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A reassessment of the genome size–invasiveness relationship in reed canarygrass (*Phalaris arundinacea*)

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• **Background and Aims** Genome size is hypothesized to affect invasiveness in plants. Key evidence comes from a previous study of invasive eastern North American populations of the grass *Phalaris arundinacea*: invasive genotypes with smaller genomes had higher growth rates, and genome sizes were smaller in the invasive vs. native range. This study aimed to re-investigate those patterns by examining a broader range of North American populations and by employing the modern best-practice protocol for plant genome size estimation in addition to the previously used protocol.
• **Methods** Genome sizes were measured using both internal and pseudo-internal standardization protocols for 20 invasive and nine native range accessions of *P. arundinacea.* After a round of vegetative propagation to reduce maternal environmental effects, growth (stem elongation) rates of these accessions were measured in the greenhouse.
• **Key Results** Using the best-practice protocol, there was no evidence of a correlation between genome size and growth rates ($P = 0.704$), and no evidence for differences in genome sizes of invasive and native range accessions ($P > 0.353$). However, using the older genome size estimation protocol, both relationships were significant (reproducing the results of the previous study).
• **Conclusions** Genome size reduction has not driven increased invasiveness in a broad sample of North American *P. arundinacea*. Further, inappropriate genome size estimation techniques can create spurious correlations between genome size and plant traits such as growth rate. Valid estimation is vital to progress in understanding the potentially widespread effects of genome size on biological processes and patterns.

Key words: Invasion biology, range expansion, C-value, plant secondary chemistry, flow cytometry, wetlands, large genome constraint hypothesis, EICA hypothesis, *Phalaris arundinacea.*

INTRODUCTION

Ecologists and evolutionary biologists have worked for decades to identify traits that predict invasiveness in plants (e.g. Baker, 1965; Williamson and Fitter, 1996; Pyšek and Richardson, 2008; van Kleunen *et al.*, 2010). This task is difficult, in part because trait correlations can obscure causal linkages, different sets of traits influence success at different stages of invasion (Theoharides and Dukes, 2007) and under different conditions (Funk and Vitousek, 2007), and the fact that the eventual success or failure of an introduction can be idiosyncratic. Nevertheless, hope is held out that there may be easily quantifiable 'master traits' that correlate with the traits directly implicated in invasion success, analogous to traits emphasized in the leaf economics spectrum (Wright *et al.*, 2004) that indicate broad ecological strategies. For invasiveness, genome size is one such candidate master trait (Suda *et al.*, 2015). Genome size can refer to either holoploid genome size (total DNA content of the nucleus) or monoploid genome size (holoploid size divided by the ploidy level); herein, 'genome size' refers to holoploid size unless otherwise noted.

Rejmánek (1996) was the first to hypothesize clearly that taxa with smaller genome sizes are more invasive. Organisms within a population vary in their genome size due to insertions and deletions at multiple scales that occur during DNA replication and recombination (Bennetzen *et al.*, 2005), allowing genome size to evolve over time within a species. Variation in the volume of DNA is hypothesized to entail physical consequences for cell size and DNA replication rates (Beaulieu *et al.*, 2008), with, for example, organisms with larger genomes constrained to a longer cell cycle duration (Francis *et al.*, 2008). Through these cellular-level effects, genome size may then influence numerous higher level organismal traits: many interspecific studies (reviewed in Suda *et al.*, 2015) indicate that genome size covaries with plant traits such as timing of spring leaf emergence (Fridley and Craddock, 2015), guard cell size and stomatal characteristics (Beaulieu *et al.*, 2008), and seed characteristics influencing growth and reproductive ability (Grotkopp *et al.*, 2004). Moreover, generation time and life history appear to be constrained by the amount of nuclear DNA: a large genome may limit where a plant can grow and its ability to survive in extreme environments (Knight *et al.*, 2005; Carta and Peruzzi, 2016).

At the interspecific level, several studies have examined the relationship between genome size and invasiveness/weediness in plants (reviewed in Suda *et al.*, 2015). Unfortunately, most of these studies provide limited inferences because they do not account for the fact that species are phylogenetically non-independent; they thus apply traditional statistical approaches – assuming independence of samples – to estimate the strength of the genome size–invasiveness correlation. These approaches are invalid when closely related species have similar trait values (Felsenstein, 1985). In contrast, the best of these studies (Grotkopp *et al.*, 2004; Kubešová *et al.*, 2010; Pandit *et al.*, 2014) use some form of phylogenetic comparative approach. Grotkopp *et al.* (2004) examined 85 *Pinus* species and found no relationship between monoploid genome size and invasiveness using phylogenetically independent contrasts. Kubešová *et al.* (2010) compared 93 invasive species alien to the Czech flora with congeneric and confamilial non-invasive species, and found significantly smaller holoploid and monoploid genome sizes in the former. Finally, Pandit *et al.* (2014) examined a globally distributed set of 890 species using phylogenetic generalized least squares and found that genome size was negatively correlated with the probability of invasiveness. Importantly, chromosome number was simultaneously included as a predictor and had a positive relationship with invasiveness (despite its positive correlation with genome size). Thus, the overall pattern from reliable interspecific studies indicates a broad association between small genome size and invasiveness, even after controlling for the effect of polyploidy.

While these cross-species correlations are intriguing, species differ in many other traits besides genome size. Such correlations do not prove that genome size causally affects invasiveness, and could instead arise if genome size and invasiveness are both driven by some third trait. Therefore, studies of genome size and invasiveness within a single species (e.g. Lavergne *et al.*, 2010; see also Meyerson *et al.*, 2016) should be quite valuable because they reduce the confounding factors; individuals differing in genome size will otherwise be similar, and thus any genome size–invasiveness relationship detected has a better chance of being causal.

To date, a study by Lavergne *et al.* (2010) represents the most widely cited intraspecific study examining genome size and invasiveness. This study concluded that invasive populations of the grass *Phalaris arundinacea* in eastern North America have smaller genome sizes than native European populations; and, using phylogenetic reconstructions, that the invasive genotypes have evolved smaller genome sizes than their Eurasian ancestors. Further, Lavergne *et al.* (2010) report a negative correlation between genome size and growth rate in the invasive populations of *P. arundinacea*; they suggest that fast growth is indicative of competitive ability and, therefore, invasion success. However, because multiple studies that purport to show intraspecific variation in plant genome size have been disproven using improved methodology (reviewed in Šmarda and Bureš, 2010), the conclusions of Lavergne *et al.* (2010) have recently been questioned (Akiyama *et al.*, 2015; Bock *et al.*, 2015). Lavergne *et al.*'s (2010) investigation relied on a pseudo-internal standard protocol for genome size estimation, a technique that can lead to substantial error in plants (see Box 1) but which is still in widespread use (e.g. Meeks and Chandra, 2015; Staton *et al.*, 2015).

BOX 1. METHODOLOGICAL ISSUES IN PLANT GENOME SIZE ESTIMATION

General principles. Flow cytometry has become the most common method to quantify DNA in cell nuclei due to its capacity for highly reliable estimates (Bennett and Leitch, 1995, 2011). Flow cytometry in plants begins with the preparation of a solution of suspended nuclei by disrupting fresh (or sometimes dried) leaf tissue, followed by application of a DNA stain such as propidium iodide (PI) (Doležel *et al.*, 1998). The stained nuclei are then run through a flow cytometer, which subjects them individually to a laser and records relative fluorescence. The ratio of sample fluorescence to standard fluorescence, where the latter is the fluorescence of stained nuclei from a species of known genome size, is then used to estimate absolute genome size of the sample (Doležel and Bartoš, 2005). Ideal standards are expected to have a similar genome size to the sample species (Doležel and Bartoš, 2005) and similar chromatin structure: because chromatin structure affects DNA staining, animal standards such as chicken erythrocyte nuclei (CEN) are not recommended for use in plant genome size estimation (Doležel and Greilhuber, 2010). Recommended plant species for use as standards are discussed in Doležel *et al.* (1992) and at http://olomouc.ueb.cas.cz/plant-dna-cytometry-standards.

Standardization protocols. Importantly, when and how the standard is exposed to the stain can have significant effects on the genome size estimate (Doležel and Bartoš, 2005). In plants, secondary compounds in the cytosol can interfere with the binding of stain to DNA (e.g. Price *et al.*, 2000; Loureiro *et al.*, 2006), a complication first identified 30 years ago as the 'self-tanning' effect (Greilhuber, 1988) when tannins are responsible. In some early protocols, the standard was stained separately from the sample and the two preparations of nuclei were run separately through the flow cytometer (*external standard sensu* Doležel and Bartoš, 2005). Under such protocols, because the standard DNA is not exposed to the secondary compounds contained in the sample (and vice versa), the genome size estimate can be skewed. For example, if secondary compounds in the sample cytosol reduce stain binding (but no such compounds exist in the standard), the sample DNA will have artificially lowered fluorescence relative to that of the standard DNA, and its genome size will be underestimated. Therefore, the modern best-practice protocol (Greilhuber, 1988; Doležel *et al.*, 1992, 1998; Price *et al.*, 2000) dictates that the standard and sample must be processed together before stain is applied; the mixture of nuclei is then run through the flow cytometer (*internal standard*). In this way, the stain–DNA interaction occurs in the same chemical environment for both standard and sample. Finally, some researchers continue to use an intermediate methodology: nuclei of the standard and sample are exposed to the stain separately, before being mixed and run simultaneously through the flow cytometer (*pseudo-internal standard*). A pseudo-internal standard does not solve the problem of differential interference of secondary compounds and, thus, like an external standard, can produce unreliable estimates of genome size (Doležel and Bartoš, 2005).

Here, we investigated the generality of the genome size–invasiveness relationship in *P. arundinacea* by examining a broad set of samples from North American populations, and by employing the current best-practice (internal standard) method of genome size estimation. Specifically, we ask: are there consistent differences in genome size between native (Eurasian) and invasive populations from across North America? Is there a negative correlation between genome size and growth rate; and, if not, is this departure from previous findings explained by our use of a more accurate flow cytometric technique?

MATERIALS AND METHODS

Study species

Phalaris arundinacea (reed canarygrass) is a cool-season, tetraploid perennial grass noted for its ability to reproduce both sexually by seeds and asexually through rhizomes and tillers (Kercher *et al.*, 2007). It possesses many characteristics associated with invasive potential, including the capacity for early season growth, rapid vegetative spread, high growth potential, wide physiological tolerance and high architectural plasticity (reviewed by Lavergne and Molofsky, 2004; Zedler and Kercher, 2004). We note that diploid and hexaploid forms also occur within a broadly proscribed *P. arundinacea* species complex (Baldini, 1995) but, because these cytotypes are more restricted in their geographical distribution and occupy unique niches, we follow previous authors in considering them distinct species (Baldini, 1995; Jakubowski *et al.*, 2011) and do not examine them in this study.

Phalaris arundinacea is native to North America and Eurasia (Merigliano and Lesica, 1998; Jakubowski *et al.*, 2013; Nelson *et al.*, 2014). Eurasian genotypes were introduced to North America in the 1850s for use as a forage crop and in restoration of degraded soils (Lavergne and Molofsky, 2004; Galatowitsch *et al.*, 2000). Introductions have continued to the present day, with recent interest in using it as a biofuel source (Ramstein *et al.*, 2015). Since their introduction, Eurasian genotypes of *P. arundinacea* have formed aggressively invasive populations in many wetlands throughout North America (Lavergne and Molofsky, 2004; Jakubowski *et al.*, 2013), although the species can also be found in upland sites due to its drought resistance (Nelson and Anderson, 2015). Despite evidence that native North American genotypes dominated herbarium samples collected in the late 1800s and early 1900s (Jakubowski *et al.*, 2013), North America is now dominated by invasive Eurasian genotypes, with 98.7 % of 235 localities and >99 % of individuals sampled showing Eurasian genetic signatures (Jakubowski *et al.*, 2014).

Plant material

We gathered 29 accessions of *P. arundinacea* from North America and Europe (Table 1). We collected 17 of these accessions as rhizomes from field localities in seven US states, from

TABLE 1. *Collection information for the* Phalaris arundinacea *accessions used in this study*

Accession	Locality	Range	Latitude	Longitude	NPGS accession	*n*
EKS 01	Arizona, USA	Invasive	35.14931°	–112.17598°		3
EKS 03	Oregon, USA	Invasive	45.46820°	–122.66429°		2
EKS 04	Oregon, USA	Invasive	45.60485°	–122.69351°		1
EKS 05	Oregon, USA	Invasive	45.65231°	–122.83412°		1
EKS 08*	Ontario, Canada	Invasive			PI 372558	3
EKS 09*	Alaska, USA	Invasive			PI 371754	3
EKS 10	Canada	Invasive			PI 278706	3
EKS 11	Austria	Native			PI 251842	3
EKS 12*	Austria	Native			PI 251841	3
EKS 13*	Switzerland	Native			PI 235485	3
EKS 14	Switzerland	Native			PI 235484	3
EKS 15*	Switzerland	Native			PI 235483	3
EKS 16*	Switzerland	Native			PI 235482	3
EKS 17*	Germany	Native			PI 235023	3
EKS 19	Denmark	Native			PI 235551	3
EKS 21*	Former Serbia and Montenegro	Native			PI 251426	2
EKS 22	Iowa, USA	Invasive	41.01319°	–93.32528°		3
EKS 23	Iowa, USA	Invasive	41.01150°	–93.32712°		3
EKS 24	Ohio, USA	Invasive	39.71851°	–84.00584°		3
EKS 25	Ohio, USA	Invasive	39.82877°	–84.00275°		3
EKS 26	Ohio, USA	Invasive	39.80829°	–84.00593°		3
EKS 27	Ohio, USA	Invasive	39.27456°	–84.73417°		3
EKS 28	Ohio, USA	Invasive	39.42069°	–84.03993°		3
EKS 29	New Mexico, USA	Invasive	36.53189°	–105.71186°		3
EKS 30	North Carolina, USA	Invasive	35.09836°	–83.15057°		3
EKS 31	North Carolina, USA	Invasive	35.19188°	–83.38254°		5
EKS 32	North Carolina, USA	Invasive	35.31863°	–83.50633°		7
EKS 33	Georgia, USA	Invasive	34.99358°	–83.38090°		3
EKS 34	North Carolina, USA	Invasive	35.11055°	–83.10157°		3

Range = whether the accession is from the native or the invasive range; NPGS accession = US National Plant Germplasm System Accession number (for further collection details for each accession, see http://www.ars-grin.gov/npgs/); *n* = number of individuals grown for the current study.

*Accession tested by Jakubowski *et al.* (2011, 2014) and confirmed to be Eurasian via 15 simple sequence repeat markers

the Pacific Northwest to North Carolina, to ensure a broad representation of the invasive range. Within a locality, individual rhizomes were collected at >10 m spacing to ensure a high probability that independent genets were sampled. We obtained the remaining 12 accessions as seeds from the US National Plant Germplasm System (https://www.ars-grin.gov/npgs/). Of the 20 North American accessions, two were of confirmed Eurasian origin (Jakubowski *et al.*, 2014; see Table 1), and the rest were highly likely to be of Eurasian origin given their distribution (with reference to Jakubowski *et al.*, 2014). For purposes of replication, we also attempted to obtain access to the 12 *P. arundinacea* accessions examined in Lavergne *et al.* (2010), but were unable to do so. However, our three North Carolina accessions (EKS30–EKS32, Table 1) were collected from the same locality ('near Franklin, centered on 35°19N, 83°38W'; Lavergne and Molofsky, 2007) as the three North Carolina accessions of Lavergne *et al.* (2010). Thus, half of the invasive range accessions from the previous study have locality matches in our data set.

We grew a mean of 3.0 individuals (range 1–7 individuals) from each of the 29 accessions, resulting in a total of 87 individuals. These plants were grown in the UNM research greenhouses (Albuquerque, NM, USA) in gallon pots with Sun Gro Metro Mix 360 potting soil (Sun Gro Horticulture, Agawam, MA, USA). The temperature in the greenhouse was maintained between 19.6 and 25.5 °C. Pots were watered to saturation three times daily using a drip irrigation system.

Estimation of DNA content

We obtained three nuclear genome size estimates for each individual, two using an internal standard and one using a pseudo-internal standard to understand potential causes of differences from previous estimates. The general chopping and flow cytometric methods were developed by Galbraith *et al.* (1983). Genome sizes are presented as holoploid (2C) values in picograms (pg), where 1 pg is approximately equal to 10^9 bp (for the exact relationship, see Doležel *et al.*, 2003).

Our internal standard protocol followed Baack *et al.* (2005). As a standard, we used rye, *Secale cereale* 'Dankovske' (2C genome size 16.19 pg); seeds were from a line specifically curated as a flow cytometric standard (Doležel *et al.*, 1998) and obtained from the Centre of Plant Structural and Functional Genomics of the Institute of Experimental Botany, Czech Republic. Seeds of *S. cereale* were germinated and grown in the greenhouse. Approximately 50 mg of *P. arundinacea* leaf tissue and 70 mg of *S. cereale* leaf tissue were chopped together (i.e. simultaneously and in the same Petri dish) using a razor blade in 1 mL of cold nucleus isolation buffer (NIB; Bino *et al.*, 1992; see Supplementary Data Table S1). Once the leaf tissue was homogenized, an additional 1 mL of NIB was added to the slurry. Using Miracloth (CalBiochem, Pasadena, CA, USA), the solution was filtered and then purified by centrifugation (15 682 × *g*, 12 s). After removing the supernatant, 0.6 mL of staining solution (Arumuganathan and Earle, 1991; see Supplementary Data Table S2) was added to the isolated pellet. Thus, the binding of the propidium iodide stain to the sample DNA and the standard DNA occurred in the same chemical environment. The nuclei solution was then vortexed until the pellet was no longer visible and placed on ice until it was loaded on the flow cytometer.

Our pseudo-internal standard protocol followed Lavergne *et al.* (2010) in using chicken erythrocyte nuclei as a standard (BioSure® Product Number 1006, Grass Valley, CA, USA; 2C genome size of 2.50 pg) and in separately staining the sample and standard nuclei. Approximately 50 mg of *P. arundinacea* leaf tissue was chopped using a razor blade in 1 mL of cold NIB. As above, once the leaf tissue was homogenized, an additional 1 mL of NIB was added to the slurry. As above, the solution was filtered and then purified by centrifugation. After removing the supernatant, 0.6 mL of staining solution was added to the isolated pellet. Next, 0.25 mL of stained pseudo-internal standard solution (one drop of chicken erythrocyte nuclei and 0.45 mL of staining solution) was added to the pellet. Thus, the binding of the propidium iodide stain to the sample DNA and the standard DNA occurred independently. As above, the nuclei solution was vortexed until the pellet was no longer visible and placed on ice until it was loaded on the flow cytometer.

Genome size was measured using an Attune NxT Acoustic Focusing Cytometer (Applied Biosystems, Carlsbad, CA, USA). Fluorescence values at maximum peak height for each G1 peak (one for *P. arundinacea* and one for the standard) were used to compute the genome size of each *P. arundinacea* individual using a custom Python script. During May to August 2015, for each individual, a pair of samples prepared with the pseudo-internal vs. internal protocols was run on the cytometer (on the same day in 72 % of cases to minimize possible noise from temporal variation in sample preparation and cytometer performance). During August to November 2015, all samples were run again with the internal standard protocol to evaluate its consistency ('internal standard run 2'). Representative histograms for the pseudo-internal and internal protocols are given in Supplementary Data Figs S1 and S2.

Growth rate measurements

We measured growth rates on transplants of each of the 87 individuals. We removed two 6 cm pieces of rhizome from each individual on 14 and 15 August 2015 to reduce maternal environmental effects on growth. Each rhizome was planted at a depth of 0.5 cm in its own 10.2 × 10.2 cm pot filled with potting soil (as above). This transplant generation was placed in a greenhouse under the same temperatures as the parent plants (19.6–25.5 °C) and watered to saturation three times a day under a mister spraying system. Following Lavergne *et al.* (2010), we measured growth rate as stem length increment in centimetres (summed across all tillers within a plant) between day 15 and 78 of growth. Growth rate measurements were averaged within individuals and then within accessions for a sample size of 29 accession-level growth rates.

Statistical analyses

For all analyses except the comparison of genome sizes in native vs. invasive accessions (see below), we averaged across individuals to arrive at a single genome size estimate per accession per protocol/run. While these estimates were based on all

87 individuals for the pseudo-internal and internal standard round 1 protocols, missing data (corrupted files for one individual each for EKS8 and EKS9) meant that the genome sizes for internal standard round 2 were based on 85 individuals. All statistics were done in R version 3.2.5 (R Core Team, 2016) running in R Studio (http://www.rstudio.com/). Residuals were checked and met analysis of variance (ANOVA) assumptions for normality and homogeneity of variances.

Genome size estimates by protocol and range (native vs. invasive). We first asked whether genome size (GS) differed according to standardization protocol (ANOVA, model GS ~ protocol), comparing the internal standard round 1 and round 2 results with the pseudo-internal standard results in two separate analyses. We then asked whether genome size differed between native and invasive range accessions for each protocol/run (internal standard run 1, internal standard run 2, pseudo-internal standard). For comparison with previous results (Lavergne *et al.* 2010), the data were genome sizes for individuals rather than accession averages, and we employed a likelihood ratio test based on a linear mixed-effect model with accession (population) as a random effect and range (invasive vs. native) as a fixed effect. This was implemented in R package 'nlme' (Pinheiro *et al.* 2017).

Genome size and growth rate. We then examined the correlation between genome size and growth rate separately for the internal and pseudo-internal standard protocols [analyses of covariance (ANCOVAs), model: growth rate ~ GS + range + GS × range; R package 'lm']. We first averaged the two internal standard rounds to obtain a single internal standard genome size estimate for each accession. If the GS × range term was significant, we then ran simple linear regressions by range to examine whether slopes differed significantly from zero for the invasive and native range accessions (model: growth rate ~ GS).

Weighted analyses. Finally, we performed a complementary set of analyses (parallel to those described above) in which the contribution of each individual genome size estimate was weighted by its quality (for full methods and results, see Supplementary Data Appendix S1). Quality estimates were based on percentage coefficient of variation (CV%), derived by processing the cytometer output files with the R package 'flowPloidy' (Smith, 2017). The CV% is related to the width of the sample and standard peaks in the histogram representing fluorescence of nuclei; narrower peaks (lower CV%) represent greater quality. Because some cytometer files (34 of 259 or 13 %) were lost during two data storage equipment failures between the time of genome size estimation (2015) and later CV% estimation (November 2017), the average CV% values reported here are based on subsets of the data (see Supplementary Data Appendix S1).

All data sets associated with this project are available from the Open Science Framework repository at DOI 10.17605/OSF.IO/JZ6EU.

RESULTS

Genome size estimates by protocol and range (native vs. invasive)

Over all accessions, estimated *P. arundinacea* mean genome size (± s.e.m.) differed substantially (by >1.3 pg) between the internal and pseudo-internal standard protocols (Table 2). Differences were highly significant ($F_{1,56}$ = 292, P < 0.0001 round 1 internal vs. pseudo-internal and $F_{1,56}$ = 268, P < 0.0001 round 2 internal vs. pseudo-internal). Genome size estimates for all accessions are presented in Supplementary Data Table S3.

When using an internal standard protocol, invasive and native accessions of *P. arundinacea* did not differ in genome size (Fig. 1; $LR_{3,4}$ = 0.86 and 0.35, P = 0.353 and 0.555 for internal standard rounds 1 and 2, respectively). When using the pseudo-internal standard protocol, invasive accessions of *P. arundinacea* had significantly smaller genome sizes than native accessions (Fig. 1; $LR_{3,4}$ = 4.23, P = 0.040). Weighted analyses reproduced both of these patterns (Supplementary Data Appendix S1), with the exception that the second result (smaller genome sizes in invasive accessions when using pseudo-internal standardization) became marginally significant (P = 0.096).

Genome size and growth rate

When using an internal standard to obtain genome size estimates (Fig. 2A), there was no evidence of an effect of genome size on growth rate in either invasive or native accessions (genome size, $F_{1,25}$ = 0.148, P= 0.704; range, $F_{1,25}$ = 0.048, P = 0.828; genome size × range, $F_{1,25}$ = 0.034, P = 0.856). In contrast, when using a pseudo-internal standard protocol (Fig. 2B), the relationship between genome size and growth rate differed depending on accession origin (GS × range interaction, $F_{1,25}$ = 11.43, P = 0.002). For native accessions, growth rate increased with increasing genome size ($F_{1,7}$ = 6.912, P = 0.034). For invasive accessions, there was a trend ($F_{1,18}$ = 4.034, P = 0.060) for growth rate to decline with increasing genome size. Weighted analyses reproduced all four of these patterns (Supplementary Data Appendix S1), with the exception that the last result (the trend for growth rate to decline with increasing genome size in invasive accessions) went from marginally significant to significant (P = 0.038).

TABLE 2. *Mean 2C genome size estimates and quality metrics for 29* P. arundinacea *accessions under different standardization protocols*

Standardization protocol	2C genome size (pg)	s.e.	2C range (pg)	Mean peak quality (CV%)		*n*
				Sample	Standard	
Pseudo-internal	9.11	0.07	8.1–11.5	7.45	5.15	68
Internal (round 1)	10.45	0.04	9.2–11.0	5.46	5.21	72
Internal (round 2)	10.48	0.03	9.5–11.2	5.70	5.02	85

n = number of individual genotypes for which peak quality was estimated.

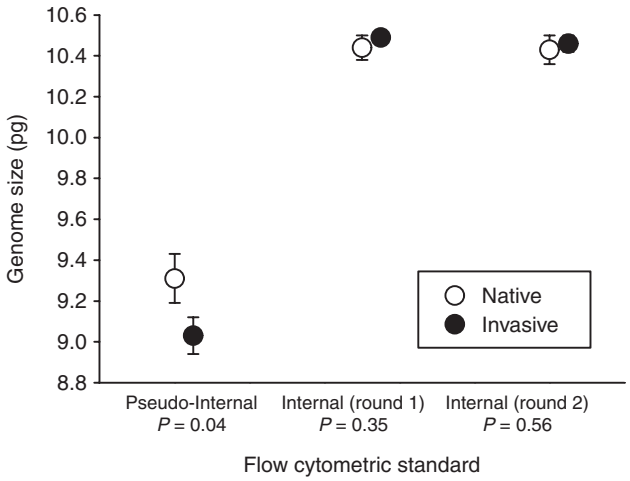

FIG. 1. Genome size estimates (mean ± s.e.) from invasive (n = 20) and native (n = 9) accessions of *P. arundinacea* using different flow cytometric protocols. Genome size estimates were averaged across individuals to obtain mean genome size for an accession, and then accession means were averaged to obtain the grand means plotted here. *P*-values are from likelihood ratio tests for the effect of range (native vs. invasive); see the Materials and methods. Pseudo-internal standard protocol: invasive mean 9.03 pg; native mean 9.31 pg. Internal standard protocol round 1: invasive mean 10.49 pg; native mean 10.44 pg. Internal standard protocol round 2: invasive mean 10.46 pg; native mean 10.43 pg.

DISCUSSION

Our results reveal several key points. We found no differences in genome size between native and invasive range accessions of *P. arundinacea*, and no relationship between genome size and growth rates in our broad sample of North American populations. We suspect that the differences between our findings and those of previous studies may be traced to our use of the best-practice flow cytometric protocol for genome size estimation in plants, rather than to differences in population sampling: we were able to replicate previous patterns by using the older pseudo-internal protocol. Our results thus suggest that a reduction in genome size is not a driving force behind invasiveness in North American populations of *P. arundinacea.*

An updated genome size estimate for P. arundinacea

Because we recorded genome sizes from multiple *P. arundinacea* accessions throughout its native and introduced range, we are able to provide an updated estimate for average genome size in the species. Our best estimate of the 2C genome size of tetraploid *P. arundinacea* is 10.46 ± 0.03 pg (combining internal standard rounds 1 and 2). This agrees well with another recent estimate using internal standards of 10.35 ± 0.03 pg (17 accessions collected in Japan; range 10.15–10.57 pg; calculated from Table 1 of Akiyama *et al.*, 2015). Previous estimates using older techniques tend to be lower: 9.26 pg (pseudo-internal standard, Lavergne *et al.*, 2010) and 8.25 pg (Feulgen densitometry, Kadir 1974, as reported in Bennett and Smith, 1976).

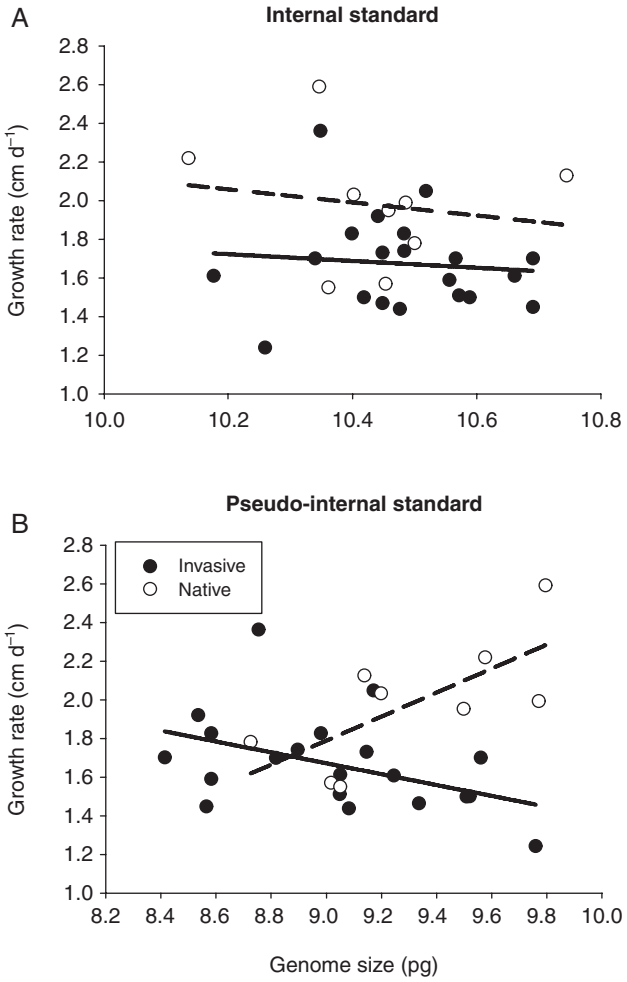

FIG. 2. Relationships between estimated genome sizes and growth rates for *P. arundinacea* in a greenhouse experiment. Each data point represents a mean across individuals (genotypes) within an accession. Note that the *x*-axis scale differs between panels. (A) When using an internal standard protocol (average of rounds 1 and 2), there was an approx. 0.6 pg range in genome size. Origin did not matter (genome size × range, P = 0.856) and there was no correlation (P = 0.704) between genome size and growth rate. (B) When using a pseudo-internal standard, there was an approx. 1.4 pg range in genome size. Genome size and growth rate were significantly correlated: origin mattered (genome size × range, P = 0.002); the correlation trended negative (but not quite significant, P = 0.060) for invasive accessions (solid line) and was positive (P = 0.034) for native accessions (dashed line).

Comparison with previous studies of P. arundinacea *genome size and invasiveness*

We expanded the range of geographic sampling relative to previous genome size studies of North American and European *P. arundinacea*. The 12 accessions examined in Lavergne *et al.* (2010) were from France and the Czech Republic (native range, four and two accessions, respectively) and North Carolina and Vermont (invasive range, three accessions from each state), while our 29 accessions came from five European countries and 11 US states/Canadian provinces (including North Carolina; see Table 1).

While we failed to detect genome size–invasiveness patterns using best-practice genome size estimation methodologies, we

were able to repeat the earlier key findings (Lavergne *et al.* 2010) by using the older flow cytometric protocol. First, under the pseudo-internal protocol, mean genome size of *P. arundinacea* in both studies was slightly over 9 pg (our value, 9.11 ± 0.07 pg; 2010 value, 9.26 pg). Secondly, as in Lavergne *et al.* (2010), we found a small but significant reduction in genome size between native and invasive range accessions when using the pseudo-internal protocol (our value, 3.1 % reduction; 2010 value, 1.7 % reduction, based on averages from Table 1 of Lavergne *et al.*, 2010). Thirdly, we found similar genome size–growth rate relationships when using the pseudo-internal protocol: the relationship between genome size and growth rate differed depending on accession origin (native range vs. invasive range); growth rate declined with increasing genome size in invasive accessions (though the trend was marginally significant in our study and significant in the 2010 study); and for native accessions in both studies, growth rate increased with increasing genome size (though it was significant in our study but not in the 2010 study). Significance levels may differ due to different choices during statistical analysis: we chose to average genome size estimates of each individual within an accession prior to analysis in order to account for non-independence, while the 2010 study may not have done so.

Overall, the different pools of *P. arundinacea* genotypes used in the two studies had similar biological characteristics (i.e. they showed nearly identical relationships between growth rates and estimated genome sizes using pseudo-internal protocols), despite being sampled from mostly different geographic locations. Thus, we feel confident that the major differences in results between the two studies arise from our addition of an internal standard protocol rather than differences in the plant materials examined.

Potential mechanisms causing apparent variation in internal vs. pseudo-internal genome sizes

We hypothesize that *P. arundinacea* contains foliar secondary compounds that interfere with genome size estimation (e.g. 'self-tanning', Greilhuber, 1988). This hypothesis is based on (1) the general demonstration of interference in several other plant species (see Box 1) and (2) the disparity between internal and pseudo-internal results in *P. arundinacea* (Figs 1 and 2). The relationship between pseudo-internal genome sizes and growth rates in *P. arundinacea* (Fig. 2; also Lavergne *et al.*, 2010) might also suggest the interference of secondary compounds. We wonder if, instead of small genomes causing fast growth (Lavergne *et al.*, 2010), fast growth may cause the *appearance* of small genomes under this inaccurate flow cytometric protocol. If growth rate is correlated with plant chemistry, tissues of fast- vs. slow-growing individuals might have differing amounts of secondary compounds, a result that has been shown in other plant species for many classes of secondary compounds (e.g. phenolics; Close and McArthur, 2002). We further note that correlated evolution of growth rate and plant chemistry is well documented at the interspecific level and indeed forms the basis for a major theory of why slow- vs. fast-growing plant species are differentially defended against herbivores: the growth–defense trade-off hypothesis (Coley *et al.*, 1985; Coley, 1988; Huot *et al.*, 2014). If some of these growth rate-associated compounds interfere with the binding of the staining solution to DNA (Box 1; Greilhuber, 1988; Noirot *et al.*, 2003), growth rate differences could cause variation in pseudo-internal genome size estimates among individuals with identical genome size. Further work is clearly needed to test this hypothesis in *P. arundinacea* and in other species.

Reduction in genome size is not the driving force behind invasiveness in a broad sample of North American P. arundinacea

Our results indicate that genome size is not correlated with invasiveness in *P. arundinacea*, either within populations or when comparing between Eurasian (native range) and North American (invasive range) populations. We note that Akiyama *et al.* (2015) found that three populations considered invasive in Japan had 1.4 % smaller genome sizes than 14 native Japanese populations. However, there was clear overlap, in that the invasive populations ranked second, third and eighth in ascending order of genome size within the 17 populations. Therefore, we suggest that general explanations for invasiveness of *P. arundinacea* must reside elsewhere.

Our results from *P. arundinacea* are similar to those of the only other study of intraspecific genome size variation and invasiveness-relevant traits of which we are aware, Meyerson *et al.*'s (2016) study of the wetland invader *Phragmites australis*. Variation in monoploid genome sizes (estimated using internal standards) was used to predict the values of several traits while controlling for variation in ploidy level across a common garden of 166 accessions. Of greatest interest for our purposes are stem height and stem number (as proxies for growth rate); interestingly, the only significant relationship for these variables was a positive relationship between genome size and stem height within the higher ploidy (8*x*) cytotype, while the relationship within the 4*x* cytotype trended negative but was non-significant (Meyerson *et al.*, 2016). Thus, the *Phragmites* results do not support the general prediction that smaller genomes should lead to higher values of invasiveness traits.

If genome size reduction does not explain invasiveness of Eurasian *P. arundinacea* in North America, what does? The recent confirmation of the existence of rare, persistent native North American lineages of *P. arundinacea* (Jakubowski *et al.*, 2013, 2014) offers the potential for powerful comparative ecological experiments investigating the factors that have led to invasiveness of Eurasian genotypes. Traits present in Eurasian genotypes but absent from North American genotypes would be prime candidates for investigation (Jakubowski *et al.*, 2014). For example, the idea that plants from the Old World may be better adapted to anthropogenic disturbances than plants in the New World (and therefore 'pre-adapted' for weediness) is an old one (Gray, 1879), and *P. arundinacea* may be well suited for testing this hypothesis directly. Many traits that have previously been implicated as key for invasiveness in *P. arundinacea* could fit the pre-adaptation hypothesis, including its highly plastic response to nutrients (Martina and von Ende, 2013), wide ecophysiological range (Nelson and Anderson, 2015) and flexible reproductive strategy (Kercher *et al.*, 2007). Common garden experiments could determine whether traits expected to confer success in disturbed environments actually are more prevalent in Eurasian than in native North American genotypes, as predicted by the pre-adaptation hypothesis.

Moving from a consideration of pre-adaptation to post-introduction evolution, interestingly, we found that native range accessions grew faster than those collected from the invasive range (Fig. 2; 1.98 ± 0.11 vs. 1.67 ± 0.06 cm d^{-1}, $F_{1,27} = 7.79$, $P = 0.010$). Thus, at least with respect to early stem elongation rates, *P. arundinacea* apparently does not match the predictions of the EICA (evolution of enhanced competitive ability) hypothesis, which posits that release from enemies in the invasive range often allows evolution towards greater competitive ability, including higher growth rates (Blossey and Notzold, 1995).

It has been suggested that *P. arundinacea* has higher genetic diversity and phenotypic variation in its invasive populations relative to native Eurasian populations (Lavergne and Molofsky, 2007), though this result has not been uniformly found [e.g. Jakubowski *et al.* (2014) found comparable allelic richness at 15 microsatellite loci]. If diversity levels are indeed higher in invasive populations, the pattern suggests a history of multiple introductions and admixture, which in turn could have increased invasiveness via hybridization (Ellstrand and Schierenbeck, 2000; Lavergne and Molofsky, 2007; Hovick and Whitney, 2014). So far, evidence suggests that hybridization between North American and Eurasian genotypes is extremely rare (Jakubowski *et al.*, 2013), but hybridization between different Eurasian sub-populations has been widespread in Europe (Jakubowski *et al.*, 2011). Although such mixing in the native range might limit the benefits of admixture in North America (Jakubowski *et al.*, 2011), the existence of diagnosable Eurasian sub-populations means that it is still possible for novel post-introduction genotypes to be formed. Common garden experiments (see Hovick and Whitney, 2014) comparing the performance of genotypes sampled from Eurasia vs. hybrids could indicate whether post-introduction admixture could have boosted invasiveness in North America.

Future directions: genome size and invasiveness

How do we reconcile our findings of no genome size–invasiveness relationship within *P. arundinacea* with cross-species results (Kubešová *et al.*, 2010; Pandit *et al.*, 2014) indicating a general association between small genome size and invasive behaviour in plants? We have three suggestions about how to move forward.

First, any biological claim regarding genome sizes in plants, from correlations of genome size with invasiveness to claims of environmental regulation of genome structure (e.g. Price and Johnston, 1996; Hidalgo *et al.*, 2015), should make use of the most reliable methods of estimation. As we demonstrate here, unreliable methods can lead to spurious correlations between genome size and organismal traits, and a misunderstanding of the drivers of invasiveness.

Secondly, further investigation of the relationship between genome size and invasiveness is needed at the intraspecific level. By reducing the confounding factors found in cross-species analyses, such studies have the potential to discover robust genome size–invasiveness relationships, should they exist. In addition to the *P. arundinacea* (this study; Lavergne *et al.*, 2010) and *Phragmites australis* (Meyerson *et al.*, 2016) systems, other intraspecific study systems of potential value could be identified by cross-referencing the Šmarda and Bureš (2010) list of species with well-documented intraspecific genome size variation with global lists of weeds and invaders. The latter lists include the Global Compendium of Weeds (http://www.hear.org/gcw/), the Global Invasive Species Database (http://www.iucngisd.org/gisd/) and the Global Invasive Species Information Network (www.gisin.org). For example, cross-referencing Šmarda and Bureš (2010) with the Global Compendium of Weeds results in several candidate species including *Festuca rupicola*, *Dasypyrum villosum*, *Bituminaria bituminosa*, *Curcuma longa*, *Lagenaria siceraria*, *Picris hieracioides*, *Silene latifolia* and *Lolium* spp.

Finally, cross-species studies would benefit from inclusion of greater numbers of traits (in addition to genome size) in order to ferret out the strongest predictors of invasiveness. Structural equation modelling (SEM) could also be used to understand the complex pathways linking genome size and ploidy level to the many intermediate traits that actually determine invasiveness. A promising new development is a set of tools for building SEMs while accounting for phylogenetic non-independence of taxa (Lefcheck, 2016); this technique is key, as the data points in cross-species analyses are necessarily phylogenetically non-independent.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: protocol for preparation of nucleus isolation buffer (NIB). Table S2: protocol for preparation of staining solution. Table S3: genome size estimates (pg) for the *Phalaris arundinacea* accessions used in this study. Figure S1: representative histogram, pseudo-internal standard protocol. Figure S2: representative histogram, internal standard protocol. Appendix S1: weighted analyses of genome size–invasiveness relationships in *Phalaris arundinacea*.

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