

Genome size as a marker for identifying the invasive alien taxa in *Fallopia* section *Reynoutria*

Velikost genomu umožňuje odlišení invazních druhů křídlatek (*Fallopia* sekce *Reynoutria*)

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DAPI and propidium iodide flow cytometry were used to determine the variation in genome size in 166 samples and of all taxa and ploidy levels of *Fallopia* section *Reynoutria* (knotweeds) recorded in the Czech Republic. Significant differences were detected in the amount of nuclear DNA, associated with the ploidy levels and taxonomic identity of the material. At each ploidy level, *F. sachalinensis* showed the lowest and *F. japonica* the highest fluorescence intensities. The fluorescence values for the hybridogenous *F. xbohemica* were located in-between these two levels. In most cases, there was at least a four-percent gap in fluorescence values between the nearest neighbours belonging to a different taxon. Intraspecific variation in genome size was very low in all taxa except hexaploid *F. xbohemica*; this could be due to the complex evolutionary history of this taxon. Our results indicate that the amount of nuclear DNA can be used as a reliable marker for the identification of homoploid knotweed species and their hybrids. Different evolutionary pathways for the origin of high polyploids and/or hybridogenous taxa are proposed based on genome size.

Keywords: flow cytometry, genome size, homoploid groups, hybridization, invasion, knotweed, nuclear DNA amount, ploidy

Introduction

Invasive plant species, i.e. non-indigenous species that successfully spread in their new environment, pose a serious threat to biodiversity worldwide. They adversely affect the habitats they invade in a multitude of ways by altering ecosystem functioning, degrading resources, changing species composition and transforming food webs (Elton 1958, Drake et al. 1989, Vitousek 1994, Williamson 1996, Lambdon et al. 2008, Chytrý et al. 2009). As a result, biological invasions are considered to be the second most important cause of native species endangerment after habitat loss (Drake et al. 1989). Some of the most serious alien plants in the European flora are species from the section *Reynoutria* (Houtt.) Ronse Decr. of the genus *Fallopia* Adans. (*Polygonaceae*), sometimes treated as a separate genus *Reynoutria* Houtt. Four taxa are found in Europe, including: two varieties of Japanese knotweed [*F. japonica* (Houtt.) Ronse Decr. var. *japonica* and var. *compacta* (Hook. f.) J. P. Bailey], giant knotweed [*F. sachalinensis* (F. Schmidt Petrop.) Ronse Decr.] and Bohemian knotweed [*F. xbohemica* (Chrtek et Chrtková) J. P. Bailey], a hybrid of the previous two taxa. These species are robust herbaceous perennials with an extensive

system of rhizomes. They are native to eastern Asia and were introduced into Europe in the mid-nineteenth century as ornamental plants (Bailey & Conolly 2000). Currently, knotweeds have colonized various man-made and seminatural habitats (particularly along river banks; Mandák et al. 2004), and significantly reduce plant species diversity (Bímová et al. 2004). Their effect is so great that they are classified as transformer species, as they can change the character, condition, form, or nature of an ecosystem (Richardson et al. 2000).

Over the last decade, knotweeds have been the subject of a comprehensive investigation using a number of biosystematic tools in Europe, including the Czech Republic (Bailey & Stace 1992, Bailey et al. 1995, Hollingsworth et al. 1998, 1999, Hollingsworth & Bailey 2000a, b, Mandák et al. 2003, 2004, 2005, Pyšek et al. 2003). The taxa differ dramatically in terms of genetic diversity estimated using isozyme markers (Mandák et al. 2005). While the nominate variety of *F. japonica* is genetically uniform (the same female clone occurs across Europe; Hollingsworth & Bailey 2000a, Mandák et al. 2005), the hybridogenous taxon *F. ×bohemica* shows the highest degree of genetic variation. This could be the result of multiple origins and/or occasional sexual reproduction occurring in the latter taxon (Hollingsworth & Bailey 2000b, Pashley et al. 2003, Mandák et al. 2005). Although vegetative regeneration is widely accepted as the main mode of reproduction in invaded regions, hybridization has dramatically influenced the sexual reproduction and furthered the invasive success of knotweeds (Bailey et al. 2007, Tiébré et al. 2007a, b). Some *Fallopia* populations in the secondary areas can have a quite complex evolutionary history and processes such as bidirectional hybridization and introgression have been identified using molecular markers (Gammon et al. 2007, Grimsby et al. 2007). A large-scale survey of ploidy variation using flow cytometry (FCM) revealed the incidence of intraspecific cytotype differentiation in *F. sachalinensis* and *F. ×bohemica*, both of which include tetraploid ($2n = 4x = 44$), hexaploid ($2n = 6x = 66$) and octoploid ($2n = 8x = 88$) chromosomal races (Mandák et al. 2003). In contrast, the varieties of *F. japonica* are cytologically uniform: tetraploid and octoploid in var. *compacta* and var. *japonica*, respectively. During a previous FCM study, variation in fluorescence intensity among different plant accessions of the same ploidy level was observed. Because the high-resolution histograms were achieved that are supportive of genuine variation, a targeted investigation of the patterns of genome size in invasive knotweed species was initiated.

Our interest in genome size was fuelled by the fact that this marker is associated with various cellular, tissue and organismal level characteristics (i.e. the ‘nucleotype theory’; Bennett 1972, see also Loureiro et al. 2010). For example, positive correlations were found between genome size and nuclear and cell volumes, mitotic cycle duration and duration of meiosis (Leitch & Bennett 2007). Genome size is also significantly associated with minimum generation time in herbaceous plants. On average, ephemerals have the smallest and obligate perennials the largest genomes. It is not surprising that weedy species generally possess smaller genome sizes than their non-weedy counterparts (Bennett et al. 1998). Similarly, the amount of nuclear DNA is a characteristic that can be successfully used to predict invasive behaviour in plants (Rejmánek 1996, Grotkopp et al. 1998). In fact, Rejmánek (2000) lists genome size among the eight best predictors of plant invasiveness. In plant taxonomy, the amount of nuclear DNA often facilitates the delineation of taxa at various taxonomic ranks and may therefore guide taxonomic decisions (Kron et al. 2007). Moreover, genome size can indicate the genomic constitution of allopolyploid taxa and/or reveal the putative parents of interspecific hybrids (Suda et al. 2007).

In this study, the variation in genome size in knotweed plants from the Czech Republic was analyzed. Using flow cytometry, the following questions were addressed: (i) How the genome size varies within and between species? (ii) Can genome size be used for making taxonomic decisions? (iii) Can genome size be used to infer the evolutionary history/mode of origin of certain taxa/cytotypes?

Material and methods

Plant material

Samples of *Fallopia* were collected from 166 localities in the Czech Republic during 1995–2000 (see Electronic Appendix 1 for locality details) and transplanted to the experimental garden of the Institute of Botany, Academy of Sciences, Průhonice, Czech Republic (49°59'30"N, 14°34'00"E, ca 320 metres above sea level). Rhizomes were grown in 12 l plastic pots filled with garden soil and the regenerated plants subjected to FCM analyses. The number of cytotypes/individuals per species was as follows: *F. japonica* (2 ploidy levels/59 plants), *F. sachalinensis* (3/48) and *F. ×bohemica* (3/59), which represents the overall variation in ploidy levels recorded in our previous study (Mandák et al. 2003). Herbarium vouchers of all the taxa analyzed are deposited in the herbarium of the Institute of Botany at Průhonice (PRA).

Flow cytometry

Relative fluorescence intensities and genome sizes were determined by flow cytometry using a Partec PA II and CyFlow instrument (Partec GmbH., Münster, Germany) equipped with a mercury arc lamp and 532 nm solid state laser as the sources of the excitation light, respectively. The analyses were performed during 2002–2005. Sample preparation followed a two-step procedure using Otto buffers (Doležel et al. 2007). *Glycine max* (L.) Merr. 'Polanka' (2C = 2.50 pg; Doležel et al. 1994) and *Zea mays* L. 'CE-777' (2C = 5.43 pg; Lysák & Doležel 1998) were selected as appropriate reference standards for analyses with DAPI and propidium iodide (PI), respectively. Young intact leaf tissue of a *Fallopia* sample (ca 1 cm²) and the internal reference standard were chopped using a sharp razor blade in a Petri-dish containing 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The suspension was filtered through a 42-µm nylon mesh and centrifuged (150 g for 5 min). The supernatant was removed and the pellet re-suspended in 100 µl of fresh ice-cold Otto I buffer. Samples were incubated for 15 min at room temperature and stained with 1 ml of Otto II buffer (0.4 M Na₂HPO₄·12 H₂O) supplemented with β-mercaptoethanol (2 µl/ml) and a fluorochrome. DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 4 µg/ml was used for the determination of relative genome size (relative fluorescence intensity); PI + RNase IIA (both at concentrations 50 µg/ml) were used for the determination of genome size in absolute units (DNA picograms). Samples were stained for 5 min at room temperature and analyzed using the flow cytometer. A fluorescence intensity of 5000 particles was recorded. Only histograms in which both peaks are of a similar height were considered. The threshold for accepting the analyses was set as follows: coefficients of variation (CVs) of G₀/G₁ peak of the *Fallopia* sample below 3.0% and 5.0% for the DAPI and PI staining, respectively. Each plant was measured twice in

DAPI acquisitions, while genome size values were calculated on the basis of at least three estimates performed on different days. Because *Fallopia* plants contain high levels of polyphenolic compounds (Vaher & Koel 2003), which may bind to DNA and distort FCM analyses, AT-selective fluorochrome DAPI was favoured over intercalating propidium iodide. DAPI is less sensitive to the chromatin state, shows high DNA selectivity and has a high increase in quantum efficiency after binding (Shapiro 2003). Therefore, DAPI usually produces histograms with high accuracy, resolution and reproducibility and is particularly useful for the detection of small differences in the amount of nuclear DNA. As a result, all samples were analyzed with DAPI, while absolute genome size was only determined for particular individuals (see Electronic Appendix 1). Reliability of FCM measurements (i.e., between-plant differences) was repeatedly confirmed in simultaneous runs of two or more *Fallopia* samples. The genome size terminology follows Greilhuber et al. (2005).

Statistical analyses

A General Linear Model (procedure GLM available in SAS 8.1; SAS Institute, Cary, NC, USA) was used to test for differences in fluorescence intensities among species/ploidy levels (this procedure was chosen for the analysis of variance because of unbalanced data design). A box-and-whisker plot was drawn using Statistica 8.0 (Statsoft Inc., Tulsa, OK, USA).

Results

DAPI staining yielded high-resolution histograms with little background noise and low coefficients of variation. Mean CV of *Fallopia* samples was 2.41%, which suggests that the analyses were not negatively affected by secondary metabolites. FCM runs of 166 samples resulted in eight significantly different ($F = 35440.8$, $P < 0.0001$) non-overlapping groups of fluorescence intensities, corresponding to ploidy levels and taxonomic identity of the material (Fig. 1). Table 1 presents the average fluorescence intensities and standard deviations for individual cytotypes/taxa (setting tetraploid *F. japonica* var. *compacta* as the unit value). At each ploidy level, *F. sachalinensis* showed the lowest fluorescence intensities, while *F. japonica* exhibited the highest; the fluorescence values for the hybridogenous *F. ×bohemica* are located between these values. This differentiation in genome size indicates that the amount of nuclear DNA is a taxon-specific marker. Indeed, there was mostly at least a four-percent gap in fluorescence values between the nearest neighbours belonging to different taxa. The only exception was the octoploid plants of *F. ×bohemica* and *F. japonica*, whose recognition on the basis of genome size may be quite challenging in some cases because they differ only slightly (~ 1.2%) from their nearest neighbours (but note that the mean values differed by about 2.7%). Intraspecific variation in fluorescence intensity per ploidy level was very low (< 2.6%) for all but the hexaploid *F. ×bohemica* plants; in the latter taxon the difference between max/min values is ca 4%. Simultaneous analyses of plants differing by at least 5% (i.e., about twice the average CV) yielded a bifurcated peak or two distinct peaks (Fig. 2A), which is regarded as robust proof of a genuine variation in genome size.

A subsample of 22 *Fallopia* plants, representing all taxa and cytotypes, was subjected to genome size estimation in absolute units. Analyses with PI are generally more demand-

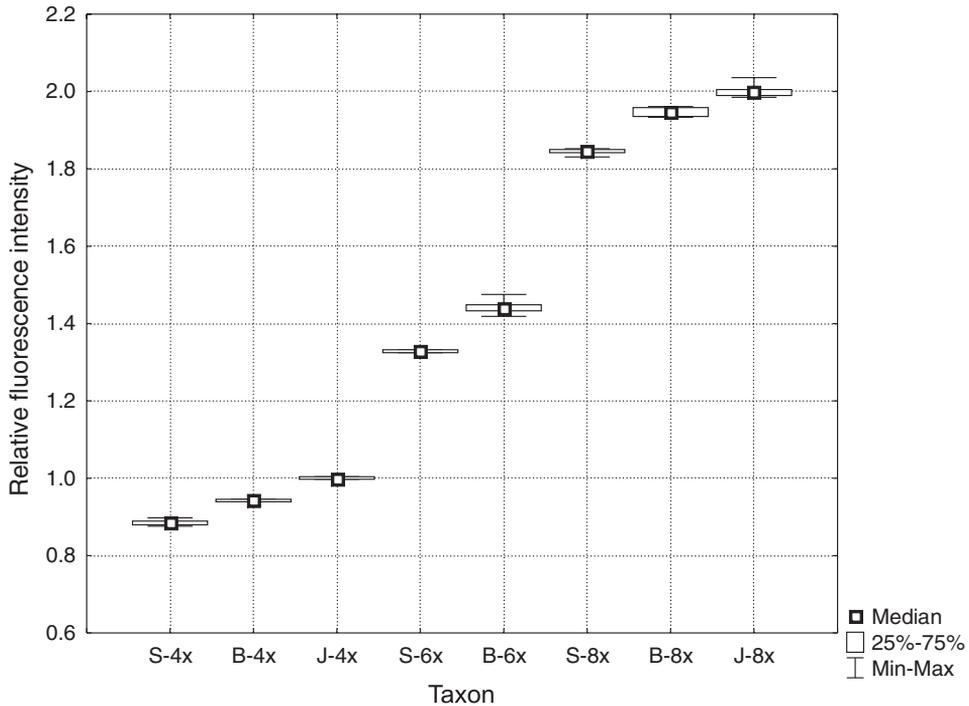


Fig. 1. – Box-and-whisker plots showing relative fluorescence intensities of eight groups of *Fallopia* samples (corresponding to species and ploidy levels). The number of samples is the same as in Table 1. The first letter designates the species (B – *F. ×bohemica*, J – *F. japonica*, S – *F. sachalinensis*), followed by the ploidy level (4x–8x). Mean fluorescence of tetraploid *F. japonica* var. *compacta* was set as the unit value.

Table 1. – Relative fluorescence intensities (means ± SD) of 166 *Fallopia* samples determined using DAPI flow cytometry and *Glycine max* 'Polanka' as an internal reference standard. Mean fluorescence of tetraploid *Fallopia japonica* var. *compacta* was set as the unit value. Numbers of individuals analyzed are shown in parentheses.

Taxon	Tetraploid	Hexaploid	Octoploid
<i>F. sachalinensis</i>	0.885 ± 0.006 (n=36)	1.329 ± 0.004 (n=2)	1.844 ± 0.007 (n=10)
<i>F. ×bohemica</i>	0.942 ± 0.003 (n=2)	1.442 ± 0.014 (n=53)	1.947 ± 0.012 (n=4)
<i>F. japonica</i>	1.000 ± 0.003 (n=3)	–	1.999 ± 0.011 (n=56)

ing; nevertheless reasonably low CVs (mean value = 3.42%) were achieved even with this intercalating fluorescent dye (Fig. 2B). The variation in absolute genome sizes was consistent with the pattern of DNA amounts observed in DAPI staining and confirmed the between-species divergences. The holoploid genome sizes (2C-values) for all taxa/ploidy levels are summarized in Table 2. Monoploid genome sizes (Cx-values; 2C-value/ploidy level) for individual species were as follows: *F. sachalinensis* (1Cx = 1.07–1.11 pg), *F. ×bohemica* (1Cx = 1.16 pg) and *F. japonica* (1Cx = 1.21 pg). Except for the octoploid *F. sachalinensis*, which has higher Cx-values than its 4x and 6x counterparts, the monoploid genome sizes were stable across ploidy levels within the same species.

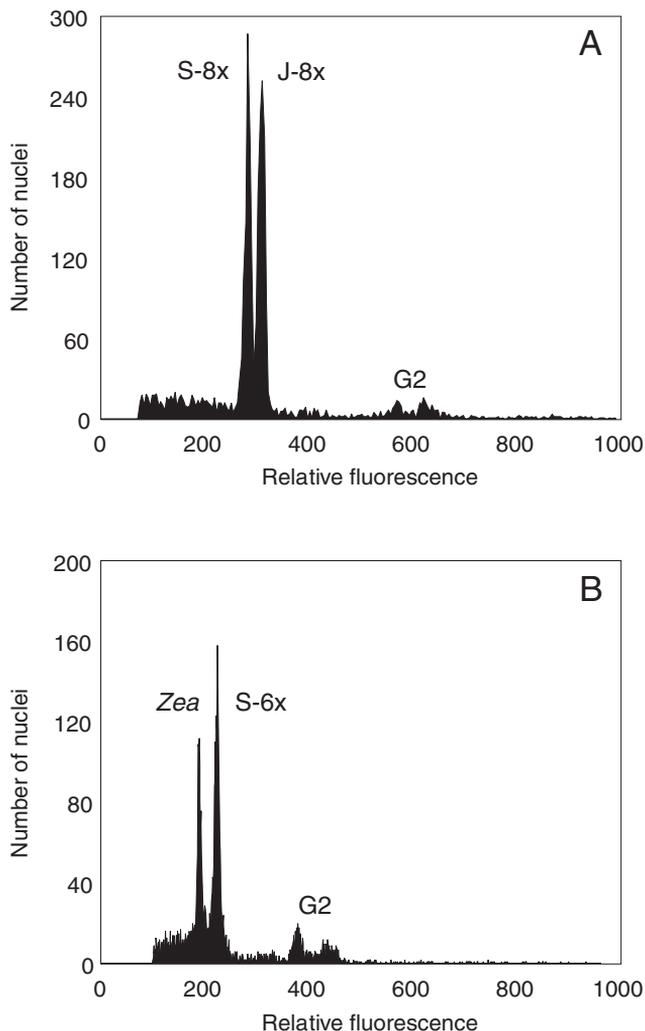


Fig. 2. – Flow cytometric fluorescence histograms: (A) Simultaneous analysis of DAPI-stained nuclei isolated from octoploid *F. sachalinensis* (S-8x) and octoploid *F. japonica* (J-8x), documenting the difference in genome sizes. (B) Estimate of the genome size of the hexaploid *F. sachalinensis* (S-6x) using *Zea mays* ‘CE-777’ as an internal standard. Nuclei were stained with propidium iodide.

Table 2. – Genome sizes (2C-values in DNA pg; means±SD) of 22 *Fallopia* samples determined using propidium iodide flow cytometry. *Zea mays* ‘CE-777’ (2C = 5.43 pg) was used as an internal reference standard. Numbers of individuals analyzed are shown in parentheses.

Taxon	Tetraploid	Hexaploid	Octoploid
<i>F. sachalinensis</i>	4.32±0.04 (n=3)	6.42 (n=1)	8.88±0.03 (n=4)
<i>F. ×bohemica</i>	4.63±0.04 (n=2)	6.93±0.03 (n=4)	9.25±0.07 (n=3)
<i>F. japonica</i>	4.85±0.01 (n=2)	–	9.64±0.05 (n=3)

Discussion

In this study, the variation in genome size was assessed in a representative set of knotweed plants from the Czech Republic. The most important result was that flow cytometry detected taxon-specific amounts of nuclear DNA. This finding facilitates easy, rapid and reliable recognition of invasive species and hybrids of *Fallopia*, which is often quite challenging using a conventional phenotype-based approach. With the aid of FCM, it is now possible to determine individuals at an early ontogenetic stage, plants with poorly developed morphological characteristics or even plant fragments, which would be difficult to achieve as quickly or cheaply using other cytogenetic or molecular techniques. In addition, it is now possible to determine the population structure of mixed stands of *Fallopia*, detect individuals with chromosomal irregularities (such as aneuploids) and easily identify novelty in this highly variable group of alien taxa. FCM can also be used as a powerful tool for exploring the taxonomic and karyological diversity of knotweed plants.

Generally, two prerequisites must be satisfied before genome size is employed as a taxonomic marker: (i) low intraspecific variation and (ii) sufficient divergence among different species (see also Loureiro et al. 2010). Section *Reynoutria* of the genus *Fallopia* meets both these criteria. Apart from the hexaploid *F. ×bohemica*, the variation in fluorescence intensities per species and cytotype was negligible and ranged from 0.6% (in hexaploid *F. sachalinensis*) to 2.6% (in octoploid *F. japonica*). Because there is only one genotype in the population of the latter species throughout Europe (Hollingsworth & Bailey 2000a, Mandák et al. 2005), the recorded variation in genome size should be regarded as an artefact, caused, for instance, by instrument drift and/or secondary metabolites interfering with the DNA staining. The variation in the hexaploid *F. ×bohemica* approached 4.0%, which is still reasonably low, and does not compromise the utility of genome size in taxonomic decision-making. At a particular ploidy level, the average interspecific differences in relative genome size ranged from 2.7% (octoploid *F. ×bohemica* – *F. japonica*) to 8.5% (hexaploid *F. sachalinensis* – *F. ×bohemica*) and the five-percent threshold was passed in all but one case. In practice, the divergence between nearest neighbours belonging to different taxa is of crucial importance. Once again, only octoploid plants of *F. ×bohemica* and *F. japonica* possessed similar relative genome sizes (divergence 1.2%), whereas all other species pairs showed reasonable differences (4.4–6.5%). Therefore, genome size can be used to identify homoploid knotweed taxa despite the fact that the discrimination between 8x *F. ×bohemica* and *F. japonica* might prove difficult.

The interspecific differentiation in genome size can be utilized for determining the evolutionary history of high polyploids and/or hybrid taxa (see Mahelka et al. 2005, Leong-Škorničková et al. 2007, Suda et al. 2007). There is only one way of accounting for the origin of the following taxa/cytopypes: tetraploid *F. ×bohemica* (i.e. hybridization between 4x *F. sachalinensis* and 4x *F. japonica* var. *compacta*), hexaploid *F. sachalinensis* (i.e. fusion of non-reduced and reduced gametes of the tetraploid counterpart) and octoploid *F. japonica* (i.e. autopolyploidization of the tetraploid var. *compacta*). Relative fluorescence intensities provide strong support for these scenarios. In all cases, there was virtually no difference between the theoretical (= inferred from the data on putative parents) and actual (= estimated by FCM) values. However, the evolutionary history of the higher polyploids of the hybridogenous taxon *F. ×bohemica* may be more complex. Disregarding the back-crosses, hexaploid and octoploid cytopypes may theoretically have origi-

nated by three and five pathways, respectively. The best agreement between theoretical and actual values for hexaploid plants is when it is assumed they are hybrids of 4x *F. sachalinensis* and 8x *F. japonica*. In addition, these cytotypes are the dominant ploidy levels in both parental species. Reciprocal hybrids (i.e. 4x *F. japonica* var. *compacta* and 8x *F. sachalinensis*) are much less likely because of the scarcity of either of the putative parents; the same is true for the genesis of hexaploids *via* the fusion of 2n and n gametes of 4x *F. ×bohemica*. Nevertheless, it should be noted that FCM results do not rule out any of the above-mentioned alternative pathways (the average differences between the theoretical and actual values are 1.4% and 2.1%, respectively). Perhaps a certain intraspecific variation (~4%) in fluorescence intensities among 6x plants of *F. ×bohemica* indicates multiple origins of this taxon, including the involvement of different parental combinations. In fact, the high degree of genetic differentiation of *F. ×bohemica* indicated by isozyme analyses (Mandák et al. 2005) provides further support for the complex evolutionary history of this hybridogenous taxon. In addition, Berchová-Bímová et al. (unpublished data) recently recorded successful crossing within a population of hexaploid hybrids, which may account for the high genetic variability found in a relatively small geographic area. The results also document the ability of an invasive species to rapidly evolve outside its native geographical range and increase in genetic variability, which might be an indication of an increasing adaptability. Octoploid cytotypes of *F. ×bohemica* most likely originated *via* hybridization between the corresponding cytotypes of *F. sachalinensis* and *F. japonica* (the average difference between theoretical and actual fluorescence values was 1.3%). Other evolutionary pathways (see Bailey & Wisskirchen 2006) are less likely either due to the paucity of respective parental taxa/cytotypes and/or lower agreement between the theoretical and actual relative genome sizes. The lowest correspondence with regards to the FCM results was observed in octoploid cytotypes of *F. sachalinensis*. Although autopolyploidization of their tetraploid counterparts is the most plausible mode of octoploid origin, the actual fluorescence intensity was about 4% higher than the values inferred from the putative parentage. Perhaps the higher activity of mobile genetic elements after polyploidization (Baumel et al. 2002) triggered an increase in the amount of nuclear DNA in octoploid *F. sachalinensis*.

Previously, the genome sizes of a tetraploid cytotype of *F. sachalinensis* and tetraploid and octoploid cytotypes of *F. japonica* were estimated, using Feulgen microdensitometry (Bailey & Stace 1992). The amounts of DNA recorded in this study are much smaller (by ca 30–50%) than the C-values determined in our study. Because Feulgen densitometry is more sensitive to experimental conditions (especially when hot hydrolysis is used; see Greilhuber 2005), we are convinced that our flow cytometric results are more accurate. In fact, even the authors (Bailey & Stace 1992) treated their values with caution and mostly assessed only the relative between-species differences.

See <http://www.preslia.cz> for Electronic Appendix 1.

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Souhrn

Pomocí průtokové cytometrie byla studována variabilita v obsahu jaderné DNA u invazních zástupců křídlatek (*Fallopia* sekce *Reynoutria*), vyskytujících se na území České republiky. Analýzy všech dosud nalezených druhů a ploidních úrovní odhalily výrazné mezidruhové rozdíly v množství jaderné DNA. Křídlatka sachalinská (*F. sachalinensis*) se vyznačuje nejmenší monoploidní velikostí genomu, naproti tomu křídlatka japonská (*F. japonica*) má monoploidní genom největší. Hybridogenní křídlatka česká (*F. ×bohemica*) vykazuje v tomto směru intermediární postavení. Rozdíly v obsahu jaderné DNA mezi jedinci různých druhů zpravidla překračovaly čtyřprocentní hranici. Vnitrodruhová variabilita ve velikosti genomu byla většinou zanedbatelná, jedinou výjimku představoval hexaploidní cytotyp křídlatky české. Výrazné mezidruhové rozdíly v obsahu jaderné DNA spolu se stabilitou v rámci téhož taxonu otevírají možnost pro použití velikosti genomu jako spolehlivého determinačního znaku (dovoluje odlišit i druhy se stejným počtem chromozómů). Na základě množství jaderné DNA jsou diskutovány pravděpodobné způsoby vzniku polyploidních a hybridogenních zástupců křídlatek.

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