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Species boundaries and hybridization in central-European *Nymphaea* species inferred from genome size and morphometric data

Diagnostické znaky a mezidruhová hybridizace středoevropských leknínů, zjištěné na základě cytometrických a morfometrických analýz

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Aquatic plants often pose considerable taxonomic problems. The genus *Nymphaea* (water lily) in central Europe is a good example of this in that their morphological similarity blurs the boundaries between species, which in addition are highly phenotypically plastic and possibly hybridize. The situation is further complicated by the occurrence of many garden cultivars. We used DNA flow cytometry and multivariate morphometrics (both distance-based and geometric) to obtain an insight into their phenotypic variation, identify taxon-specific characters and assess the frequency of hybridization in water lilies collected from 72 localities in the Czech Republic. For comparative purposes, we also included 34 garden cultivars. Flow cytometric measurements revealed a 45% difference in the holoploid genome sizes of *N. alba* and *N. candida*, which makes it easy to reliably separate them. In addition, the great majority of garden cultivars have distinctly smaller genomes than their native counterparts. Interspecific hybridization under natural conditions was quite rare (only ~1.8% of the individuals cytotyped corresponded to *N. ×borealis*), and involved both reduced and unreduced gametes. Discriminant analyses revealed cultivar- and species-specific morphological characters, which allow accurate determination of the samples. Gynoecium and stamen characters had the greatest taxonomic value. The recognition of *N. ×borealis* on the basis of morphological characters is uncertain. Our study shows that genome size may help to resolve the long-standing taxonomic complexities in this important component of the temperate aquatic flora.

Keywords: aquatic plants, Czech Republic, flow cytometry, genome size, interspecific hybridization, multivariate morphometrics, *Nymphaea*, species determination, taxonomy, water lily

Introduction

Due to their high phenotypic plasticity and simplified morphology, many aquatic plants pose considerable taxonomic problems (Schmid 1992). Differences in water depth and chemistry, light intensity and nutrient conditions of sediment can lead to the genesis of distinct morphotypes, which are not genetically determined and change rapidly with change in environmental conditions. Formal recognition of environmentally-induced morphotypes has often resulted in a deluge of evolutionary unjustified and morphologically intergrading taxa (Kaplan 2002). Extensive geographical ranges of many species of aquatic plants (Hultén & Fries 1986) present another challenge as usually only a part of the entire distributional range can be studied. The genetic make-up of populations of aquatic plants may be greatly affected by the discrete and patchy nature of aquatic habitats and the directional transport of propagules in running water (Barrett et al. 1993). In addition, clonal propagation supports the establishment and spread of unique genotypes and/or hybrids, further contributing to the complexity of populations.

Water lilies (*Nymphaea* L.) are among the showiest aquatic plants and have long attracted the attention of botanists, horticulturists and plant enthusiasts. About 50 species are recognized worldwide (Borsch et al. 2007), four of which are native to Europe (Tutin & Webb 1993). While *N. alba* L. and *N. candida* J. Presl are widespread in Europe, *N. tetragona* Georgi only grows in Europe in Finland, Belarus and Russia (Uotila 2009), and *N. lotus* L. is restricted to Romanian and Hungarian hot springs [as the supposedly endemic var. *thermalis* (DC.) Tuzson] (Masters 1974). *Nymphaea alba* occurs throughout most of Europe (except northern Scandinavia) and in northernmost Africa, while *Nymphaea candida* has been reported from central and northern Europe, from where it extends further eastwards (Meusel et al. 1965); its southern distribution remains a moot question (Muntendam et al. 1996, Nowak et al. 2010, Ejankowski & Małysz 2011). Native populations of both *N. alba* and *N. candida* are rapidly declining in many European countries (e.g. Tomšovic 1988, Ejankowski & Małysz 2011).

Despite the low number of indigenous European species, their high morphological polymorphism and plasticity have triggered a continuous dispute concerning the boundaries between the taxa, in particular between the widespread *N. alba* and *N. candida*. A number of species-specific morphological characters are reported, although their usefulness is often questioned. *Nymphaea alba* and *N. candida* should differ in the pattern of their leaf venation, shape of flower base (cup base), shape of the innermost stamens, shape and colour of stigma disc, number of carpellary teeth (also referred to as carpellary appendages), and pollen size and sculpture (Heslop-Harrison 1955, Tomšovic 1988, Muntendam et al. 1996, Wayda 2000, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011). Some differences in habitat requirements are also recorded. While *N. alba* tolerates eutrophic waters, *N. candida* prefers mesotrophic conditions in central Europe (Neuhäusl & Tomšovic 1957, Szańkowski & Kłosowski 1999). The recognition of typical individuals of both species usually presents few problems, but it is the occurrence of transient morphotypes or plants with a mosaic-like combination of characters that challenge the identification of water lilies in Europe.

Morphological similarities at least partly stem from close evolutionary relationships of *N. alba* and *N. candida*. Volkova et al. (2010) show that the latter species is of allopolyploid origin, with *N. alba* and *N. tetragona* as putative parental taxa. This hypothesis, in addition to AFLP fingerprints, cpDNA and ITS sequences, is also supported by data on the size of its nuclear genome. The sum of relative nuclear DNA amounts of *N. alba* and *N. tetragona* fits very well the mean value for *N. candida*. While the authors report significant interspecific differences in genome size (~40% divergence between *N. alba* and *N. candida*), the variation at the intra-specific level is negligible, indicating that the amount of nuclear DNA is a suitable species-specific marker (Loureiro et al. 2010). The most commonly reported numbers of somatic chromosomes for European populations of *N. alba* and *N. candida* are $2n = 84$ and $2n = 112$, indicating hexa- and octoploidy, respectively, based on $x = 14$ (Májovský 1976, Pellicer et al. 2013). Several other chromosome numbers (from $2n = 48$ to $2n = 160$) are reported in the literature (Bolkhovskikh et al. 1969, Goldblatt & Johnson 1979 onwards, Gupta 1980). However, they must be viewed with caution because of frequent misidentifications, different species circumscriptions and/or problems with karyological analyses (Heslop-Harrison 1955).

Individuals with intermediate morphologies are often interpreted as interspecific hybrids (Heslop-Harrison 1955, Ejankowski & Małysz 2011) although their hybrid status is rarely supported by molecular or cytogenetic markers. A few exceptions include crosses between *N. alba* and *N. candida* (= *N.* ×*borealis* Camus) at several sites in Germany and Sweden confirmed by AFLP fingerprinting (Werner & Hellwig 2006), and Indian plants originally determined as "*N. alba* var. *rubra*", which based on chloroplast and ribosomal DNA sequence data are hybrids between *N. alba* and *N. odorata* Aiton (Dkhar et al. 2012). In general, interspecific hybridization in water lilies seems to be quite extensive as indicated by the great number of horticultural crosses (Slocum 2005). The nothotaxon *N.* × *borealis* is reported from different geographic regions where both parental taxa co-occur, including the Czech Republic (Neuhäusl & Tomšovic 1957, Tomšovic 1988), Poland (Ejankowski & Małysz 2011) and Russia (Komarov 1970). In addition to unusual combinations of morphological characters, the authors also mention low pollen fertility and reduced seed set as indicators of hybrid origin (Heslop-Harrison 1955). However, considering the non-trivial recognition of parental taxa and the lack of any clear morphological discontinuities, published records of interspecific hybrids should not be accepted uncritically.

The great popularity of water lilies as ornamental plants also raises other specific issues. Long-term horticultural selection and targeted breeding have resulted in the development of several hundreds of hardy cultivars (Hříbal 1985, Slocum 2005) that are often collectively referred to as *N. hybrida* hort. Although the origin of many of these cultivars, especially the old ones, is uncertain (Conard 1905), white-flowered fragrant *N. odorata* [incl. subsp. *tuberosa* (Paine) Wiersema & Hellq.] and yellow-flowered *N. mexicana* Zucc. are among the extra-European species that were most commonly used for hybridization (Hříbal 1985). Garden cultivars were repeatedly introduced (either accidentally or intentionally) into natural habitats where they can survive for long periods and potentially interact (competition, mating interactions) with native plants. Reliable discrimination between escaped white-flowered cultivars and native species on the basis of morphological traits is difficult, if not impossible, and the occurrence of garden plants makes the study of European water lilies even more difficult.

There have been several attempts in recent years to elucidate the taxonomic composition of *Nymphaea* populations in Europe, by determining the frequency of interspecific hybridization and/or revealing diagnostic morphological characters (Muntendam et al. 1996, Volkova & Shipunov 2007, Ejankowski & Małysz 2011). Although the authors often used sophisticated morphometric approaches, a major limitation to their studies was the lack of any straightforward discriminating marker, resulting in subjective identification of the samples analysed. The findings of Volkova et al. (2010) nonetheless suggest that the amount of nuclear DNA can serve as a species-specific trait, which allows not only *N. alba* and *N. candida* but also their hybrids to be reliably recognized. We therefore built on their study and assessed the variation in genome size in water lilies occurring in the Czech Republic (incl. some garden cultivars) and subjected these cytologically-proven plants to morphometric analysis.

Specifically, we addressed the following questions: (i) What is the variation in the amount of nuclear DNA and can some distinct genome size groups be recognized?

(ii) Does the variation in genome size reflect phenotypic variation in water lilies? Which characters can be considered as species- and/or cultivar-specific? (iii) What is the incidence of interspecific hybrids and which morphological characters do the crosses share?

Material and methods

Plant material

Samples were collected in the Czech Republic during 2009–2013. More than 150 historical localities listed mainly in the Flora Database of the Czech Republic (www.florabase.cz) and identified on the basis of information supplied by local botanists were visited, and the occurrence of water lilies at 72 of them was confirmed (Fig. 1, Electronic Appendix 1). Whenever possible depending on population size and phenology, one mature leaf and one fully developed flower from each of 10 individuals (range 1–56; Electronic Appendix 1) were sampled per locality. The sampling strategy was designed to (i) include the phenotypic variation present at the localities, and (ii) collect putatively different genotypes (i.e. distantly-spaced individuals). In total, 619 *Nymphaea* individuals sampled in situ of both native species and putative garden cultivars were included to this study. This dataset was supplemented by 34 hardy garden cultivars originating from collections of the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice. Herbarium vouchers are deposited in PRC.

Plant samples were kept wet and processed within two days of collecting. Abaxial side of leaves was scanned using an A3 scanner (for large leaves or leaves with overlapping lobes, only one flank of the lamina was scanned). Flowers were dissected and pictures of individual parts (cup base, outer sepal, outer petal, innermost stamen, median section of the gynoecium) were taken, together with an appropriate ruler, under standardized conditions using a Pentax Optio W80 camera (Fig. 2). Before imaging, both sepals and petals were flattened and attached by adhesive transparent tape to a sheet of paper. Because water lilies are protogynous (Wiersema 1988), we noted the phenological stage (using a 5-point scale) of each flower in order to assess potential temporal changes in floral characters. In order to assess pollen viability, samples of pollen from selected populations (of native species, interspecific hybrids and garden cultivars) were stained following the protocol detailed by Peterson et al. (2010) and examined using an Olympus BX-61 light microscope. Stamens from three individuals per population were usually pooled, dissected and 100 pollen grains evaluated.

Flow cytometry

Variation in genome size (2C-values) was estimated using DNA flow cytometry (FCM). Leaf petioles were used for isolating nuclei rather than laminas because they provided histograms of better quality (more uniform fluorescence, lower background signals). Approximately 1 cm of an upper part of the leaf petiole was chopped together with an appropriate volume of the internal reference standard using a sharp razor blade in a Petri-dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto 1990). *Glycine max* (L.) Merr. ‘Polanka’, 2C = 2.50 pg (Doležel et al. 2007) served as the primary reference standard (which has a similar, but not overlapping genome size with that

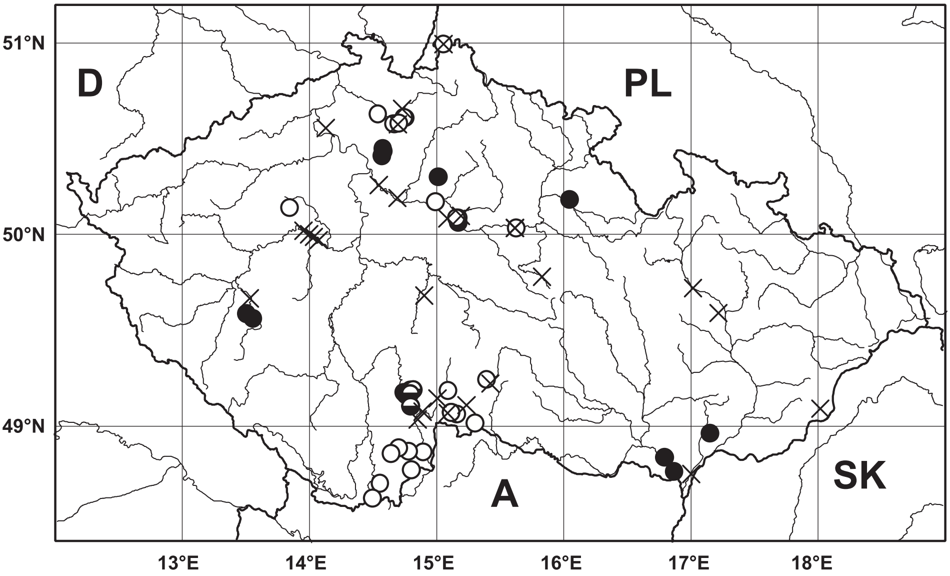


Fig. 1. – Map showing the localities where water lilies were sampled in the Czech Republic. ● *Nymphaea alba*, ○ *N. candida*, ◒ *N.* ×*borealis*, × garden cultivars.

of most samples). Garden cultivars were re-analysed using *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al. 2007) as a standard due to their similarities in genome sizes with that of *Glycine*. The crude suspension was filtered through a 42-μm nylon mesh and incubated for ~15 min at room temperature. Isolated nuclei were stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) supplemented with ß-mercaptoethanol (2 μl/ml), DNA-selective fluorochrome propidium iodide and RNase A, type IIA (both at a final concentration of 50 μg/ml). Shortly after staining, fluorescence intensities of 5000 particles were recorded using a Partec CyFlow instrument (Partec GmbH, Münster, Germany) equipped with a green diode-pumped solid state laser (Cobolt Samba, 532 nm, 100 mW output power). A subset of 147 samples was analysed using DAPI flow cytometry. In this protocol modification, the staining solution consisted of 1 ml of Otto II buffer, ß-mercaptoethanol (2 μl/ml) and AT-selective fluorochrome DAPI (4 μg/ml) and the samples were analysed using a Partec ML flow cytometer equipped with a UV diode chip set as the light excitation source. Histograms were evaluated using FloMax software, ver. 2.4d. To ensure the comparability of results, fluorescence values obtained by DAPI staining were re-calculated to propidium iodide values using a calibration set consisting of 37 individuals from all taxonomic groups that was measured using both fluorescent stains.

Geometric morphometrics

Six parts of plants with potential taxonomic value were subjected to a detailed analysis of the variation in their shape. On each part, several landmarks (with fixed positions) and

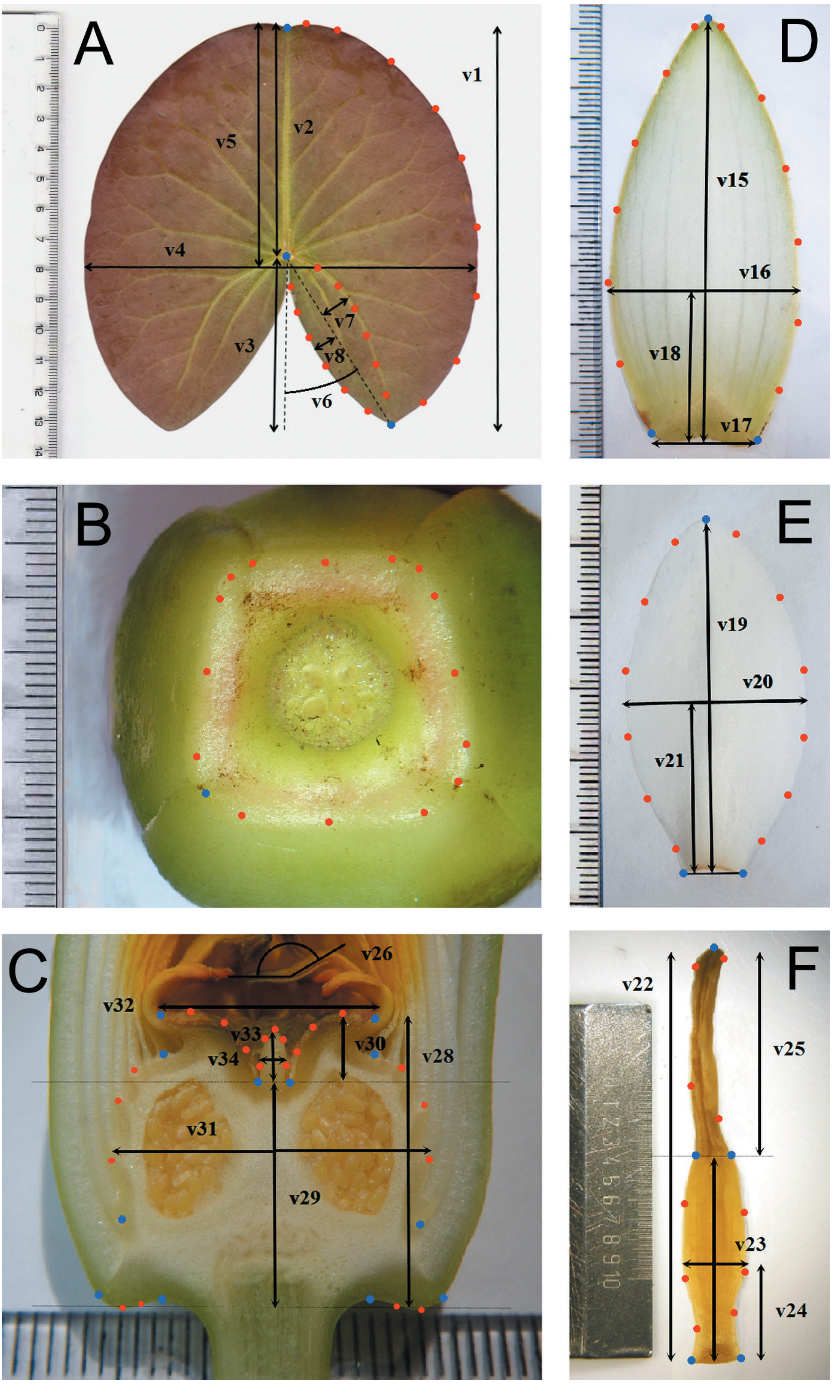


Fig. 2. – Pictures of the vegetative and generative parts used in the morphometric analysis. (A) leaf lamina, (B) cup base, (C) median section of the gynoecium, (D) sepal, (E) petal, (F) stamen. Landmarks in blue, semilandmarks in red. Variables measured in distance-based morphometrics are also shown (see Table 1 for descriptions of variables).

semi-landmarks (allowing the sliding along the abscissa connecting adjacent landmarks) were designated. Specifically, the numbers of landmarks and semi-landmarks designated on individual organs were as follows: right flank of leaf lamina – 3+21, cup base – 1+15, sepal – 3+12, petal – 3+12, innermost stamen – 3+12 and median section of the gynoecium – 12+21 (see Fig. 2). Four taxonomic groups were delimited on the basis of FCM results (i.e. *N. alba*, *N. candida*, interspecific hybrids and garden cultivars) and individual plants were used as operational taxonomic units (OTUs). Because some of the individuals cytotyped were not flowering, the numbers of OTUs for which vegetative and generative characters were analysed differed (Electronic Appendix 2).

The TPS-series software (available at http://life.bio.sunysb.edu/morph) was employed to manage the morphometric data (Neustupa et al. 2010, Viscosi & Cardini 2011). Positions of (semi-)landmarks on each plant organ were digitized in tpsDig 2.16 and the (semi-) landmark configurations were superimposed by generalized Procrustes analysis in tpsRelw 1.49. This procedure standardizes the size of the objects and optimizes their rotation and translation (Bookstein 1991). The resulting dataset was analysed using PAST 2.16 (Hammer et al. 2001). To obtain an insight into the phenetic relations among the OTUs studied, principal component analyses (PCA) were done for each plant organ. Canonical discriminant analyses and classificatory discriminant analyses of relative warp scores (i.e. deviations from the consensus shape) were performed in PAST to test for differences in shape among the a priori defined taxonomic groups and to assess the power of discrimination (i.e. the proportion of correctly classified OTUs), respectively. The deformation grids illustrating differences in shape along the discriminant axes were obtained using tpsRegr 1.38. Because of the low number of interspecific hybrids, this group was omitted from discriminant analyses.

Distance-based morphometrics

In total 68 quantitative, qualitative and ratio characters were measured and scored, including 11 primary leaf characters, 24 primary floral characters, 28 ratios and five colour characters (Table 1). This character set was chosen on the basis of the results of geometric morphometrics, published determination keys, flora handbooks and our own observations. Whenever possible, size variables were calculated from the digitized images using tpsDig 2.16 software.

Data were analysed in SAS 9.3 statistical package (SAS Institute, Cary, NC, USA) following the methodology of Rosenbaumová et al. (2004). Basic statistical measures, including minimum and maximum values, 5% and 95% percentiles were computed (procedure UNIVARIATE) for each character and taxon. Pearson and Spearman correlation coefficients (procedure CORR) were calculated on the pooled data matrix of all samples and on data matrices of each group to assess the relationships among variables and to identify the tightly correlated ones. Potential temporal changes in floral characters were also assessed separately in each species by analysing correlations between a phenological stage and character values. Principal component analysis (PCA) based on correlation matrices (procedure PRINCOMP), canonical discriminant analysis, CDA (procedure CANDISC) and classificatory discriminant analysis (procedure DISCRIM) were performed in order to visualize relationships among the OTUs studied, identify group-specific characters and determine the success in discriminating between OTUs. Because the

Table 1. – List of the quantitative and qualitative morphological characters analysed and corresponding contributions of individual characters to the first (Can1) and second (Can2) canonical axes in the canonical discriminant analysis. Three taxonomic groups (*Nymphaea alba, N. candida*, garden cultivars) represented by 361 individuals were analysed. Five characters with the highest absolute loadings for each axis are presented in bold. Numbers in parentheses are ranks of the strength of the correlation of each variable with the canonical axes. Ten closely correlated characters and qualitative colour characters were not included in the discriminant analysis (marked with asterisk).

No.	Character description	Unit	Can 1	Can2
Leaf characters				
v1	Lamina length	cm	–0.528880 (20)	0.153720 (35)
v2	Midrib length	cm	*	*
v3	Length of leaf notch (v1–v2)	cm	*	*
v4	Lamina width	cm	*	*
v5	Distance of the widest part of leaf lamina from lamina tip	cm	*	*
v6	Angle between lobe axis and vertical axis	degree	0.099186 (48)	0.188051 (31)
v7	Maximum distance between lobe axis and main lobe vein	cm	0.238307 (40)	0.168081 (33)
v8	Maximum distance between lobe axis and lobe margin	cm	–0.035832 (53)	0.494708 (5)
v9	Leaf lobe width (v7+v8)	cm	0.100678 (47)	0.396312 (11)
v10	Shape of lamina tip	1 (sharp) – 5 (round)	–0.171442 (43)	0.453132 (8)
v11	Shape lobe tip	1 (sharp) – 5 (round)	–0.218252 (41)	–0.214640 (26)
Floral characters				
v12	Number of air channels in flower peduncle	number	–0.284177 (34)	–0.200955 (29)
v13	Number of sepals	number	–0.309134 (33)	0.120416 (37)
v14	Number of sepal veins	number	–0.102582 (46)	–0.378203 (15)
v15	Sepal length	cm	–0.399280 (26)	–0.343321 (17)
v16	Sepal width	cm	–0.058814 (50)	**–0.532267 (2)**
v17	Sepal width at the base	cm	0.408490 (25)	0.246117 (23)
v18	Distance of the widest part of sepal from its base	cm	–0.542271 (19)	–0.326932 (19)
v19	Petal length	cm	–0.631755 (12)	–0.391256 (13)
v20	Petal width	cm	–0.493601 (23)	–0.411211 (10)
v21	Distance of the widest part of petal from its base	cm	*	*
v22	Stamen length	mm	0.309901 (32)	–0.256705 (21)
v23	Stamen width	mm	0.696040 (8)	0.239937 (24)
v24	Distance of the widest part of stamen from its base	mm	0.281805 (35)	0.430680 (9)
v25	Anther length	mm	–0.326339 (30)	**0.555285 (1)**
v26	Anther bending	degree	**0.897960 (1)**	–0.063472 (42)
v27	Number of carpels	number	**–0.843275 (2)**	0.055534 (45)
v28	Gynoecium length (height)	cm	–0.387066 (27)	–0.135167 (36)
v29	Ovary length (height)	cm	–0.337941 (29)	–0.062359 (43)
v30	Stigma length (height) (v28–v29)	cm	0.646149 (10)	–0.236619 (25)
v31	Ovary (= gynoecium) width	cm	–0.593565 (15)	0.038417 (47)
v32	Stigma width	cm	**–0.732438 (6)**	0.183488 (32)
v33	Length of stigma projection	mm	–0.198461 (42)	0.246801 (22)
v34	Width of stigma projection	mm	**–0.793353 (3)**	0.113898 (38)
v35	Number of stamens potentially filling the gap at the top of the ovary	number	0.761477 (5)	0.012685 (52)

No.	Character description	Unit	Can 1	Can2
Ratios				
v36	Leaf lamina length/width (v1/v4)		0.360412 (28)	**0.530439 (3)**
v37	Midrib length / leaf notch length (v2/v3)		*	*
v38	Midrib length / leaf lamina length (v2/v1)		–0.278958 (36)	–0.018743 (49)
v39	Distance of the widest part of leaf lamina from lamina tip / lamina length (v5/v1)		–0.248325 (38)	–0.041271 (46)
v40	Distance of the widest part of leaf lamina from lamina tip / midrib length (v5/v2)		0.058079 (51)	0.036213 (48)
v41	Maximum distance between lobe axis and main lobe vein / maximum distance between lobe axis and lobe margin (v7/v8)		0.091194 (49)	**–0.509065 (4)**
v42	Leaf lobe width / lamina length (v9/v1)		0.564681 (16)	0.203880 (27)
v43	Leaf lobe width / length of leaf notch / (v9/v3)		*	*
v44	Distance of the widest part of leaf lamina from lamina tip / leaf lobe width (v5/v9)		*	*
v45	Maximum distance between lobe axis and lobe margin / length of leaf notch (v8/v3)		0.325765 (31)	0.391368 (12)
v46	Maximum distance between lobe axis and main lobe vein / length of leaf notch (v7/v3)		0.619114 (14)	0.016986 (51)
v47	Sepal length / sepal width (v15/v16)		–0.525567 (21)	0.354063 (16)
v48	Distance of the widest part of sepal from its base / sepal length (v18/v15)		–0.495411 (22)	–0.094882 (39)
v49	Sepal width at the base / sepal length (v17/v15)		0.551262 (18)	–0.059793 (44)
v50	Sepal width at the base / sepal width (v17/v16)		0.258575 (37)	**0.493132 (6)**
v51	Sepal length / petal length (v15/v19)		0.558063 (17)	0.078431 (41)
v52	Petal length / petal width (v19/v20)		–0.246054 (39)	0.080753 (40)
v53	Distance of the widest part of petal from its base / petal length (v21/v19)		–0.128796 (44)	–0.201221 (28)
v54	Stamen length / stamen width (v22/v23)		*	*
v55	Stamen length / anther length (v22/v25)		*	*
v56	Gynoecium width / length (v31/v28)		–0.639304 (11)	0.271418 (20)
v57	Ovary width / length (v31/v29)		–0.630910 (13)	0.157981 (34)
v58	Stigma width / length (v32/v30)		–0.470322 (24)	0.380821 (14)
v59	Length/width of central stigma projection (v33/v34)		**0.768744 (4)**	–0.002369 (53)
v60	Width of stigma projection / stigma width (v34/v32)		–0.714614 (7)	–0.018543 (50)
v61	Length of stigma projection / stigma length (v33/v30)		0.056307 (52)	0.476861 (7)
v62	Ovary length / stigma length (v29/v30)		0.124951 (45)	0.195709 (30)
v63	Stigma width / ovary width (v32/v31)		–0.693130 (9)	0.335979 (18)
Qualitative colour characters				
v64	Colour of abaxial side of leaf lamina (0 – green, 1 – reddish, 2 – red)		*	*
v65	Colour of interior surface of sepals (0 – white, 1 – pink tinge, 2 – light pink, 3 – pink)		*	*
v66	Colour of petals (0 – pure white, 1 – pink tinge, 2 – pink)		*	*
v67	Colour of stigma (1 – yellow, 2 – reddish, 3 – red)		*	*
v68	Colour of carpellary teeth (1 – light yellow, 2 – deep yellow, 3 – orange, 4 – reddish, 5 – red)		*	*

distribution within the groups was not multivariate normal, non-parametric k-nearest-neighbour method was employed in the classificatory analysis. The discriminant power was determined by cross-validation. Several modifications of the discriminant analysis (e.g. all taxonomic groups, cultivars vs. native species including hybrids, parental species vs. natural hybrids, *N. alba* vs. *N. candida*) were performed. In addition to a pooled set of all morphological traits, leaf and flower characters were also analysed separately in order to discriminate sterile and fertile individuals, respectively.

Results

Variation in genome size

The FCM analysis of 653 samples resulted in five distinct groups of holoploid genome sizes (Fig. 3). Disregarding two cultivars originating from the water lily collection at Průhonice (‘Firecrest’ and ‘Virginalis’) and one individual from northern Bohemia (loc. 49; Electronic Appendix 1) whose genome sizes overlapped those of *N. alba*, there were

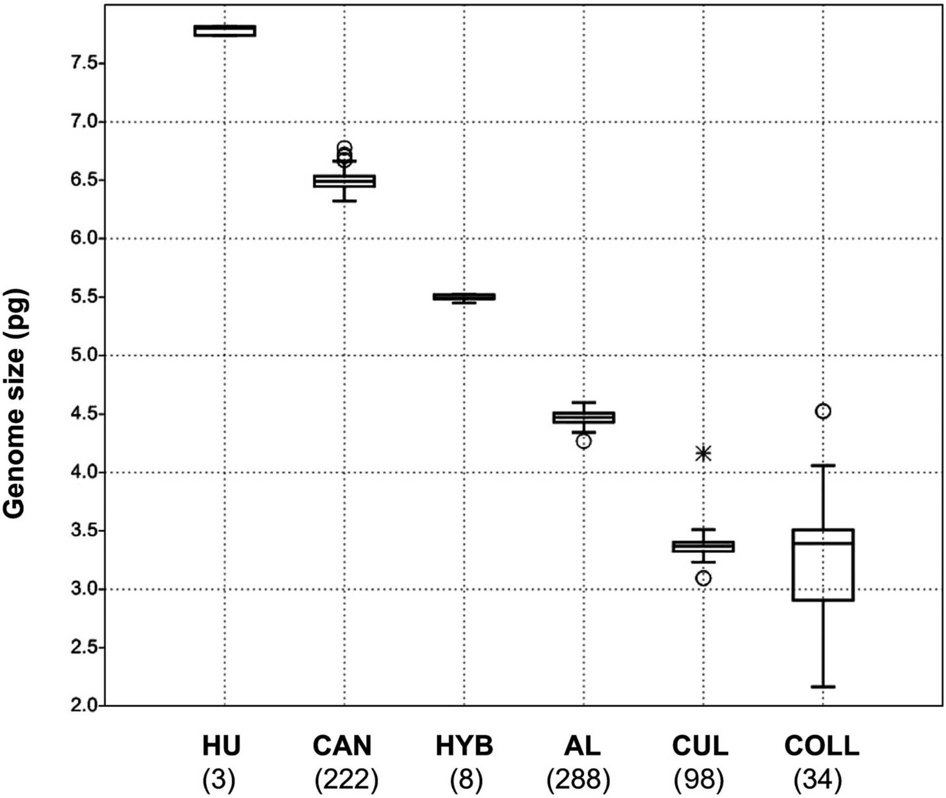


Fig. 3. – Box-and-whisker plots of the variation in 2C-values of six groups of *Nymphaea* samples, corresponding to *N. alba* (AL), *N. candida* (CAN), two types of interspecific hybrids that originated via two reduced gametes (HYB) and unreduced gamete of *N. alba* + reduced gamete of *N. candida* (HU), cultivars from natural habitats (CUL) and cultivars from a garden collection (COLL). Number of individuals analysed is given in parentheses.

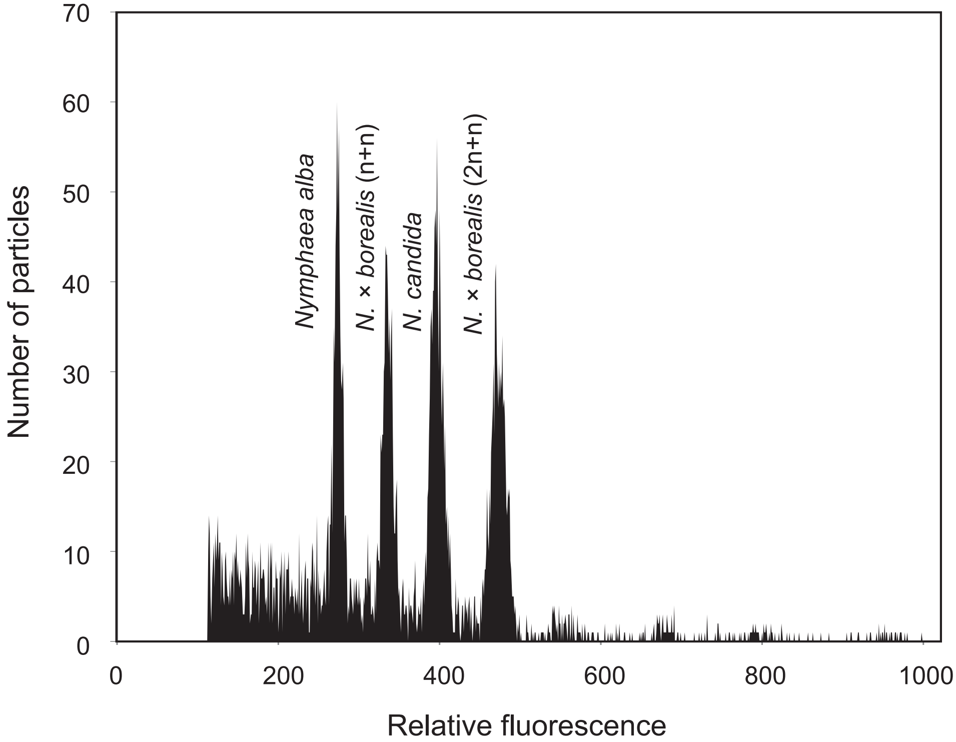
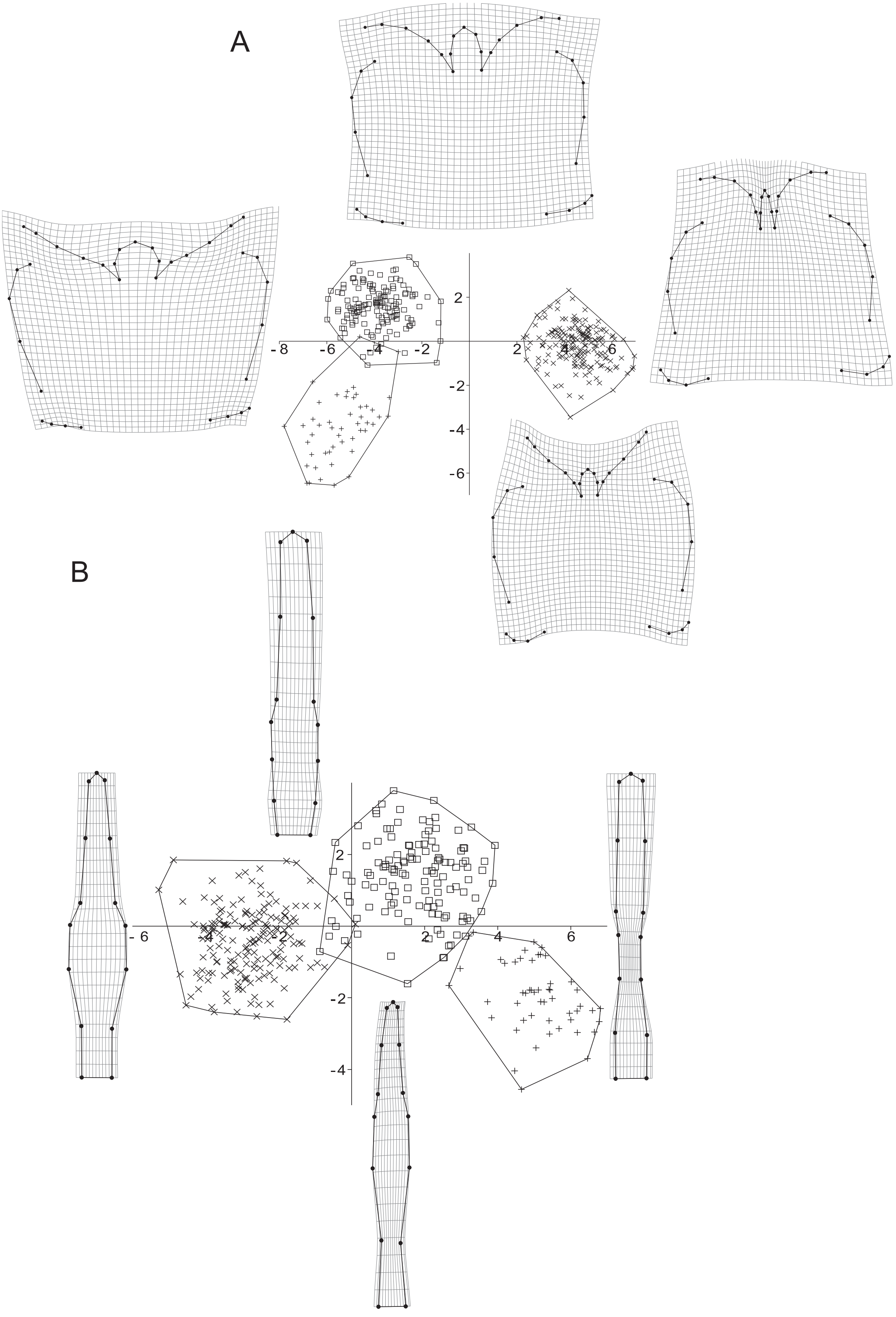


Fig. 4. – Histogram of the results of the flow cytometric fluorescence showing simultaneous analysis of DAPI-stained nuclei isolated from *Nymphaea alba*, *N. candida* and two types of interspecific hybrids (originating via two reduced gametes and 2n gamete of *N. alba* + n gamete of *N. candida*, respectively).

clear discontinuities in nuclear DNA contents. Average 2C-values of samples tentatively determined as *N. alba* (mean 2C = 4.47 pg) and *N. candida* (mean 2C = 6.50 pg) differed 1.45-fold, so both of these species could be reliably separated.

Eight samples from two sites in the Třeboň basin, southern Bohemia (ponds Fejmárek and Pohořelec; Electronic Appendix 1) otherwise occupied by *N. alba* had genome sizes intermediate between *N. alba* and *N. candida* and are classified as F1 interspecific hybrids. The greatest amount of nuclear DNA was recorded in three samples (which possibly represent only one individual) from the Skopaný pond in the same geographical region. These samples are interpreted as interspecific hybrids, originating by a syngamy of an unreduced gamete of *N. alba* and a reduced gamete of *N. candida.* While there was little variation in the 2C-values of both native species of *Nymphaea* studied and their crosses, the genome sizes of garden cultivars varied greatly, ranging from 2.16 pg/2C to 4.53 pg/2C (Fig. 3, Electronic Appendix 1). A simultaneous FCM analysis of both native *Nymphaea* species and two types of interspecific hybrids is shown in Fig. 4.

Of the 72 localities at which the occurrence of water lilies was confirmed, 17 were inhabited by *N. alba*, 25 by *N. candida* and 26 by cultivars. Sympatric growths of *N. alba* + *N.* ×*borealis* and *N. candida* + hardy cultivars were recorded at three and one site, respectively.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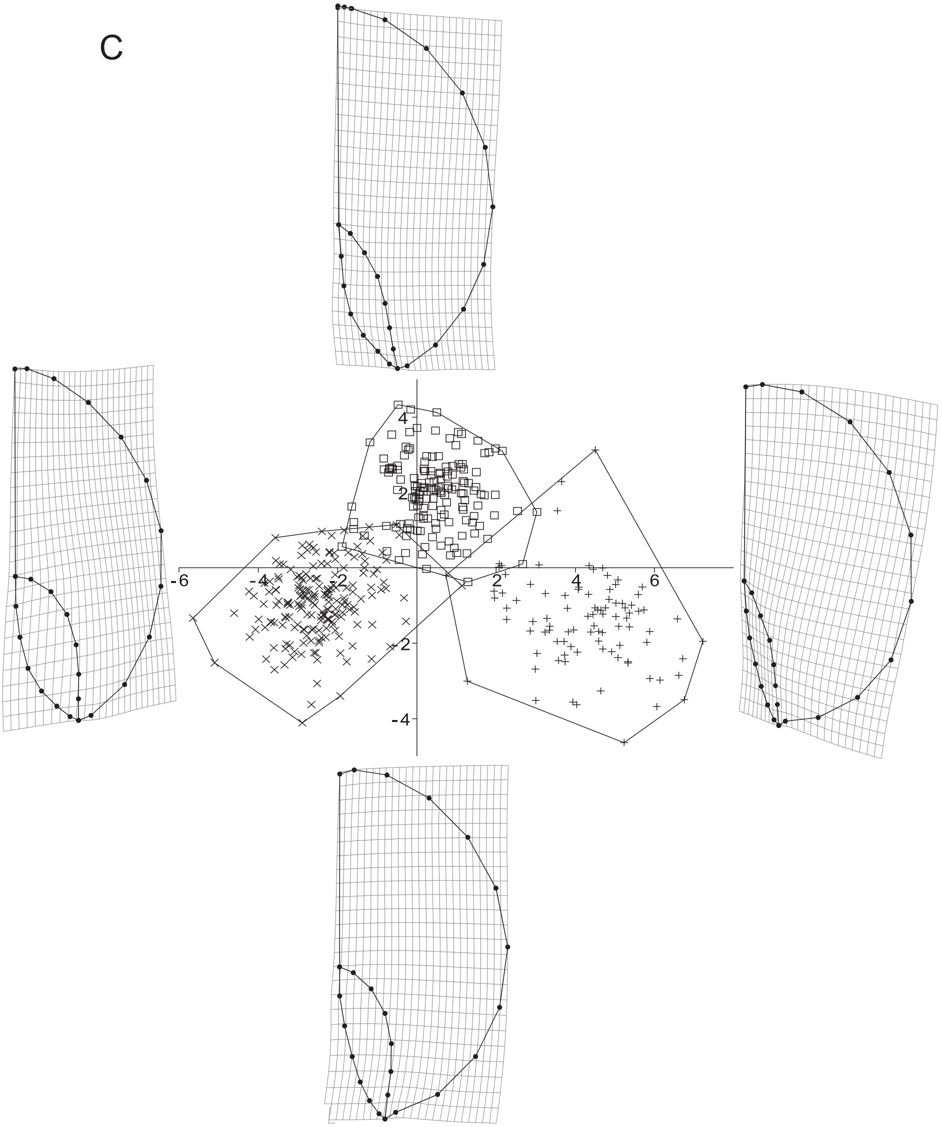

Fig. 5. – Results of the canonical discriminant analysis of the three taxonomic groups corresponding to □ *Nymphaea alba*, × *N. candida* and + garden cultivars using characteristics of the shapes of (A) gynoecium (median section), (B) stamens and (C) leaf lamina (right flank). The thin-plate spline deformation grids illustrate the changes in shape correlated with the canonical axes.

Geometric morphometrics

The most pronounced differences in shape among the three groups of taxa (*N. alba*, *N. candida*, garden cultivars) were detected in the median section of the gynoecium (Fig. 5A) and the shape of inner stamens (Fig. 5B), which allowed correct classification of 99.5%

and 97.5% of individuals, respectively (Table 2). The leaf shape (Fig. 5C) had similar discriminating power (96.6% of individuals correctly classified; Table 2), whereas the shape of sepals and cup base were more similar among the taxa and their application resulted in the misclassification of about 15% of the samples analysed (Electronic Appendix 3). Of the six parts studied the shape of petals had the least taxonomic value (Electronic Appendix 3).

Table 2. – Results of classificatory discriminant analysis of *Nymphaea* samples assigned to the three taxonomic groups (two indigenous species and garden cultivars) using characteristics of the shapes of the gynoecium, stamens and leaves.

		Predicted group membership		
Gynoecium shape (n = 365)	actual group membership	garden cultivars	*N. alba*	*N. candida*
	garden cultivars	46 (95.8%)	2 (4.2%)	0
	N. alba	0	141 (100%)	0
	N. candida	0	0	176 (100%)
Stamen shape (n = 355)	actual group membership	garden cultivars	*N. alba*	*N. candida*
	garden cultivars	49 (96.1%)	2 (3.9%)	0
	N. alba	3 (2.3%)	121 (93.1%)	6 (4.6%)
	N. candida	0	2 (1.1%)	172 (98.9%)
Leaf shape (n = 435)	actual group membership	garden cultivars	*N. alba*	*N. candida*
	garden cultivars	88 (93.6 %)	6 (6.4 %)	0
	N. alba	1 (0.7 %)	149 (98.0%)	2 (1.3%)
	N. candida	0	6 (3.3%)	183 (96.7%)

The phenotype of garden cultivars was usually closer to *N. alba*, which is considered to be one of the parental species (Fig. 5, Electronic Appendix 3). Although the low number of hybrid individuals (10 for leaf characteristics and four for floral characteristics) precluded their inclusion in the discriminant analysis, PCA scatterplots indicated intermediate positions of most characters (Electronic Appendix 4). Average shapes of five taxonomically informative characters for the four groups recognized are illustrated in Fig. 6.

Distance-based morphometrics

Principal component analysis of 365 individuals, including 143 samples of *N. alba*, 169 samples of *N. candida*, four natural interspecific crosses and 49 garden cultivars, revealed three partially overlapping groups of OTUs (Electronic Appendix 5). Garden cultivars formed the most distinct cluster, while natural hybrids overlapped with *N. alba*. The main contributions to the first and the second PCA axes came from gynoecium and leaf characters, respectively (Electronic Appendix 5).

Discriminant analyses were employed to select a set of characters that gave the best separation of taxonomic groups, which were defined a priori on the basis of genome size data, and determine the proportion of correctly classified individuals. In total, ten characters (v2–v5, v21, v37, v43, v44, v54, v55; Table 1) were excluded from the discriminant analyses because of their high correlation (Pearson r > 0.95) with other characters. None of the floral characters was highly correlated with the ontogenetic stage of a flower.

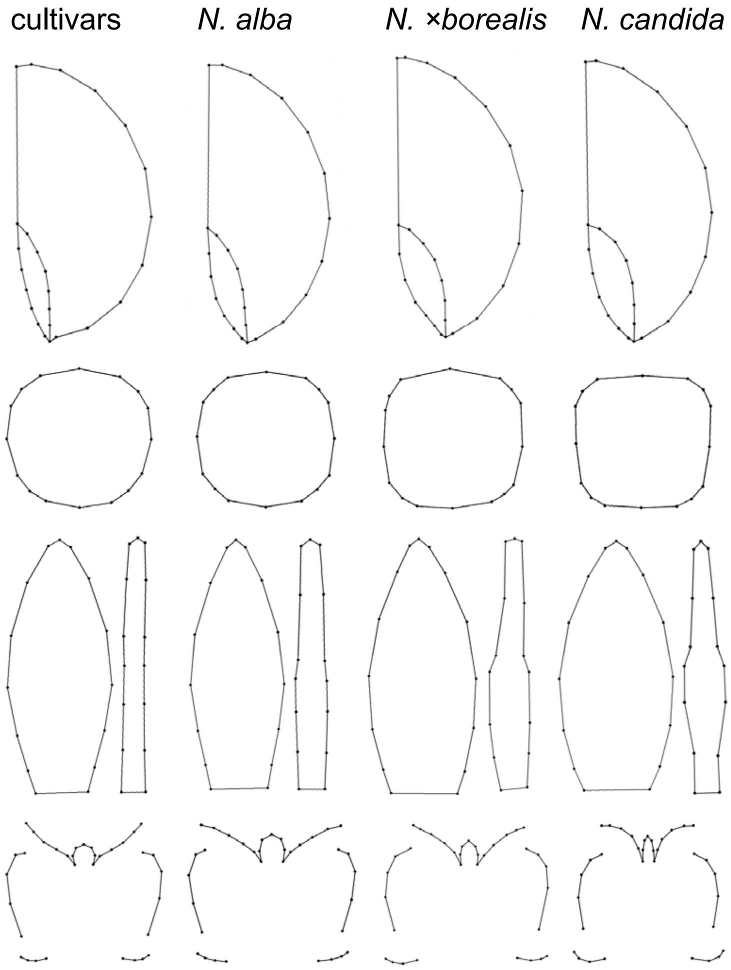

Fig. 6. – Mean shapes of five taxonomically informative characters in the four groups of *Nymphaea* samples recognized. From top: leaf lamina (right flank), cup base, sepal and stamen, median cross-section of gynoecium.

CDA of three groups (interspecific hybrids were excluded due to low number of individuals) using the remaining 54 quantitative and ratios of characters resulted in a complete separation of the groups and 100% of the individuals correctly classified (Fig. 7). Characters most closely correlated with the first canonical axis (separating *N. candida* from the group including *N. alba* and garden cultivars) were the degree of anther bending (v26), number of carpels (v27) and width of the stigma projection (v34), while anther length (v25), sepal width (v16) and leaf length/width (v36) contributed most to the division along the second canonical axis, which separated garden cultivars from *N. alba* (Table 1). The value of characters was further assessed by discriminant analyses of two groups of objects; our aim was to identify a small set of characters with the highest discrimination power and easy to use in determination keys. Major differences between native water lilies (both parental species

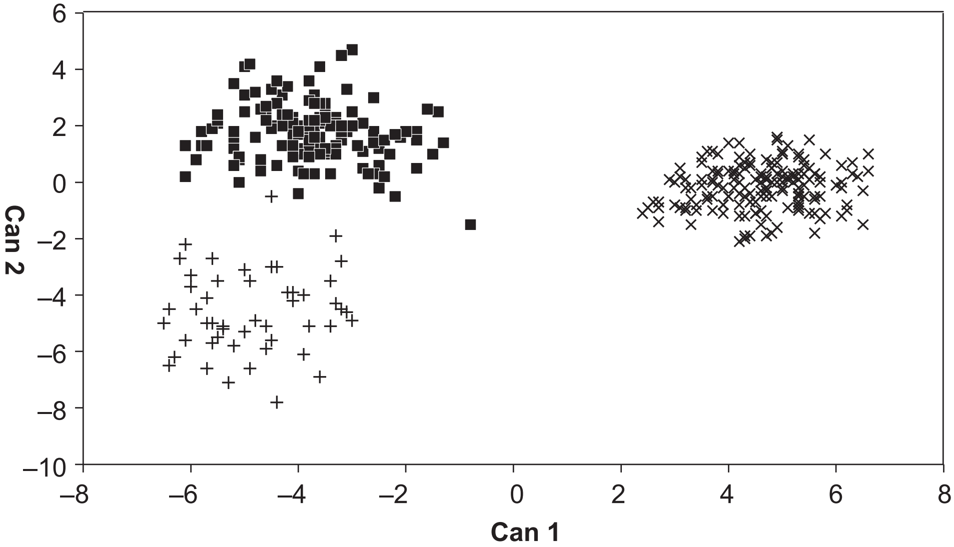

Fig. 7. – Results of the canonical discriminant analysis of the three taxonomic groups corresponding to *Nymphaea alba*, *N. candida* and garden cultivars using 53 quantitative characters (see Table 1). ☐ *N. alba* (n = 143), × *N. candida* (n = 169), + garden cultivars (n = 49).

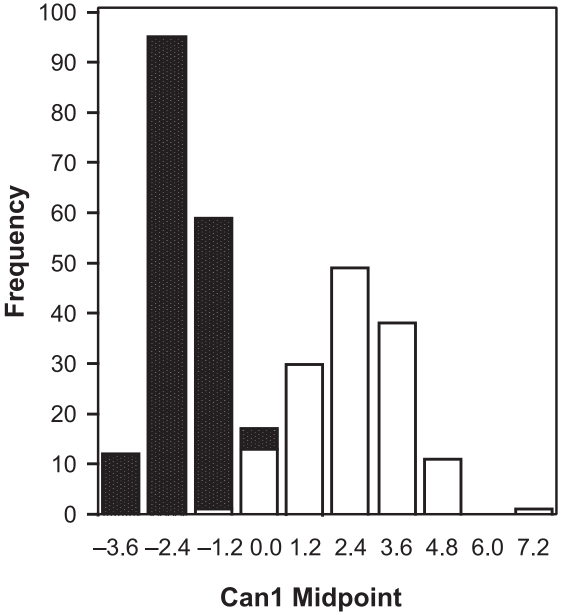

Fig. 8. – Results of the canonical discriminant analysis of *Nymphaea alba* and *N. candida* using the four quantitative characters with the highest discriminating power (degree of anther bending, number of carpels, width of the stigma projection, and stigma width). Only two individuals of *N. alba* were misclassified. ☐ *N. alba* (n = 143), ■ *N. candida* (n = 169).

and hybrids) and garden cultivars were found in leaf length/width (v36), petal size (both length – v19, and width – v20) and stamen width (v23); combination of these four characters resulted in 4.2% of the individuals being misclassified. Quantitative characters best discriminating *N. alba* and *N. candida* were largely similar to those identified by the CDA of three groups (see above) and included the degree of anther bending, number of carpels, width of the stigma projection and stigma width; their combination resulted in only 0.7% of the samples being misclassified (Fig. 8). Recognition of sterile individuals of *N. alba* and *N. candida* was less successful; a combination of five leaf characters with the highest discrimination power (v46, v44, v1, v42, v43; see Table 1) resulted in 7.4% of the individuals being misclassified (Electronic Appendix 6). Very small numbers of natural hybrids precluded meaningful discrimination from their parental species; nonetheless, in general, hybrids had larger sepals and petals, and their ovary and gynoecium heights were greater than those of their parental species.

Colours and pollen fertility

Qualitative differences in colour are not suitable for morphometric analyses and were therefore assessed separately. Proportions of samples of a particular colour are summarized in Table 3. Colour of petals, stigma disc and carpellary teeth were identified as the most important taxonomic qualitative characters.

Pollen fertility of the garden cultivars analysed was very low (5–18%, n = 12), whereas most of the pollen of both native species was fertile (stainable) (99–100%, n = 12 and 97–100%, n = 12 for *N. alba* and *N. candida*, respectively). Pollen fertility of four individuals of *N. ×borealis* analysed varied considerably and ranged between 50–99%.

Table 3. – Proportions of individuals with five plant characters of a particular colour in the four taxonomic groups of *Nymphaea* recognized.

Character	*N. alba* (n = 143)	*N. ×borealis* (n = 4)	*N. candida* (n = 169)	cultivars (n = 49)
Lamina undersurface	green (32.9%) reddish (51.7%) red (15.4%)	reddish (75%) red (25%)	green (5.9%) reddish (53.9%) red (40.2%)	green (8.2%) reddish (46.9%) red (44.9%)
Inner surface of sepals	white (55.2%) pink tinge (39.9%) light pink (4.9%)	white (25%) pink tinge (25%) light pink (50%)	white (41.4%) pink tinge (44.4%) light pink (13.6%) pink (0.6%)	white (14.3%) pink tinge (26.5%) light pink (34.8%) pink (22.4%)
Petals	pure white (99.3%) pink tinge (0.7%)	pure white (100%)	pure white (94.7%) pink tinge (4.7%) pink (0.6%)	pure white (34.7%) pink tinge (24.5%) pink (40.8%)
Stigma	yellow (100%)	yellow (50%) reddish (50%)	yellow (8.3%) reddish (62.1%) red (29.6%)	yellow (83.7%) reddish (12.2%) red (4.1%)
Carpellary teeth	light yellow (22.4%) deep yellow (77.6%)	deep yellow (25%) orange (50%) reddish (25%)	light yellow (0.6%) deep yellow (5.3%) orange (25.4%) reddish (55.1%) red (13.6%)	deep yellow (75.5%) orange (24.5%)

Discussion

In this study, we assessed the variation in the morphology of water lilies occurring in the Czech Republic and identified taxon-specific characters. Unlike previous studies, which exclusively used subjective criteria for species delimitation (e.g. Muntendam et al. 1996, Wayda 2000, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011), we used genome size, which is a more reliable way of assigning samples to a particular taxon.

The value of karyological data for delineating taxa

Karyological variation is widespread in the plant kingdom and differences in ploidy level, number of somatic chromosomes and/or genome size may have detectable effects on phenotypic and/or reproductive traits (Levin 2002, Husband et al. 2013). Consequently, karyological data are often used as an important criterion guiding taxonomic delineation in plants (Stace 2000). While the accurate determination of the number of chromosomes is time- and labour-intensive and therefore impractical for large-scale population studies, genome size can serve as a proxy for chromosome numbers. The last decade has seen an increasing number of studies that used genome size data for taxonomic decision-making, including delimitation of species boundaries and detection of interspecific hybrids in both heteroploid and homoploid plant groups (Kron et al. 2007, Ekrt et al. 2010, Loureiro et al. 2010, Suda & Pyšek 2010).

Volkova et al. (2010) provide strong evidence that *N. candida* is an allopolyploid in which the genomes of *N. alba* and *N. tetragona* are combined and its relative genome size equals the sum of parental 2C-values. On average, genetically-confirmed samples of *N. alba* and *N. candida* from Russia and surroundings differ by 40% in their nuclear DNA amounts. We observed very comparable differences in genome size between typical morphotypes of both species collected in the Czech Republic. Fluorescence values of less certainly identified white-flowered water lilies usually matched genome sizes of either *N. alba* or *N. candida* and were therefore assigned to the corresponding species.

A few white-flowered individuals collected in situ possessed genomes with sizes either intermediate between those of *N. alba* and *N. candida* or substantially larger than that of the latter species. Although they were not readily identified by visual inspection in the field, FCM results clearly demonstrated their hybrid origin. We were unable to determine the exact number of chromosomes in putative natural crosses as we were unable to obtain rhizomes and our attempts to use young leaves failed. Nonetheless, we are convinced that hybridization is well supported by the genome size data as the differences between theoretical and actual 2C-values are only 0.6% and 1.2% for crosses originating by syngamy of two reduced gametes of parental species, and 2n gamete of *N. alba* + n gamete of *N. candida*, respectively. The available evidence indicate that most hybridization events are not accompanied by any dramatic changes in nuclear DNA content and genome sizes of hybrids can be simply inferred from the values for their putative parents (Kron et al. 2007, Loureiro et al. 2010). All hybrid individuals occurred as minorities in populations otherwise formed by *N. alba* in the Třeboň basin, which is one of the centres of water lily distribution (with the presence of both species) in the Czech Republic (see Fig. 1). Although the second parent (*N. candida*) has not been recently recorded at localities of *N.* ×*borealis*, it is very likely it grew there in the past (cf. floristic records of Laně 1981 and Kurka 1996).

All but two of the garden cultivars (‘Firecrest’ and ‘Virginalis’) from the water lily collection in Průhonice had distinctly smaller genomes than any native species. The cultivars with 2C-values similar to (or even overlapping) that of *N. alba*, however, were clearly recognizable on the basis of morphological characters (e.g. leaf shape and in the case of ‘Firecrest’ also lavender-pink flowers) and thus do not compromise the value of genome size data. The great majority of plants collected in the field for which a garden origin was suspected had small genomes (3.29–3.48 pg/2C) that fall within the range of C-values measured for cultivars. Despite the fact that the vast majority of cultivars investigated had genome sizes dissimilar to native species, our screening of garden plants was by no means exhaustive and it is possible that their variation may actually be more complex. The small genomes of garden cultivars are not surprising because exotic species that supposedly participated in their origin (e.g. *N. mexicana* and *N. odorata*) have lower 2C-values than their native European counterparts (Diao et al. 2006).

Phenotypic variation and taxon-specific characters

The last two decades have seen several attempts to find morphological characters that are reliable for identifying *Nymphaea* plants growing in (central) Europe (Neuhäusl & Tomšovic 1957, Tomšovic 1988, 1995, Muntendam et al. 1996, Wayda 2000, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011). Perhaps the most comprehensive analysis of the phenotypic variation of the *Nymphaea alba-candida* complex is that done in the Netherlands (Muntendam et al. 1996). The authors report major interspecific differences in the dimension of the stigma projection, sepal width, stigma diameter, number of carpellary teeth and pollen characteristics. In addition, both species also differ in the shape of some organs, including that of fully opened flowers, cup base and/ or colour of stigma, carpellary teeth and undersurface of leaves. A morphometric study of material from the European part of Russia indicates that cup shape, filament shape of inner stamens, number of carpellary teeth and leaf position (floating or raised above the water surface) are the main diagnostic characters of *N. alba* and *N. candida*, but questions the relevance of pollen characteristics (Volkova & Shipunov 2007).

We built on these studies and assessed the value of both quantitative (using discriminant analyses) and qualitative (by calculating the proportion of samples with a particular state of the variable) morphological characters using karyologically verified samples of water lilies from the Czech Republic. In addition, we used geometric morphometrics to objectively quantify the variation in shape of particular generative and vegetative parts, an approach that has only rarely been used previously (Volkova & Shipunov 2007, Volkova et al. 2007). Our analyses confirmed the high discriminant power of gynoecium characters previously used for identifying *N. alba* and *N. candida*, including the number and colour of carpellary teeth, shape of stigma projection and dimension of stigma disc (Tables 2, 3, and Figs 5, 6). It is noteworthy that the interspecific differences in gynoecium characteristics were emphasised by J. S. Presl who described *N. candida* (Presl 1822, 1823). Shape of filaments of inner stamens (linear in *N. alba*, lanceolate in *N. candida*) is another species-specific character. Quite surprisingly, the best discriminating character in our analyses was the degree of anther bending (best seen in the median section of the flower; Electronic Appendix 7), which has never been previously considered to be taxonomically important. Determination of non-flowering plants is more challenging; our results indicate that the most important clue is offered by the shape of the main vein of the leaf lobe, which is consistent with results of previous studies (Neuhäusl & Tomšovic 1957, Tomšovic 1988, Ejankowski & Małysz 2011). Additional support for the identification of native water lilies in the field can be provided by examining the overall habit of the plants. In accordance with some other authors (e.g. Volkova & Shipunov 2007), leaves of all the populations of *N. candida* in the Czech Republic analysed were flat and floating whereas those of *N. alba* occasionally emerged above the water surface and their margins bent upwards. The same was true for flowers (floating or partly submerged in *N. candida*, occasionally raised above the surface in *N. alba*; see also Muntendam et al. 1996, Nowak et al. 2010).

Our statistical analyses of clearly delimited species of *Nymphaea* challenged the value of some morphological characters traditionally used in determination keys. In particular, the shape of the cup base (round in *N. alba* vs rounded-quadrangular in *N. candida*) has been commonly used for distinguishing between species of *Nymphaea* (Neuhäusl & Tomšovic 1957, Tomšovic 1988, Muntendam et al. 1996, Volkova & Shipunov 2007, Nowak et al. 2010, Ejankowski & Małysz 2011). Although the cup base does show some interspecific differences in shape, these are quite difficult to grasp objectively and the incidence of intermediate morphotypes further blurs the picture. In comparison with other characters in which the variation in shape was assessed in our study (e.g. leaf venation pattern, filaments, gynoecium), the cup base yielded a distinctly lower proportion of correctly classified individuals in the discriminant analysis.

A specific challenge that accompanies the determination of *Nymphaea* plants in central Europe is a frequent in situ occurrence of garden cultivars. Although the correct recognition of cultivars can be as difficult as that of native species this issue has been completely neglected in previous studies. According to our analyses, garden cultivars can be distinguished by larger and usually distinctly coloured petals, filament dimensions and leaf shape characteristics, the most easily measurable of which is the leaf length/width ratio. Similarly to *N. alba*, leaves of cultivars occasionally emerge above the water surface. Although only a few populations of water lilies were examined for pollen fertility, this character may in some cases guide determination. The fertility of the garden cultivars analysed was dramatically low as the plants produced mostly sterile grains.

Interspecific hybridization

Morphotypes with intermediate values of characters and/or a mosaic-like combination of characters are usually interpreted as interspecific hybrids (Tomšovic 1988, Volkova & Shipunov 2007, Ejankowski & Małysz 2011), although this is not based on any evidence other than morphology. Some authors (e.g. Ejankowski & Małysz 2011) even report the prevalence of individuals identified as *N.* ×*borealis* over typical morphotypes of parental species. Our results, however, indicate that interspecific hybridization under natural conditions is quite rare (at least in the Czech Republic) and hybrid origin was confirmed for only eleven out of 619 cytotyped samples collected in situ (~1.8%). In addition to reduced gametes, unreduced gametes also participated in the origin of some hybrids, a situation which is not uncommon in interspecific hybridization (Mahelka et al. 2005, Krahulcová et al. 2011). The small number of natural crosses detected precluded a detailed assessment of their morphological variation. Nonetheless, on average, the floral parts of hybrids are larger, suggesting heterosis for these traits (Baack & Rieseberg 2007). Pollen fertility of natural crosses varied considerably and were generally between the values recorded for garden cultivars and parental species. Reduced pollen fertility is also recorded in some other European *Nymphaea* populations (Heslop-Harrison 1955, Volkova & Shipunov 2007) and usually considered to indicate interspecific hybridization. Although pollen fertility seems to be taxonomically valuable, more intensive investigation using karyologically-proven samples is needed before any firm conclusions can be drawn.

Determination key

The following determination key is based on the results of both distance-based and geometric morphometrics; qualitative characters such as colour of plant organs were also considered. It should, however, be pointed out that the very small number of individuals of *N.* ×*borealis* included in this study makes determination of this natural interspecific hybrid uncertain. Values for quantitative characters are usually expressed as (minimum–) 5 percentile – 95 percentile (–maximum).

1a Filaments of the innermost stamens (4.4–) 4.6–6.6 (–7.0) times longer than wide, petals usually of different shades of pink (not pure white), (5.3–) 5.8–10.0 (–11.2) cm × (2.2–) 2.4–4.4 (–5.2) cm, leaf lamina length/width ratio (0.88–) 0.95–1.12 (–1.13) .... **garden cultivars**
1b Filaments of the innermost stamens (2.1–) 2.6–4.8 (–5.6) times longer than wide, petals white or near white (rarely with a faint pinkish tinge), (2.7–) 3.8–6.9 (–8.4) cm × (1.4–) 1.6–3.2 (–3.9) cm, leaf lamina length/width ratio (0.98–) 1.04–1.21 (–1.25) **2**
2a Sepals 7.5–9.5 cm × 3.1–4.2 cm, petals 6.0–8.4 cm × 3.2–3.9 cm, gynoecium 2.2–2.7 cm high, ovary 1.6–2.0 cm high, pollen fertility often low (usually < 75%) .... ***N.* ×*borealis* Camus**
2b Sepals (2.9–) 4.3–7.9 (–9.3) cm × (1.5–) 1.9–3.5 (–4.2) cm, petals (2.7–) 3.8–6.9 (–8.2) cm × (1.4–) 1.6–3.2 (–3.7) cm, gynoecium (1.0–) 1.2–2.3 (–2.6) cm high, ovary (0.7–) 0.9–1.6 (–2.0) cm high, most pollen grains fertile (usually > 95%) **3**
3a Anthers of the innermost stamens strongly bent [angle (83–) 89–135 (–144) degrees], their filaments with nearly parallel margins, carpellary teeth light to deep yellow, (10–) 12–22 (–24) in number, stigma disc yellow, (1.0–) 1.3–2.7 (–3.1) cm in diameter, stigma projection spherical to broadly conical, (1.6–) 2.0–5.2 (–6.3) mm wide, ovary covered up to the top by stamens (or scars of fallen stamens), primary vein on the leaf lobe only slightly bent in the proximal half, leaves and flowers occasionally emerge above the water surface .... ***N. alba* L.**
3b Anthers of the innermost stamens slightly bent to almost straight [angle (133–) 141–166 (–180) degrees], their filaments distinctly dilated in central part, carpellary teeth usually reddish to red, (5–) 7–13 (–15) in number, stigma disc usually orange to red, (0.6–) 0.7–1.6 (–1.9) cm in diameter, stigma projection narrowly conical, (0.4–) 0.6–2.5 (–3.5) mm wide, ovary not covered up to the top by stamens (or scars of fallen stamens), primary vein on the leaf lobe distinctly bent in the proximal half, leaves and flowers never emerge above the water surface .... ***N. candida* J. Presl**

See http://www.preslia.cz for Electronic Appendices 1–7.

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Souhrn

Přestože jsou lekníny (rod *Nymphaea*) ve střední Evropě zastoupeny pouze dvěma původními druhy (l. bílým – *N. alba* a l. bělostným – *N. candida*), jedná se o taxonomicky poměrně obtížnou skupinu. Jejich určování komplikuje vysoká morfologická proměnlivost v závislosti na podmínkách prostředí, časté přechodné morfotypy i předpokládaná mezidruhová hybridizace. Specifický problém představují záměrně vysazované či zplaňující zahradní kultivary. Pomocí průtokové cytometrie a mnohorozměrných morfometrických technik jsme hodnotili karyologickou a fenotypovou variabilitu 72 populací leknínů z území České republiky (pro srovnávací účely bylo do studie zahrnuto i 34 pěstovaných zahradních kultivarů). Spolehlivým determinačním znakem se ukázala být velikost jaderného genomu. Valná většina zahradních kultivarů vykazovala (výrazně) menší genomy než původní druhy. Množství jaderné DNA *N. candida* bylo v průměru 1,45násobné oproti *N. alba*. Na několika místech v jižních Čechách byly nalezeny rostliny (zhruba 1.8 % studovaných jedinců), jejichž velikost genomu odpovídala mezidruhovým hybridům (*N.* × *borealis*), přičemž na vzniku kříženců se podílely jak redukované, tak i neredukované gamety rodičovských druhů. Zpětní kříženci nebyli na základě dat o velikosti genomu zjištěni. Následná morfometrická analýza cytometricky ověřených jedinců umožnila vybrat soubor taxonomicky významných znaků. Jako nejvíce informativní se ukázal být tvar pestíku a tyčinek, určité mezidruhové rozdíly lze najít i na listech. Odlišení kříženců na základě makromorfologických znaků je problematické, nejlepším vodítkem bývá snížená barvitelnost pylu. Celkově studie odhalila vhodné determinační znaky obou původních druhů i pěstovaných kultivarů, a ukázala, že mezidruhová hybridizace je ve studovaném území poměrně vzácným jevem a nepředstavuje tedy významný ochranářský problém.

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