

## COMPARATIVE ANALYSIS OF MICROSPORE SIZE VARIABILITY IN THE GENUS *AESCULUS* (HIPPOCASTANACEAE)

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**Abstract** — Pollen size varies extensively among angiosperm species and partially reflects evolutionary adaptation of each species to the pollination and fertilization environment. Size of uninuclear microspores in *Aesculus parviflora* was analyzed and compared with the size of microspores in *Aesculus hippocastanum*, *Aesculus carnea*, and *Aesculus flava*. The microspores came from closed flower buds of different size (3, 4, and 5 mm) isolated from lower (female flowers), middle (bisexual flowers), and upper (male flowers) segments of inflorescences. *Aesculus parviflora* had smaller microspores than *Aesculus carnea* and *Aesculus flava*, but larger microspores than *Aesculus hippocastanum*. All analyzed microspores showed bimodal distribution in all investigated species of the genus *Aesculus*.

**Key words:** *Aesculus*, Hippocastanaceae, pollen dimorphism, microspore diameter

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### INTRODUCTION

Pollen size varies extensively among angiosperm species. This variation partially reflects evolutionary adaptation of each species to the pollination and fertilization environment. Thus, the competitive advantages of large pollen may be outweighed by the numerous advantages of small pollen, given the specific reproductive environment of the species (Sarkissian and Harder, 2001). Pollen dimorphism has been detected in anthers of some woody species, including *Aesculus hippocastanum* (Radojević 1989, 1991; Čalić et al., 2003-2004), *Aesculus carnea* (Marinković and Radojević, 1992), and *Aesculus flava* (Čalić-Dragosavac et al., 2008). Also, scanning and transmission electron microscopy of the genus *Aesculus* confirmed significant differences in pollen size and shape (Pozhidaev, 1995). In statistics, a bimodal distribution is a continuous probability distribution with two different modes. These appear as distinct peaks (local maxima) in the probability density function.

The buckeye genus *Aesculus* is one of ornamental trees, notably the horse chestnut (*Aesculus*

*hippocastanum*), which is grown for its winter buds, large leaves, and striking inflorescence. The genus *Aesculus* has 13-19 species and is one of the most remarkable examples of intercontinental disjunction of plants in the Northern Hemisphere. *Aesculus hippocastanum* (horse chestnut) can rarely be found in woods as a cultured species, although it grows under different ecological conditions. Because of its beautiful and dense crown, horse chestnut is frequently planted either as a solitary tree or in avenues. *Aesculus carnea* is an artificial hybrid of *A. hippocastanum* L. and *A. pavia* L.; because of its beautiful red inflorescence, it is widely used as an ornamental tree. *Aesculus* species have different medicinal or cosmetic uses. The bark of the horse chestnut contains small amounts of gallic and tannic acids, which are used in industry.

Bottlebrush buckeye (*Aesculus parviflora* Walt.) is one of the most beautiful flowering shrubs in North America. *Aesculus parviflora* flowers, as well as the flowers of *Aesculus hippocastanum*, *Aesculus carnea*, and *Aesculus flava*, located in the basal part of panicle are female and fertile, while flowers in

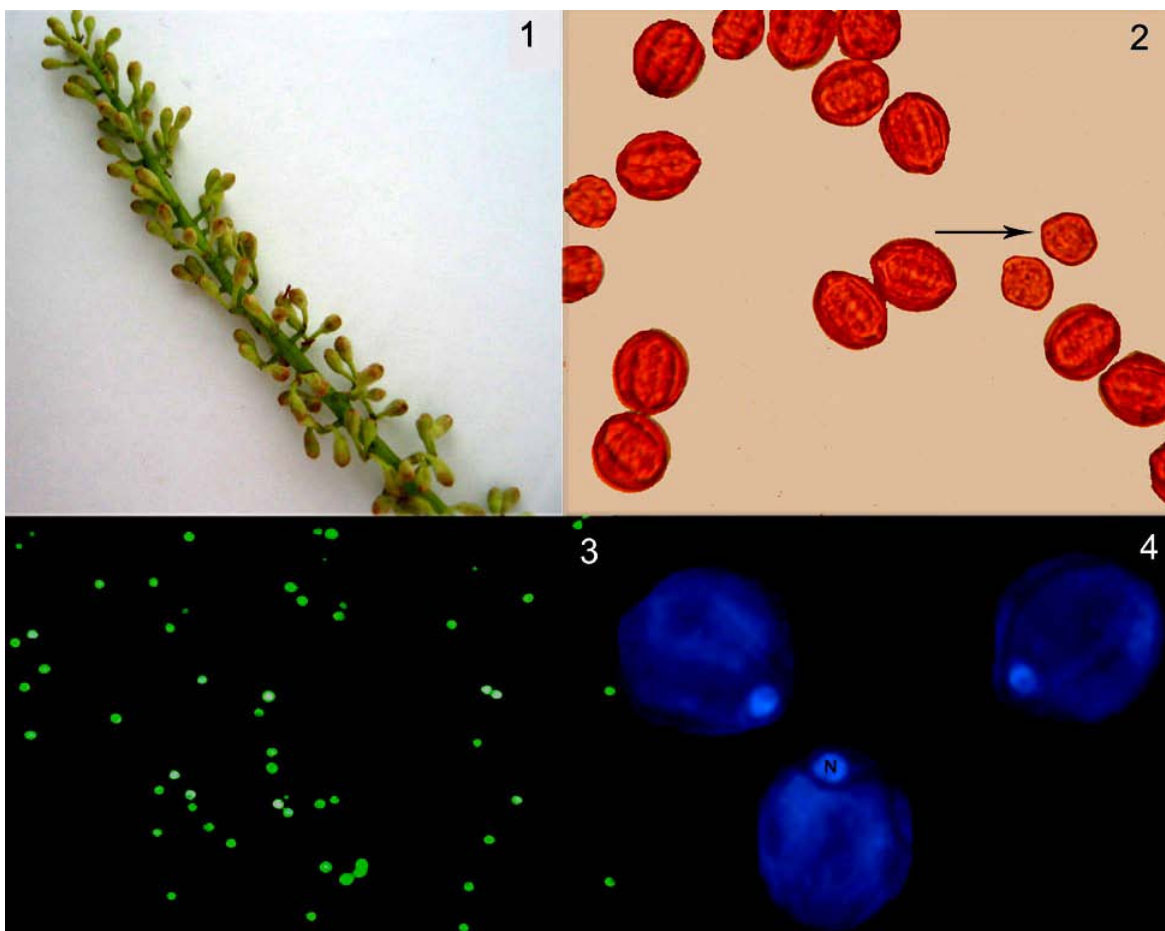
the middle are bisexual and those on top are male (Heywood, 1978). As for inflorescences, we can define three different segments independent of flower sexuality. Female flowers (segment A) have pistils and stamens, but if the anthers do not open stamens will peak prematurely. On the other hand, bisexual flowers (segment B) have normally developed and functional pistils and stamens. Male flowers (segment C) have undeveloped pistils and never form fruits (Heywood, 1978).

#### MATERIALS AND METHODS

The influence of the position of closed flower buds in relation to the axis of the inflorescence on microspore size in four species of *Aesculus* is examined

in this paper. Anthers were collected from elite *A. parviflora*, *A. hippocastanum*, and *A. flava* trees, growing in the Botanical Garden of Belgrade, as well as from an *A. carnea* Hayne tree growing in a local park. The flowers of all four species are bisexual and zygomorphic. Uninuclear microspores were isolated from closed flower buds of different size (3, 4, and 5 mm) originating from inflorescence segments A, B, and C of *A. parviflora*, *A. hippocastanum*, *A. carnea*, and *A. flava*.

Anthers were longitudinally resected and free microspores were stained with a 1% orcein solution prepared in 45% acetic acid (Fig. 1). Three hundred microspores were analyzed from each closed flower bud. Aceto-orcein-treated microspores were viewed



**Fig. 1.** 1) Inflorescence with closed flower buds of *Aesculus parviflora*. 2) Microspores of *A. parviflora* stained with aceto-orcein (small microspore – arrow; x 40). 3) Microspores of *A. parviflora* stained with fluorescein-diacetate (x 10). 4) DAPI staining uninucleate (N-nucleus) microspores (ultraviolet exciter BP340-380 and Y50 barrier filter; x 100).

with a Leica DMRB microscope (Wetzlar, Germany) and analyzed using the UTHSCSA Image Tool version 3.0 (San Antonio, USA) software program. The results were analyzed using a completely randomized design and tested according to the least significant difference (LSD) test.

A rapid method with fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison, 1970) and 4', 6-diamidino-2-phenylindole (DAPI) (Coleman and Goff, 1985) was used to determine pollen viability, as well as pollen dimorphism in all types of flowers. These reagents were also used to observe cell divisions in the microspores. Fluorescein diacetate (2 mg l<sup>-1</sup>) dissolved in acetone was diluted (1:1) with a 0.5 M sucrose solution. The contents of anthers were squeezed out and stained with a DAPI (1 µg ml<sup>-1</sup>) solution prepared in distilled water and examined as previously described. The FDA and DAPI-treated microspores were viewed with an Olympus BX 51 microscope fitted with an ultraviolet exciter filter of the B 12 or BP 340-380 type for DAPI in combination with a Y 50 barrier filter.

## RESULTS AND DISCUSSION

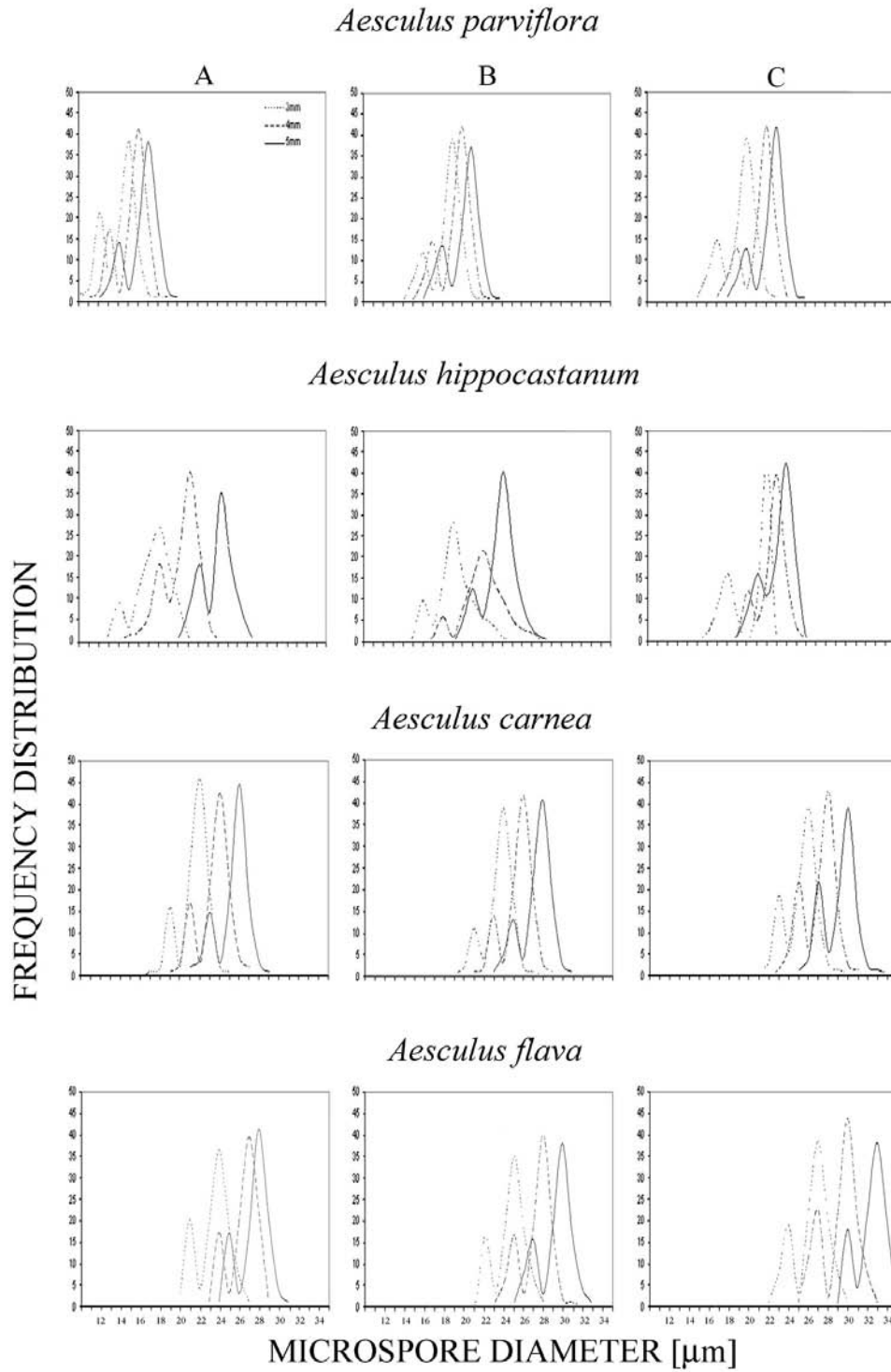
Uninuclear microspores of *A. parviflora*, as well as uninuclear microspores of *Aesculus hippocastanum*, *A. carnea*, and *A. flava*, showed differences in size, shape, staining intensity, fluorescence, and viability after treatment with fluorescein diacetate. Microspores were isolated from closed flower buds measuring 3, 4, and 5 mm in length and different segments of inflorescences (A, B, and C) (Fig. 1, part 1).

Microspores of *A. parviflora* (14.04-18.56 µm) were smaller than microspores of *A. hippocastanum* (16.90-19.29 µm), *A. carnea* (21.85-26.18 µm), and *A. flava* (23.85-27.18 µm) (Table 1). Also, microspores of all studied species can be divided into two groups: small and lightly staining (with acetoorceine and acetocarmine); and large and densely staining (Fig. 1, parts 2 and 3). Small uninuclear microspores (Fig. 1, part 4) were embryogenic, while large ones were nonembryogenic. Variability in microspore size was confirmed by the presence of bimodal distribution (Fig. 2).

**Table 1.** Means values ( $\bar{x}$ ) and standard errors (SE) of microspores size (in µm), were isolated from closed flower buds of different length size (3, 4, and 5 mm) from three segments of inflorescences: A (female flowers), B (bisexual flowers), and C (male flowers).

Species	Length of flower buds (mm)	Diameter of microspores ( $\bar{x} \pm SE$ )(µm)		
		A	B	C
<i>A. parviflora</i>	3	14.04 ± 0.19 <sup>d</sup>	16.09 ± 0.19 <sup>d</sup>	18.58 ± 0.31 <sup>c</sup>
<i>A. hippocastanum</i>		16.90 ± 0.18 <sup>c</sup>	18.98 ± 0.16 <sup>c</sup>	19.29 ± 0.16 <sup>c</sup>
<i>A. carnea</i>		21.85 ± 0.22 <sup>b</sup>	24.05 ± 0.26 <sup>b</sup>	26.18 ± 0.27 <sup>b</sup>
<i>A. flava</i>		23.85 ± 0.23 <sup>a</sup>	25.05 ± 0.28 <sup>a</sup>	27.18 ± 0.29 <sup>a</sup>
<i>A. parviflora</i>	4	18.97 ± 0.17 <sup>d</sup>	20.44 ± 0.24 <sup>d</sup>	20.84 ± 0.22 <sup>d</sup>
<i>A. hippocastanum</i>		20.46 ± 0.20 <sup>c</sup>	22.14 ± 0.17 <sup>c</sup>	22.31 ± 0.16 <sup>c</sup>
<i>A. carnea</i>		23.87 ± 0.21 <sup>b</sup>	26.02 ± 0.23 <sup>b</sup>	28.16 ± 0.20 <sup>b</sup>
<i>A. flava</i>		26.87 ± 0.22 <sup>a</sup>	28.02 ± 0.27 <sup>a</sup>	30.16 ± 0.26 <sup>a</sup>
<i>A. parviflora</i>	5	21.19 ± 0.23 <sup>c</sup>	22.63 ± 0.22 <sup>c</sup>	23.02 ± 0.26 <sup>c</sup>
<i>A. hippocastanum</i>		22.34 ± 0.17 <sup>c</sup>	22.88 ± 0.14 <sup>c</sup>	23.43 ± 0.20 <sup>c</sup>
<i>A. carnea</i>		25.34 ± 0.25 <sup>b</sup>	27.55 ± 0.29 <sup>b</sup>	30.32 ± 0.31 <sup>b</sup>
<i>A. flava</i>		28.34 ± 0.21 <sup>a</sup>	30.25 ± 0.23 <sup>a</sup>	33.32 ± 0.25 <sup>a</sup>

\*The values of different letters are significantly different at the 0.05 probability level according to protected LSD test.



**Fig. 2.** Bimodal distribution of uninuclear microspore size (in 3, 4, and 5 mm closed flower buds) from inflorescence segments A, B, and C of *A. parviflora*, *A. hippocastanum*, *A. carnea*, and *A. flava*.

The 3-mm-long flower buds contained significantly smaller microspores compared to the 4- and 5-mm-long flower buds in all (A-C) segments of the inflorescence. Depending on bud size, the smallest microspores were in segment A (from 23.85 to 28.34  $\mu\text{m}$ ), the largest in the segment C (from 27.18 to 33.32  $\mu\text{m}$ ). In addition, average size of microspores varied depending on bud size and segment, by about 10  $\mu\text{m}$ . The two characteristic peaks had different values for microspores derived from flower buds of the same length, but from different segments.

The presence of variability and bimodal distribution of size in uninuclear microspores of four investigated species of the genus *Aesculus* (Fig. 2) was in correlation with the results of Nageli (1998) and ali et al. (2003-2004). Our findings that uninuclear microspores isolated from closed flower buds showed differences in size, shape, staining intensity, fluorescence, and viability in four species of *Aesculus* are in agreement with results on *Aesculus hippocastanum* (Radojevi, 1989, 1991; Radojevi et al., 2000; ali et al., 2003-2004), *Aesculus carnea* (Marinkovi and Radojevi, 1992), and *Aesculus flava* (ali-Dragosavac et al., 2008). Our results confirm differences of microspore size in 13 species of the genus *Aesculus* (Pozhidaev, 1995).

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**REZIME**

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Ово истраживање је имало за циљ да се анализира присуство мутације у кодону 12 гена K-ras у панкреасном ткиву пацијената са карциномом панкреаса и да се процени да ли ова мутација представља потенцијални молекуларни маркер за карцином панкреаса у српској популацији. Истраживање је обухватило анализу 40 узорака ткива панкреаса пацијената

са клиничком дијагнозом карцинома панкреаса. Присуство мутације у кодону 12 гена K-ras анализирано је методом PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism). Резултати истраживања указују да је мутација у кодону 12 гена K-ras у ткиву панкреаса присутна са високом учесталošћу (66 %) код пацијената са карциномом панкреаса.