry, about a five times higher value of nuclear DNA content was determined in *A. hippocastanum*, namely, 1·200 pg $2C^{-1}$ (Hanson *et al*., 2002 in Bennett and Leitch, 2005). Evidently, this value, although still indicating a rather small nuclear DNA amount in *Aesculus*, is considered to be more likely correct than that published previously (Bennett and Leitch, 2012).

Aesculus consists of perennial trees or shrubs reproducing by rather robust seeds (horse chestnuts) that are commonly eaten and dispersed by mammals. In *A. hippocastanum*, seed dormancy is released by storage for several months in moist and cold conditions. The seeds are recalcitrant, which means they are short lived and sensitive to desiccation (Obroucheva and Lityagina, 2007). Seed recalcitrance was also found in *A. parviflora* Walter, and most other *Aesculus* species are expected to possess this attribute (Seed Information Database, 2008). Two starchy cotyledons constitute the main mass of the seed (Beger, 1924; Hardin, 1957*b*; Obroucheva and Lityagina, 2007). What makes the genus *Aesculus* unique is that several of its species possess the highest seed masses not only in the family Sapindaceae and order Sapindales (besides *Xylocarpus granatum*, Meliaceae), but also among most of the angiosperm orders with the exception of Arecales, Ericales, Fabales and Laurales (Linkies *et al*., 2010).

TABLE 1. *Four major clades in* Aesculus *recognized by Harris et al. (2009) and sections described by Hardin (1957a, b, 1960)*

Clada	Taxa involved
1	Asian clade (sect. *Calothyrsus* excl. *A. californica*): *A. assamica*, *A. chinensis*, *A. indica**, *A. polyneura*, *A. tsiangii*, *A. wangii*, *A. wilsonii*
2	Sect. *Macrothyrsus* + *A. californica*: *A. californica**, *A. parviflora**
3	Sect. *Pavia* + sect. *Parryana*: *A. flava*, *A. glabra**, *A. glabra* var. *arguta**, *A. hankensii*, *A. pavia**, *A. parryi**, *A. sylvatica*
4	Sect. *Aesculus*: *A. hippocastanum**, *A. turbinata**

*Taxa included in this study.

TABLE 2. *Origins, sources and chromosome numbers of ten* Aesculus *taxa*

Taxon	Clade	Area of origin	Source	Chromosome number* Reported	Counted
A. californica (Spach) Nutt.	2	California	A	$n = 20$[a]	$2n = 40$
A. glabra Willd. var. *glabra*	3	Midwestern USA	B	$n = 20$[b]	$2n = 40$
A. glabra Willd. var. *arguta* (Buckley) B.L. Rob.	3	Texas to Kansas	C	–	$2n = 40$
A. hippocastanum L.	4	Balkan Peninsula	B	$2n = 40$[c,d,e]	–
A. indica (Camb.) Hook I	1	Nordwest Himalaya	D	–	$2n = 40$
A. indica (Camb.) Hook II	1	Nordwest Himalaya	E	–	$2n = 40$
A. parryi A. Gray	3	Baja California	F	–	$2n = 40$
A. parviflora Walter	2	Southeast USA	B	$n = 20$[b]	$2n = 40$
A. pavia L.	3	Southeast USA	B	$n = 20$[b]	$2n = 40$
A. turbinata Blume	4	Japan	C	–	$2n = 40$

Sources: A, collected in Stebbins Cold Canyon, California; B, Průhonice Park, Czech Republic; C, Cusanelli Seed Company, Beachwood, NJ; D, University of California, Davis Arboretum; E, Quarry Hill Botanical Garden, CA; and F, collected 12 km south of El Rosario, Baja California.

*Reported chromosome numbers in the following references:

[a]Ornduff and Lloyd (1965).

[b]Skovsted (1929).

[c]Dobeš and Vitek (2000).

[d]Měsíček and Javůrková-Jarolímová (1992).

[e]Pogan *et al*. (1980).

TABLE 3. *Genome size and seed mass of ten* Aesculus *taxa*

Taxon	Clade	2C nuclear DNA content (pg ± s.e.)		*n*	Mean seed mass (g ± s.e. or range)
		Reported	Measured		
A. californica (Spach) Nutt.	2	–	1·023 ± 0·004	5	39·7 ± 2·6*
A. glabra Willd. var. *glabra*	3	–	1·275 ± 0·004	5	7·8 (6·7–9·4)[†]
A. glabra Willd. var. *arguta* (Buckley) B.L. Rob.	3	–	1·273±0·010	3	11·4 (9·6–14·1)[†]
A. hippocastanum L.	4	1·20[§]	1·122 ± 0·010	3	15·6 (13·3–19·6)[†,‡]
A. indica (Camb.) Hook I	1	–	1·063 ± 0·001	4	16·7 ± 0·8*
A. indica (Camb.) Hook II	1	–	1·084 ± 0·004	4	59·8 ± 3·9*
A. parryi A. Gray	3	–	1·202 ± 0·004	3	7·2 ± 0·4*
A. parviflora Walter	2	–	0·955 ± 0·006	3	3·8 ± 0·6*
A. pavia L.	3	–	1·253 ± 0·006	5	7·5 ± 0·3*
A. turbinata Blume	4	–	1·092	1	12·4 ± 0·6*

n indicates the number of individuals analysed.

*Mean mass of 30 seeds.

[†]Bonner *et al*. (2008).

[‡]Daws *et al*. (2004).

[§]Hanson *et al*. (2002).

In general, seed mass is an essential factor influencing seed dispersal. Because the nucleotypic effects of the amount of DNA on cell size and volume are additive in multicellular structures and organs, a causal relationship between nuclear genome size and seed mass is expected (Bennett, 1987). A positive correlation between both traits was identified within species and across species from the same genus and family (Bennett, 1987; Dąbrowska, 1992; Grotkopp *et al*., 2004; Knight *et al*., 2005; Knight and Beaulieu, 2008; Kim *et al*., 2015). However, no significant correlation between genome size and seed mass was found in 92 species of the genus *Acacia*, subgenus *Phillodineae* (Gallagher *et al*., 2011), and there is no significant correlation between genome size and seed size in diploid species of palms in the tribe Cocoseae and genus *Syagrus* (Henderson *et al*., 1995; Gunn *et al*., 2015). A broad-scale evaluation of data gathered for large numbers of species across genera, families and orders showed that species with a small 2C DNA content have a wide range of seed masses, while a large 2C DNA content is associated more with large seeds (Beaulieu *et al*., 2007). Considering the seed dispersal pattern, the highest values of genome size and seed mass were found in those plants whose seeds are dispersed via endozoochory (Bai *et al*., 2013).

In this study, we sought to find out whether the potential variation in genome size among *Aesculus* species is related to variation in their seed mass. Determining the chromosome numbers, we verified the ploidy level in eight available *Aesculus* taxa. A visual karyotyping should detect the potential gross interspecific differences in total metaphase chromosome sizes that have formerly been described in *Aesculus* (Skovsted, 1929). Such differences, if verified, may be associated with variation in the nuclear DNA content.

MATERIALS AND METHODS

Plants representing nine *Aesculus* taxa, namely eight species and one additional variety of *A. glabra*, were used in this study (Tables 1 and 2). Except *A. hippocastanum*, where material from three mature trees was used, seedlings obtained from seeds provided material for assessment of chromosome number and nuclear DNA content. The seeds, originating from natural populations, Průhonice park (Czech Republic), botanical gardens or seed companies (Table 2), were obtained in autumn in 2013 and 2014. Seeds were kept for several weeks in a refrigerator at 4 °C, after which they were planted separately in pots with moist garden soil and left in a cellar (temperature range approx. 8 – 12 °C). As soon as the seeds started to germinate (predominantly from January to March), the pots were transferred to daylight at room temperature. Those remaining seeds that did not germinate in a cellar by March were transferred to an unheated greenhouse where some of them eventually started to germinate. Young plants about 40 cm tall were repotted and cultivated in an open bed in the experimental garden at Průhonice. The final number of available individuals was limited by a low germinability of seeds and poor survival of young seedlings (especially in *A. turbinata*). Voucher specimens of all taxa are deposited in the Herbarium of the Institute of Botany, Průhonice, Czech Republic (PRA).

Chromosomes were counted for one to two plants for each of the taxa studied; *A. hippocastanum* was not examined, because its chromosome number has been confirmed several times in the literature (Table 2). Root tip cuttings of cultivated seedlings were used for chromosome preparations. The material was pretreated with either a saturated solution of α-bromonaphthalene or 0·002 M 8-hydroxyquinoline for 3·5 h at room temperature, fixed overnight in a cold mixture of ethanol and acetic acid (3:1) and then stored in 70 % ethanol. After that, the root tips were hydrolysed in 1 N HCl at 60 °C for 10 min and rinsed in water, and the cut off meristematic tissue was squashed in a drop of lacto-propionic orceine (Dyer, 1963). The chromosomes were counted in at least five metaphases per plant, using both standard and phase contrast illumination (Olympus BX-51 microscope). Nuclear genome sizes were determined via flow cytometry using a Partec CyFlow instrument (Partec GmbH, Görlitz, Germany) equipped with a 532 nm solid state laser as the source of excitation light. The flow cytometric measurements were made for one to five plant individuals per taxon (Table 3). Samples were prepared by the two-step protocol described by Doležel *et al*. (2007). *Solanum pseudocapsicum* (1C = 1·295 pg; Temsch *et al*., 2010) was selected as an appropriate reference standard for analyses, and propidium iodide was used as the fluorescent dye. Young intact leaf tissue of an *Aesculus* sample and a proportional piece of the internal reference standard were processed together in each analysis (for details of the procedure, see Suda *et al*. 2010). At least three flow cytometric analyses performed on different days were conducted to avoid day to day fluctuations and used for calculation of the genome size values for included taxa. Weighing of 30 freshly mature seeds was performed for most of the species (Table 3), while the published data on seed mass for *A. glabra*, *A. glabra* var. *arguta* and *A. hippocastanum* were taken from Bonner *et al*. (2008). Statistical analyses were performed in R version 3.2.3 (R Core Team, 2015).

RESULTS

The same chromosome number, $2n = 40$, was determined in all investigated taxa (Table 2). Chromosome numbers for *A. indica*, *A. parryi* and *A. turbinata* are reported here for the first time. Chromosomes in all species are small, approx. 1 – 2 µm long (Fig. 1). Comparing the metaphase chromosome sets among taxa examined, we did not find any conspicuous differences in chromosome size.

The genome size was measured for the first time for *A. californica*, *A. glabra* var. *glabra*, *A. glabra* var. *arguta*, *A. indica*, *A. parviflora*, *A. parryi*, *A. pavia* and *A. turbinata* (Table 3). The nuclear DNA content in *Aesculus* ranges from 0·955 pg $2C^{-1}$ in *A. parviflora* to a 2C value of 1·275 pg in *A. glabra* var. *glabra* (Table 3), i.e. genome sizes differ nearly 30 % between species with the smallest and largest genomes within the genus. The pattern of variation in genome size and a post-hoc Tukey HSD test suggest a statistically distinct 2C value in most of the species analysed. Nevertheless, neither the two varieties of *A. glabra*, nor the three taxa with medium genome size (*A. indica* II, *A. turbinata* and *A. hippocastanum*), are statistically distinguishable from each other. The genome sizes seem to be distinct and non-overlapping among the four major *Aesculus*

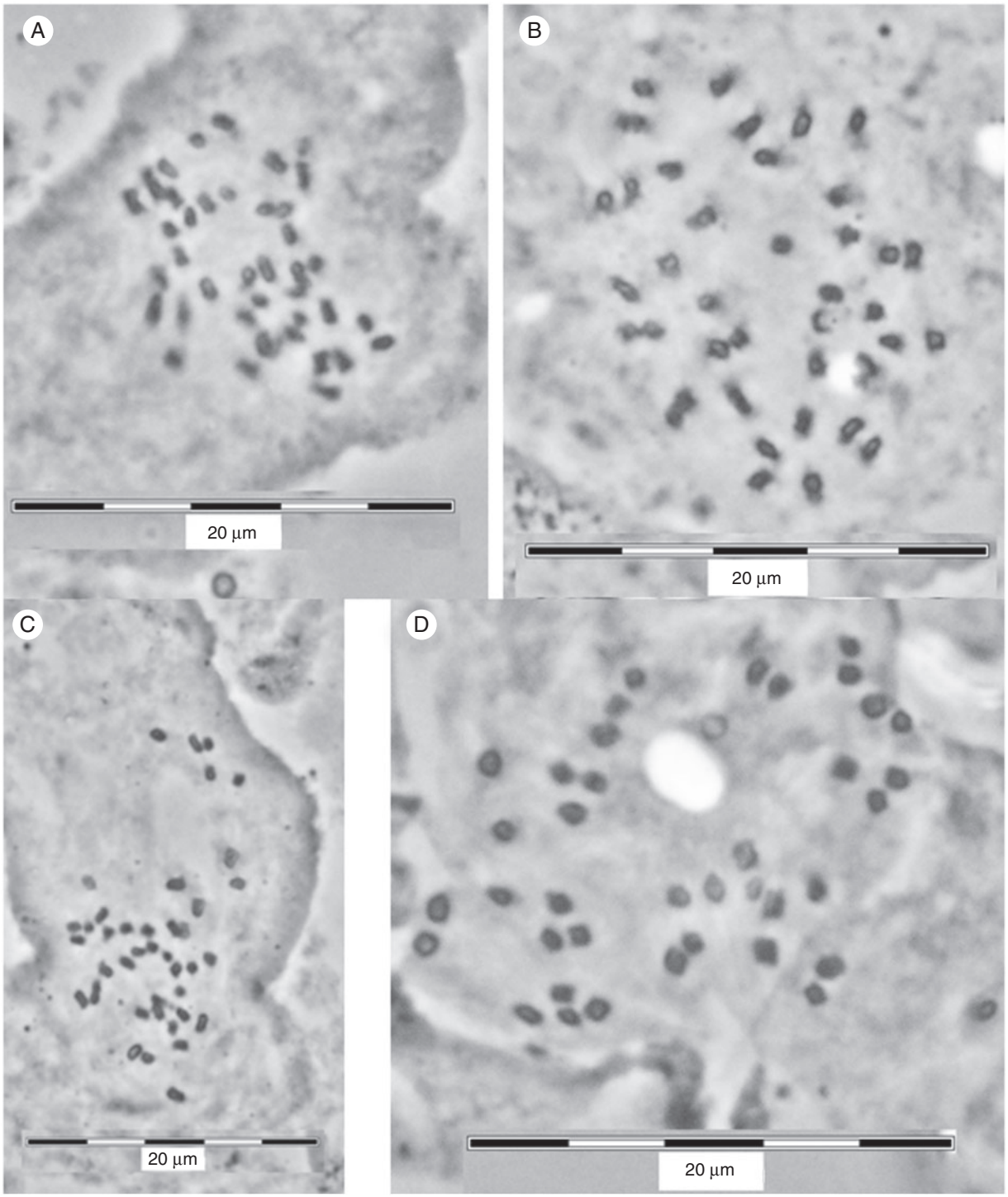

FIG. 1. Representative example of metaphase chromosomes from root tip meristem of *Aesculus* ($2n = 40$ in all four species). (A) *A. indica* II; (B) *A. parryi*; (C) *A. pavia*; (D) *A. turbinata*.

clades (Table 3; Fig. 2A, B). These distinct ranges of 2C values point to evolutionary genome size conservatism within clades. The 2C values increase from clade 2 (*A. parviflora* + *A. californica*), through clade 1 (*A. indica*) and clade 4 (*A. turbinata* + *A. hippocastanum*), to clade 3 (*A. parryi*, *A. pavia*, *A. glabra* and *A. glabra* var. *arguta*) (Fig. 2A, B). Mean seed mass values for seven examined *Aesculus* taxa ranged from 3·8 to 59·8 g (Table 3). Overall, the correlation between the 2C values and seed masses across all examined species (ten taxa) is slightly negative, but not significant ($P = 0{\cdot}35$). However, when the four major clades are treated separately, there is consistent positive association between larger genome size and larger seed

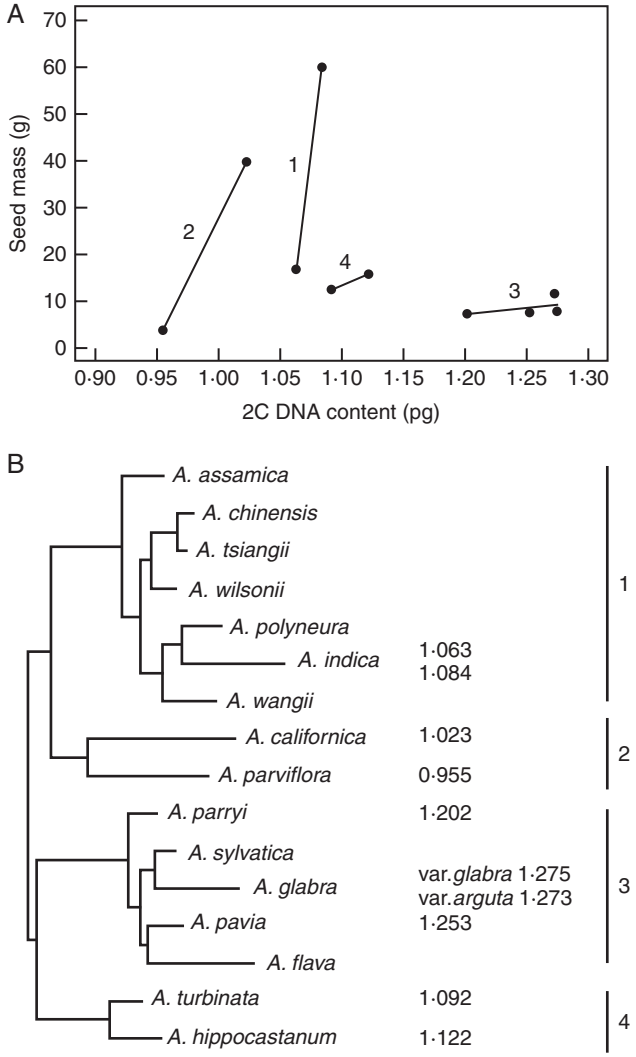

FIG. 2. (A) The relationship between seed mass and 2C DNA content in selected species of the four major lineages of *Aesculus*. 1 (from left to right): *A. indica* I–*A. indica* II; 2: *A. parviflora*–*A. californica*; 3: *A. parryi*–*A. pavia*–*A. glabra* var. *arguta*–*A. glabra* var. *glabra*; 4: *A. turbinata*–*A. hippocastanum*. (B) Phylogram of *Aesculus* based on analysis of DNA and morphology (modified, adapted from Harris *et al.*, 2009, p. 17, fig. 4A) supplemented by genome size data (pg DNA $2C^{-1}$, column in the middle). Numbering of lines (right), that delimit the four major lineages of *Aesculus*, corresponds to (A).

mass within individual clades (Fig. 2A). The sample size is too small (even after including an intraspecific relation in *A. indica*) to perform a meaningful sign test.

DISCUSSION

The chromosome number of $2n = 40$, confirmed here for four *Aesculus* species previously reported in the literature, known for five other species in the literature and reported for the first time here for three additional species (four taxa) (Table 2), seems to be conclusively the universal $2n$ number for all non-hybrid species in this genus. Interestingly, $2n = 40$ is a unique number in Sapindaceae and very rare in the order Sapindales (*Lannea* and *Mangifera* in Anacardiaceae may be the only exceptions) (Fedorov, 1969).

Original measurements of 2C values for seven *Aesculus* species (eight taxa), added to the single reliable datum for *A. hippocastanum*, confirmed the notion that the genome size in this genus with relatively large seeds is surprisingly low (0·955–1·275 pg $2C^{-1}$, compared with mean and median 2C values of angiosperms which are 11·8 and 5·0 pg, respectively; Suda *et al.*, 2015). According to the classification of DNA amounts among embryophytes by Leitch *et al.* (2005), the genome sizes in *Aesculus* species belong to the category of 'very small' ($\leq$2·8 pg $2C^{-1}$) genomes. Mean seed mass values for seven *Aesculus* species were in agreement with published data, insofar as data for studied species were available (Bonner *et al.*, 2008; Seed Information Database, 2008). The only notable exception was *A. parviflora* (mean seed mass 3·8 g; Table 3), for which the published data were higher by 2–6 g (table 5·6 in Chanon, 2005). Two extremely different seed mass values for accessions I and II of *A. indica* may represent two 'unofficial' varieties *lakut handun* (small seeded) and *budh handun* (large seeded) found in the Kashmir valley (Rafiq *et al.*, 2015).

Small genome sizes in *Aesculus* are not unusual among angiosperm woody species with large seeds. The reported 2C value for *Mangifera indica* is 1·8 pg (Bennett and Leitch, 2005). On the other hand, the 2C value for *Persea americana*, which has seed mass values comparable with some large-seeded *Aesculus* species, is somewhat larger (4·6 pg; Bennett and Leitch, 2012). Taking only temperate woody angiosperms into account, where mean and median 2C values are 2·24 and 1·6 pg, respectively (Ohri, 2005), *Aesculus* genome size is still rather small. Also, it is small within its own family, Sapindaceae (mean 2C value = 2·71 ± 0·37 pg, median = 2·02 pg, $n = 62$; Bennett and Leitch, 2012; Coulleri *et al.*, 2014).

A positive association between larger genome size and larger seed mass within individual clades (all slopes in Fig. 2A are positive) is consistent with other studies that found a positive correlation between genome size and seed mass (Bennett, 1987; Dąbrowska, 1992; Grotkopp *et al.*, 2004; Knight *et al.*, 2005; Knight and Beaulieu, 2008; Kim *et al.*, 2015). Slight differences in genome sizes between two *A. indica* accessions (I and II) are also positively associated with differences in their seed mass values (Table 3; Figs 2A and 3). Even if this is only one comparison, it is consistent with positive intraspecific correlations between genome size and seed mass in some other species (Caceres *et al.*, 1998; Chung *et al.*, 1998; Benor *et al.*, 2011; Aliyu, 2014). The results presented here support the generalization made by Beaulieu *et al.* (2007): 'Large seed masses have evolved in species with both small and large genomes, but large genome species rarely have small seed sizes … genome size may set a minimum seed mass, that increases with increasing genome size, but the maximum seed mass for any given genome size may be determined by other factors.' The range of seed masses in *A. indica* (Figs 2A and 3) is an excellent illustration of this last point. Large seeds in *Aesculus* may have several adaptive advantages evolved over relatively short evolutionary times (Leishman and Westoby, 1994; Mendoza and Dirzo, 2009).

Based on species used in this study, there is a clear division among the four major *Aesculus* clades in 2C DNA content (Fig. 2A, B). Clade 3 (sections *Pavia* and *Parryana*), with the largest

Fig. 3. Two fruit/seed morphotypes, I (UCD Arboretum) - upper row and II (Quarry Hill Botanical Garden) - lower row, of *Aesculus indica*.

genomes in the genus, seems to be isolated from other clades. Clade 2 (*A. californica* + *A. parviflora*) exhibits the lowest 2C values, and the two remaining major clades (Asian clade and sect. *Aesculus*) are situated in the middle. These results provide an extra support for the most recent reconstruction of *Aesculus* phylogeny (Harris *et al*., 2009; Fig. 2B). In spite of the fact that our sample size is too small for any statistical evaluations, a positive trend of an increase of seed mass and 2C values in individual lineages (Fig. 2A) is in agreement with many generalizations that have been proposed. Unfortunately, the exact mechanism of this relationship is still not known (Beaulieu *et al*., 2007). Finally, invasiveness (spread in areas of introduction) of seed plants is often associated with small genome sizes, similar to those we are finding in *Aesculus* (Kubešová *et al*., 2010; Pandit *et al*., 2014). Several *Aesculus* species have been introduced as ornamentals many times into many areas where they had not been native. However, there is not one *Aesculus* species among >750 invasive tree and shrub species currently known globally (Rejmánek and Richardson, 2013). This may be in agreement with the fact that small genome sizes are more reliable predictors of invasiveness in some gymnosperms and herbaceous angiosperms than in woody angiosperms (Grotkopp *et al*., 2004; Chen *et al*., 2010). Surprisingly, studies of *Aesculus* seed dispersal are rather rare (Thompson and Thompson, 1980; Hoshizaki *et al*., 1999; Irie and Tsuyuzaki, 2001) and focused mostly on the Japanese species *A. turbinata*. Lack of efficient dispersal mechanisms for relatively large seeds is probably the major reason for limited spread of introduced *Aesculus* species. Therefore, *Aesculus* species seem to be relatively safe, inconsequential introductions. Nevertheless, dispersal by vertebrates and by water remains a possibility in some areas.

Including both the data published earlier and those presented here, the uniform chromosome number of $2n = 40$ is currently known for 13 extant recently recognized *Aesculus* species. Consequently, at least 70 % of all *Aesculus* species are diploid, considering the maximum total number of 19 non-hybrid species recognized at present. In spite of invariable chromosome number detected within the genus, the nuclear DNA content varies among the eight examined species, ranging up to a 30 % difference between the species with the smallest and largest genome. The 2C values seem to be distinct and non-overlapping among the major *Aesculus* clades, supporting the most recent reconstruction of *Aesculus* phylogeny (Harris *et al*., 2009). Irrespective of detected variation, the genome sizes in *Aesculus* species rank among very small genomes that are, however, not exceptional among woody angiosperms. Although *Aesculus* is in general a large-seeded genus, there is a substantial variation in seed mass among species. The correlation between 2C values and seed masses in examined *Aesculus* species is slightly negative and not significant. If the four major clades are treated separately, a consistent positive association between larger genome size and larger seed mass is suggested within individual lineages. Although the small genome sizes may suggest that the introduced *Aesculus* species should be invasive, the relatively large seeds are probably the main reason for the lack of efficient dispersal mechanisms.

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