Comparison of Genome Size in Reed Canarygrass (*Phalaris arundinacea* L.) Exotic and Putative Native Japanese Genotypes by Flow Cytometry

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Abstract

Reed canarygrass (*Phalaris arundinacea* L.) is a cool-season perennial grass widely distributed throughout North America, Europe and Asia. *P. arundinacea* is currently considered a major invasive plant species in the USA. Despite recent concerns that *P. arundinacea* has become an invasive plant species in Japan, differences between invasive and native Japanese genotypes remain unclear. We used flow cytometry to clarify genome size differences among 14 populations of putative native Japanese *P. arundinacea* genotypes and three exotic cultivars. The average genome size of the 14 Japanese populations and the three exotic *P. arundinacea* differed significantly ($P < 0.05$) and ranged from 4963.90 to 5166.69 Mbp/C. These results implied that the sampled populations included native Japanese genotypes. These populations may be useful in discriminating between invasive and native Japanese *P. arundinacea* genotypes.

Introduction

Reed canarygrass (*Phalaris arundinacea* L.) is a cool-season perennial grass widely distributed throughout North America, Europe and Asia (Bews 1929, Hitchcock 1935). Two ploidy levels, tetraploid ($2n = 4x = 28$) and hexaploid ($2n = 6x = 42$), have been reported in *P. arundinacea* (McWilliam and Neal-Smith 1962). The creeping rhizomes of *P. arundinacea* exhibit vigorous growth, and the species exhibits a marked tolerance to changes in humidity, snow, cold and heat. In addition, because *P. arundinacea* can be propagated in various ways (seeds, tillers and rhizomes), the species has been an important source of forage in the USA since the nineteenth century, when the European cultivar was introduced, and is also expected to be a promising source of biomass material in the future (Lewandowski *et al.* 2003, Casler *et al.* 2009). However, the high propagation of *P. arundinacea* is also a potential ecological problem, and it is perceived as a major invasive species in the USA (Merigliano & Lesica 1998, Lavergne & Molofsky 2007). It is also thought that the introgression of germplasm from European cultivars into native North American genotypes could account for the increased invasiveness of the species (Merigliano & Lesica 1998).

In Japan, *P. arundinacea* has been used extensively as forage since the latter half of the twentieth century, and, as in America, is currently considered an invasive plant species (Hokkaido Government 2004, 2010). The differences between native and invasive *P. arundinacea* populations have not yet been investigated, and it is unclear whether genotypes of native Japanese *P. arundinacea* still exist in the wild, or whether these have been contaminated

by invasive genotypes. To locate genotypes of putative native Japanese *P. arundinacea*, we previously collected *P. arundinacea* specimens from areas in the Tohoku region that were far away from locations with well-established forage crop fields (Ueyama & Kubota 2009, Ueyama & Akiyama 2010).

Information about the genome size of a species is important for biodiversity and evolutionary studies because it is thought to play an important role in evolution (Gregory & Hebert 1999). Such information is also essential for genetic research, i.e. constructing DNA libraries and genetic maps, and also designing molecular markers, which explains the numerous studies conducted to date to determine the genome size of many plant species (Bennett & Leitch 1995, Bennett *et al.* 2000). While the size of tetraploid *P. arundinacea* was reported as 4009.8 Mbp/C (4.1 pg in 978 Mbp/pg equivalent (Doležel *et al.* 2003)), and that of hexaploid *P. arundinacea* was reported as 5574.6 Mbp/C (5.7 pg) and 5868 Mbp/C (6.0 pg) (Bennett & Smith 1976), the genome size of Japanese *P. arundinacea* remains indeterminate and the prevalence of tetraploidy or hexaploidy is unclear. Flow cytometry is a powerful tool for the efficient, rapid and convenient estimation of genome size in plants (Doležel & Bartos 2005).

The objectives of the current study were, therefore, to estimate and index the genome size and ploidy level of putative native Japanese *P. arundinacea* genotypes by flow cytometry, and compare the derived index with exotic *P. arundinacea* cultivars.

Materials and methods

1. Plant materials

Five individuals of each of the three most widely introduced exotic tetraploid *P. arundinacea* variety/cultivars ($2n = 4x = 28$), i.e. 'Canada common', 'Palaton' and 'Vantage', and five individuals of each of the 14 putative native Japanese population (Ueyama & Akiyama 2010, Ueyama & Kubota 2009) were analyzed in 2012 ($n = 85$) (Fig. 1, Table 1). In addition to the five individuals of the K8-12 population and 'Palaton' that were analyzed in 2012 and again in 2013, a further 14 individuals of the K8-12 and 12 individuals of 'Palaton' were analyzed in 2013, and 8 individuals of another exotic tetraploid *P. arundinacea* cultivar 'Venture' were also analyzed in 2013. All the plants, except for 'Venture' cultivated in pots, were cultivated in a field in Morioka city, Iwate prefecture, Japan in 2011-2013. *Pisum sativum* L. 'Ctirad' (4.545 pg/C, 4445.01 Mbp/C), one of the species in Doležel's set of standard plants (Doležel *et al.* 2007) was utilized as the primary standard. *Solanum lycopersicum* L. 'Stupické palní rané' (0.98 pg/C, 958.44 Mbp/C), which was also one of Doležel's set of
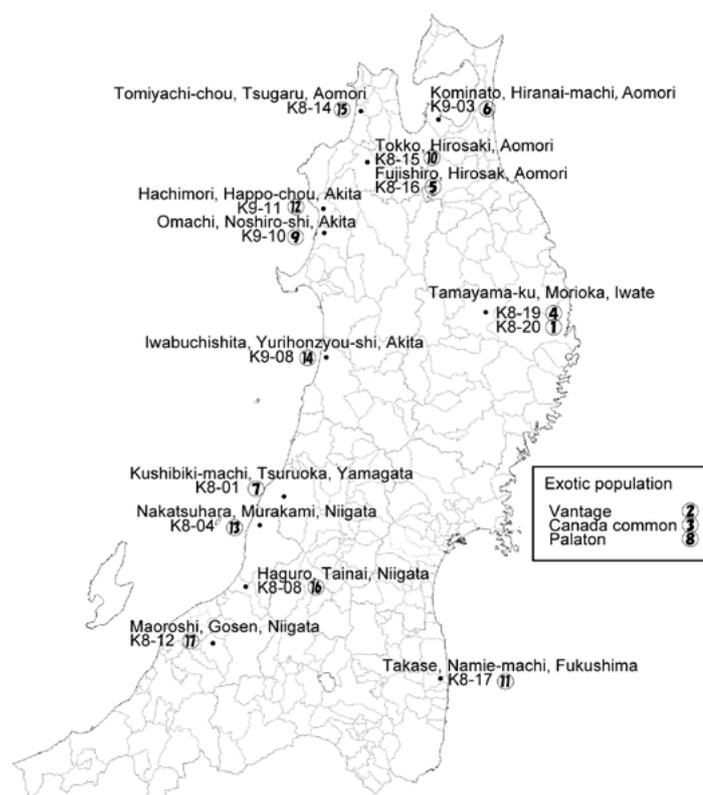

Fig. 1. Locations of putative native Japanese *P. arundinacea* genotypes collected in the Tohoku region of Japan. Circled numbers after the population indicate the rank of the genome size in Table 1.

standard plants, was used to confirm the consistency and reliability of the obtained results. The seeds of *P. sativum* and *S. lycopersicum* were imported into Japan with permission from the Minister of Agriculture, Forestry and Fisheries under the plant protection act. A Morioka Fescue Standard (MFS) clone of diploid *Festuca pratensis* Huds. 'First' (2n = 2x = 14) was used as an internal standard to estimate the genome size of *P. arundinacea*.

2. Isolation of nuclei and flow cytometry

Approximately 1.5 cm^2 of young leaf tissue from a target sample and one of the standards were placed together in a plastic Petri dish and soaked in 1.0 ml of Tris-MgCl$_2$ buffer (Pfosser *et al.* 1995). Nuclei were isolated by chopping leaf tissue with a razor blade and then filtering the resulting nucleus suspension through a 30 μm nylon mesh to remove debris. Subsequently, 1.0 ml of a Tris-MgCl$_2$ buffer containing 50 μg/mL RNase and 100 μg/mL propidium iodide (PI) was added to the nucleus suspension and the fluorescence intensities of each nucleus suspension were measured using an acoustic focusing cytometer (Attune™; Applied Biosystems LLC, Foster City CA, USA). Due to a malfunction with the cytometer used in 2012, different cytometers were used in 2012 and 2013. For each sample, an average of 2000 nuclei in the G1-phase was analyzed in triplicate and the nuclear region was gated using forward and side scatter parameters to minimize noise associated with fluorescent debris. Following Doležel and Bartos

(2005), PI data with a coefficient of variation (CV) of 5% or less for the DNA peaks of the standard and sample was used to calculate genome sizes using the formula: genome size of sample = sample peak mean / standard peak mean × genome size of standard.

Results and discussion

1. Genome size of the *F. pratensis* 'First' MFS clone

Many standard materials have been proposed for use in the estimation of plant genome size (Johnston *et al.* 1999, Suda & Leitch 2010). Dolezel *et al.* (2007) established a DNA reference standard set including eight plant species, *Allium cepa* L., *Glycine max.*, *P. sativum*, *Raphanus sativus* L., *Secale cereale* L., *S. lycopersicum*, *Vicia faba* L. and *Zea mays* L. However, Praça-Fontes *et al.* (2011) proposed *S. lycopersicum* and *P. sativum* from Dolezel's set and *Arabidopsis thaliana* (L.) Heyhn as the most adequate primary standards and *R. sativus* as the least adequate primary standard. *A. thaliana* and *P. sativum* are annual plants, while *S. lycopersicum* is a perennial plant, although in Japan, *S. lycopersicum* is a nearly annual plant due to the low temperatures experienced in winter. *F. pratensis*, meanwhile, is a perennial grass species with a high tolerance to environmental stress, implying that it is well suited for use as a standard as it can be maintained easily and utilized year-round. Therefore, we selected the MFS clone *F. pratensis* 'First' for use as an internal standard

Table 1. Genome sizes of *P. arundinacea*

Rank	Population	Average genome size of population (Mbp/C)	S.D.	Tukey's test			JP number	Location
1	K8-20	4963.90	8.05			C	236461	Tamayamaku, Morioka, Iwate
2	Vantage	4966.79	6.76			C		Exotic
3	Canada common	4984.85	7.37		B	C		Exotic
4	K8-19	5013.31	6.26		B	C	236460	Tamayamaku, Morioka, Iwate
5	K8-16	5018.92	4.51		B	C	236459	Fujishiro, Hirosaki, Aomori
6	K9-3	5026.13	6.33		B	C	247085	Kominato, Hiranaimachi, Aomori
7	K8-1	5042.59	21.93	A	B	C	236449	Katsuraaramata, Tsuruoka, Yamagata
8	Palaton	5044.85	7.47	A	B	C		Exotic
9	K9-10	5068.46	2.95	A	B	C	247088	Omachi, Noshiro, Akita
10	K8-15	5069.39	10.30	A	B	C	236458	Tokko, Hirosaki, Aomori
11	K8-17	5084.91	5.57	A	B	C	236462	Takase, Namie, Fukushima
12	K9-11	5090.73	4.62	A	B	C	-	Hachimori, Yamamotogun-Happocho, Akita
13	K8-4	5091.46	4.30	A	B	C	236452	Nakatsuhara, Murakami, Niigata
14	K9-8	5113.44	11.69	A	B		247086	Iwabuchishita, Yurihonjou, Akita
15	K8-14	5118.29	2.76	A	B		236457	Tomiyachicho, Tsugaru, Aomori
16	K8-8	5135.60	6.60	A	B		236454	Haguro, Tainai, Niigata
17	K8-12	5166.69	8.01	A			236455	Maoroshi, Gosen, Niigata

to estimate the genome size of *P. arundinacea*. A total of 10 replicates of *P. sativum* 'Ctirad' and MFS clone nuclei were prepared and analyzed. Results revealed that the size of the MFS clone genome was 3196.69 ± 22.28 Mbp/C. To confirm this result, the genome size of the MFS clone was estimated eight times with *S. lycopersicum* 'Stupické palní rané' as an internal standard. Results showed the genome size of the MFS clone to be 3259.11 ± 43.89 Mbp/C and showed significant difference between the results obtained using *P. sativum* (t-test, P < 0.01); however, the difference was quite small. *P. sativum* genome size is closer to *F. pratensis* than *S. lycopersicum*, indicating that *P. sativum* is a better internal standard. Šmarda *et al.* (2008) previously reported the genome size of *F. pratensis* as 3164.81 Mbp/C (3.236 pg/C). Based on these data, we believe that the value we obtained for the genome size of *F. pratensis* (3196.69 Mbp/C) is reliable.

2. Genome size of *P. arundinacea*

The nuclei of the MFS clone and *P. arundinacea* were analyzed in triplicate in 2012 (Table 1). The standard deviations of the measurements obtained for each individual were extremely small, ranging from a minimum of ± 1.55 to a maximum of ± 55.60, with an average range of ± 12.42 for all 85 individuals, indicating the high precision of our flow cytometry system. Moreover, when 1000 and 5000 nuclei from five individuals were analyzed in triplicate, t-test analysis showed no significant difference in the obtained results. Consequently, 1000 nuclei were considered sufficient for analysis using this system.

ANOVA analyses were then performed to compare the genome sizes of the five individuals within each population. The results showed that, except for 'Vantage', the differences in genome sizes among the five individuals in each population were significant (P < 0.01). Since *P. arundinacea* is primarily allotetraploid (Lavergne & Molofsky 2004), the obtained results may reflect the genomic complexities associated with being allotetraploid. The reason 'Vantage' showed no significant differences among individuals may be due to uniformity in the genome of the cultivar. ANOVA analysis showed that the genome sizes of the 17 analyzed populations differed significantly (P < 0.01). Tukey's test was used to compare genome sizes; however, it remained difficult to distinguish between populations based on genome size (Table 1).

The genome sizes of the three exotic *P. arundinacea* and the 14 Japanese populations ranged from 4963.90 to 5166.69 Mbp/C (Table 1), indicating that all the plants had the same ploidy level; namely, that all Japanese populations are tetraploid. T-tests showed significant difference (P < 0.05) in the average genome size between the three exotic *P. arundinacea* (4998.83 Mbp/C) and the 14 Japanese populations (5071.70 Mbp/C). Lavergne *et al.*

(2010) compared the genome size of native European and North American *P. arundinacea* genotypes collected in an area where they were exotic in the USA, and found that the North American genotypes were significantly smaller than the European genotypes. Of the exotic *P. arundinacea* analyzed in this study, 'Palaton' and 'Vantage' are bred in North America, and 'Canada common' is the most widely used cultivar in Canada. These populations and 'Venture' are mainly utilized in forage of reed canarygrass in Japan. Although 'Venture' was not analyzed in 2012, the observation of large genome size in our populations implies that our samples include native Japanese *P. arundinacea* genotypes.

Inconsistencies were observed between the results of our study and those of previous reports. Specifically, genome size in our populations ranged from 4963.90 to 5166.69 Mbp/C, but were reported elsewhere as 4009.8 Mbp/C (Bennett & Smith 1976) and 4469.46 to 4567.26 Mbp/C (Lavergne *et al.* 2010). One of the reasons for this inconsistency may be the differing genome size among cultivars, even within the same species (Akiyama *et al.* 2008), and among allogamous individuals of the same cultivar. However, the observed differences may have been too great to attribute to this reason alone. Bennett & Smith (1976) estimated genome size by densitometry utilizing the Feulgen reaction, which is considered more sensitive to inhibition by plant secondary metabolites than DNA staining with fluorochromes in flow cytometry (Greilhuber 2008). Indeed, it is possible that inhibition by secondary metabolites in *P. arundinacea* may result in the underestimation of genome size. Lavergne *et al.* (2010) used chicken erythrocyte nuclei as a standard; however, erythrocyte nuclei are clearly unsuitable for estimating plant genome size (Price *et al.* 1980, Johnston *et al.* 1999). Accordingly, the observed inconsistencies may have arisen due to differences in the standards and estimation methods used.

In terms of genome size, the genome sizes of the exotic *P. arundinacea* 'Vantage', 'Canada common' and 'Palaton' are ranked second, third and eighth, respectively, in Table 1. These results imply that *P. arundinacea* populations with genomes that are markedly larger than the exotic plants may be native Japanese genotypes. Rice agriculture is widely practiced in Niigata and Akita prefectures, where forage grass is scarcely cultivated. Conversely, livestock agriculture in the Tohoku region is most intensive in Iwate prefecture, where forage grass is widely cultivated and where it is more likely that exotic *P. arundinacea* may have invaded the grasslands (Ministry of Agriculture, Forestry and Fisheries of Japan, 2013). The *P. arundinacea* populations with larger genome size tend to be distributed in Niigata and Akita prefectures (Fig. 1). The K8-19 and K8-20 genotypes, which are ranked first and fourth in Table 1, were collected in Iwate prefecture. The results of this study, therefore, closely reflect regional differences in agriculture.

Nineteen individuals of the K8-12 population, which had the largest genome among the Japanese genotypes sampled, and 17 individuals of the 'Palaton', which had the largest genome size among the exotic *P. arundinacea* sampled in 2012, were analyzed at least six times in 2013. Additionally, 8 individuals of the 'Venture' were analyzed in 2013. Recently, only two cultivars, 'Palaton' and 'Venture' were commercially distributed in Japan. The genome sizes of K8-12, 'Palaton' and 'Venture', were estimated as 5267.76 ± 60.38 Mbp/C (n = 19), 5160.07 ± 25.67 Mbp/C (n = 17) and 5209.07 ± 18.45 (n = 8), respectively (Fig. 2). 'Venture' had the largest genome size among the exotic *P. arundinacea* in this study. Tukey analysis showed significant genome size differences among the populations (P < 0.05), and that of K8-12 significantly exceeded that of 'Venture', suggesting that the K8-12 population consisted of native Japanese genotypes.

Inconsistencies were also observed between the results obtained in 2012 and 2013. All results were analyzed by the same type of acoustic focusing cytometer (Attune™); however, the results in 2012 and 2013 were analyzed using different cytometers. Unfortunately, the cytometer used to produce the results in 2012 (Table 1) broke, and the results obtained in 2013 were analyzed using a new cytometer. In our flow cytometry system used in 2013, no statistically significant differences were observed between different operators. Doležel & Bartos (2005) recommend using a single instrument to identify small differences in genome size. According to the manufacturer, disparity of approximately 2% may arise due to differences between the two instruments. Therefore, we consider differences between the results obtained in 2012 and 2013 to be due to differences in the instruments.

In this study, we demonstrated a method for the discrimination of native Japanese genotypes of *P. arundinacea* from invasive *P. arundinacea* genotypes based on genome size. Developing a straightforward method for the screening of native Japanese genotypes would be important in the selection of Japanese genotypes for commercial *P. arundinacea* breeding programs and maintaining the natural habitat of Japanese *P. arundinacea*. Conversely, given the lack of control materials for native Japanese genotypes, further studies focusing on chromosome and molecular phylogenetic analyses (e.g. amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), etc.) should be undertaken to identify native Japanese genotypes. The K8-12 population is considered to have potential for use as a control, while other populations we collected may also be useful for identifying native Japanese *P. arundinacea* genotypes.

Acknowledgments

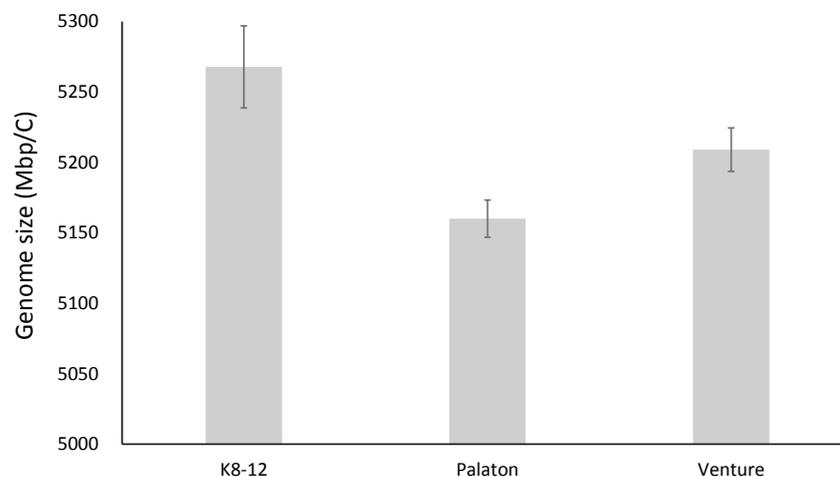**Fig. 2. Genome sizes of K8-12 (n = 19), 'Palaton' (n = 17) and 'Venture' (n = 8) *P. arundinacea* genotypes. Error bars indicate 95% confidence intervals with respect to mean genome size.**

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