Received: 14 August 2021 | Accepted: 21 October 2021

DOI: 10.1002/aps3.11452

APPLICATION ARTICLE

Applications in Plant Sciences

A dormant resource for genome size estimation in ferns: C-value inference of the Ophioglossaceae using herbarium specimen spores

Li-Yaung Kuo[1] | **Sheng Kai Tang**[1] | **Tzu-Tong Kao**[2,3] | **Atsushi Ebihara**[4] | **Susan Fawcett**[5] | **Min-Chien Hsiao**[6] | **Wataru Shinohara**[7] | **Benjamin Dauphin**[8]

[1]Institute of Molecular and Cellular Biology, National Tsing Hua University, Hsinchu City, Taiwan

[2]Department of Biology, Duke University, Durham, North Carolina, USA

[3]Institute of Plant and Microbial Biology, Academia Sinica, Taipei City, Taiwan

[4]Department of Botany, National Museum of Nature and Science, Tsukuba, Ibaraki, Japan

[5]University and Jepson Herbaria, University of California, Berkeley, California, USA

[6]Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei City, Taiwan

[7]Faculty of Education, Kagawa University, Takamatsu, Kagawa, Japan

[8]Swiss Federal Research Institute WSL, Birmensdorf, Switzerland

Correspondence

Li-Yaung Kuo, Institute of Molecular and Cellular Biology, National Tsing Hua University, No. 101, Sec. 2, Guangfu Road, East District, Hsinchu City, Hsinchu 30013, Taiwan.
Email: lykuo@life.nthu.edu.tw

Abstract

Premise: The great variation of genome size (C-value) across land plants is linked to various adaptative features. Flow cytometry (FCM), the standard approach to estimating C-values, relies mostly on fresh materials, performing poorly when used with herbarium materials. No fern C-value reports have been derived from herbarium specimens; however, the herbarium spores of some ferns remain highly viable for decades and are thus promising for further investigation. To explore this possibility, we evaluated herbarium spore collections of Ophioglossaceae ferns using FCM.

Methods: Flow cytometry was conducted on 24 spore samples, representing eight of the 12 genera of the Ophioglossaceae, using specimens ranging in age from 2.6 to 111 years obtained from five herbaria.

Results: Regardless of the genus or the source herbarium, high-quality C-value data were generated from 17 samples, with the oldest being 26 years old. Estimates of the C-values from sporophytic tissues of known ploidy did not reveal any evidence of apomixis for the species surveyed here. We also detected a pronounced genome downsizing in *Sceptridium* polyploids.

Discussion: The recent success of FCM for C-value estimation using spores provides a much more convenient method of utilizing "dry" refrigerated materials. We demonstrate here that herbarium spores of some ferns are also promising for this use, even for older specimens.

KEYWORDS

apomixis, C-value, flow cytometry, genome downsizing, genome size, Ophioglossaceae, polyploid, *Sceptridium*

Genome size is highly variable among land plants (Leitch and Leitch, 2013) and has important ecological and evolutionary implications (Mei et al., 2018; Faizullah et al., 2021; Fujiwara et al., 2021). Differences in genome size are linked to various adaptative features of plants, including their cell division rates (Šímová and Herben, 2012), reproductive modes (te Beest et al., 2012), growth form (Morgan and Westoby, 2005), ecological stoichiometry (Guignard et al., 2016), and diversification rates (Landis et al., 2018). This basic metric is also fundamental to cutting-edge genome research (e.g., Kuo and Li, 2019). To facilitate a better understanding of genome size diversity, flow cytometry (FCM) has been applied for the estimation of plant genome size (or C-value, which refers to the DNA content of a haploid genome [or "holoploid" genome, sensu Greilhuber et al., 2005]) for more than half a century (Kamentsky et al., 1965), and is still considered the most cost-effective tool for this purpose. C-value inference from FCM relies mostly on fresh materials; this technique rarely performs well when using dried samples, especially tissue

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2021 The Authors. *Applications in Plant Sciences* published by Wiley Periodicals LLC on behalf of Botanical Society of America.

from aged herbarium collections. The use of herbarium materials for FCM would enable the rapid generation of vast quantities of data, which has thus far not been feasible. Although several C-value studies have used herbarium materials (e.g., Šmarda, 2006; Roberts, 2007; Viruel et al., 2019), cases are limited outside of flowering plants. To our knowledge, no published C-values for ferns have been successfully derived from herbarium material. Nonetheless, the spores of ferns hold extraordinary potential for further genomic research. The viability of fern spores can be maintained long term at low temperatures, and the herbarium-stored spores of some species have >50% germination rates even after 30 years (Windham et al., 1986). Furthermore, previous FCM tests also demonstrated that fern spores stored at 4°C for more than 10 years can be extracted containing detectable nuclei that still satisfy the quality and quantity criteria for C-value publication (Kuo et al., 2017).

This evidence demonstrates the potential of using fern spores from herbarium specimens to infer genome size using FCM approaches. To explore this possibility in the interest of developing new techniques, we targeted the Ophioglossaceae family of ferns and performed an assessment of herbarium collections in this study. Our samples originated from five different herbaria with collection dates ranging from 1907 to 2015, covering most of the generic diversity in this family (8/12 genera, sensu PPG I, 2016; Zhang et al., 2020). In addition, we also used refrigerated spores and/or fresh leaf collections for some species to compare the effects of storage conditions, organ types, and ploidy levels. By sampling densely within the genus *Sceptridium* Lyon, we aimed to determine an upper limit for the age of herbarium specimens that can be used to accurately estimate C-values.

METHODS

Spore collections

To sample herbarium spores of Ophioglossaceae taxa, we sought promising materials in five herbaria (DUKE, TAIF, TNS, UC, and VT [Thiers, 2021]) and selected collections with 10 or more mature but unopened sporangia or an accumulation of released spores on the herbarium specimens. We collected spores using either destructive or non-destructive sampling: in the former, unopened sporangia were removed using forceps; in the latter, herbarium specimens were inverted onto white paper, and the fallen spores were then collected. We collected a total of 24 spore samples for eight genera: *Botrychium* Sw., *Cheiroglossa* C. Presl, *Helminthostachys* Kaulf., *Japanobotrychum* Masam., *Ophioderma* (Blume) Endl., *Ophioglossum* L., *Sahashia* Li Bing Zhang & Liang Zhang, and *Sceptridium*. For *Sceptridium*, we sampled collections of varying ages (two to 111 years old). The oldest specimen was collected in 1907 and preserved in the TNS herbarium. Three additional spore samples were collected from fresh spikes of *Sceptridium* plants, air-dried at room temperature for one week, and stored in 1.5-mL tubes at 4°C for up to eight years until the experiment. The detailed voucher information is provided in Appendix S1.

Flow cytometry

We followed the protocol developed by Kuo and Huang (2017) for our FCM experiments. For the buffer, we used LB01 (Doležel et al., 2007) containing 4% PVP-40, 0.5% (v/v) 2-mercaptoethanol, and RNase A (0.1 mg/mL). For the spore samples, ca. 1.2–4.7 mg of spores or unopened sporangia on a spike were used for each FCM reaction. Samples comprising dried unopened sporangia were first ground using a pellet pestle to extract the spores inside, then rinsed with LB01 buffer. These spore solutions were further spun down by 100 × *g* for 3 min to pellet the spores, and the supernatant was discarded. The wash and spin-down cycles were usually repeated twice, or until the supernatant became transparent. For each sample, the spores and 250 μL of LB01 were mixed in a 1.5-mL tube, to which 16 2.3-mm stainless steel beads (BioSpec Products, Bartlesville, Oklahoma, USA) were added. The samples were then vortexed at 1900 rpm in a Vortex-Genie 2 (Scientific Industries, Bohemia, New York, USA) for 1 min to extract the spore nuclei, and subsequently filtered through 20-μm nylon mesh (Sysmex Partec, Goerlitz, Germany) to remove large debris. Next, the samples were combined with a nuclei extraction from an internal standard with an appropriate genome size (see below), and propidium iodide (PI) solution was then added to each tube to yield a final concentration of 0.04 mg/mL. The PI staining was performed in the dark at 4°C for 1 h.

For five Ophioglossaceae species (see Table 1, taxa identified as "leaf [2*n*]" under the column "Organ [generation]" and "this study" under the column "Reference and voucher [herbarium]") and the internal standards, fresh leaf tissues were collected from either field-grown plants or plants cultivated in growth chambers. We used the same LB01 buffer and staining conditions as described above, but used a chopping method to extract the nuclei, in which a ~400-mm^2 piece of leaf tissue was chopped with a razor in a Petri dish on ice until the tissue slices were mostly <1 mm in size (Kuo and Huang, 2017). These extractions were then filtered through 30-μm nylon meshes (Sysmex Partec).

Finally, we performed the FCM analyses on a BD FACScan system (BD Biosciences, Franklin Lakes, New Jersey, USA), using three replicates per sample. For the nuclei peaks of both the samples and the internal standards, we set criteria to collect >1300 particles per peak, each with a coefficient of variation (CV) <5%. To compare the genome size differences between the samples and the CV among specimens of varying ages, we performed all statistical analyses using R (R Core Team, 2021) with the packages "dplyr" version 1.0.6 and "multcompView" version 0.1.8.

We prepared the nuclei extractions of the spore samples and internal standards from the leaf tissues separately because they required different mechanical pre-treatments

TABLE 1 Genome size (C-value) of Ophioglossaceae collections used in this study

Species	Organ (generation)	1C-value ± SD (pg)[a]	1C-value ± SD (Gbp)[a,b]	Sample CV mean (%)	Ploidy level[c]	References and voucher (herbarium)
Botrychium minganense Vict.	spore* (*n*)	25.58 ± 0.11[d]	25.02 ± 0.11[d]	3.59	4	This study; UVMVT-187865 (VT)
Botrychium minganense	leaf (2*n*)	26.84 ± 2.53	26.25 ± 2.47	—	4	Williams and Waller, 2012
Cheiroglossa palmata (L.) C. Presl	spore* (*n*)	54.70 ± 0.04[e]	53.50 ± 0.03[e]	1.58	—	This study; UC-1737361 (UC)
Helminthostachys zeylanica (L.) Hook.	spore* (*n*)	13.35 ± 0.05[f]	13.06 ± 0.05[f]	4.30	—	This study; TAIF-192355 (TAIF)
Helminthostachys zeylanica	leaf (2*n*)	11.93 ± 0.03[f]	11.67 ± 0.02[f]	3.93	—	This study; *Hung 355* (TNM)
Japanobotrychum lanuginosum (Wall. ex Hook. & Grev.) M. Nishida ex Tagawa	spore* (*n*)	14.43 ± 0.02[f]	14.11 ± 0.02[f]	3.61	—	This study; TAIF-211564 (TAIF)
Ophioderma falcatum O. Deg.	spore* (*n*)	98.88 ± 0.77[g]	96.70 ± 0.75[g]	2.52	—	This study; TAIF-463848 (TAIF)
Ophioglossum petiolatum Hook.	spore* (*n*)	72.07 ± 0.27[e]	70.48 ± 0.26[e]	2.93	—	This study; TAIF-286867 (TAIF)
Ophioglossum petiolatum	leaf (2*n*)	65.55 ± 1.93	64.11 ± 1.89	—	—	Obermayer et al., 2002
Ophioglossum petiolatum	leaf (2*n*)	55.00	53.79	—	—	Price et al., 1972
Sahashia stricta (Underw.) Li Bing Zhang & Liang Zhang	spore* (*n*)	6.17 ± 0.03[f]	6.03 ± 0.03[f]	3.76	—	This study; TAIF-458136 (TAIF)
Sahashia stricta	leaf (2*n*)	6.31 ± 0.04[e]	6.17 ± 0.03[e]	4.47	—	This study; *Shinohara 2021080501* (Kagawa University)
Sceptridium biternatum (Savigny) Lyon	spore* (*n*)	9.72 ± 0.02[h]	9.51 ± 0.01[h]	2.98	2	This study; DUKE-10003123 (DUKE)
Sceptridium dissectum (Spreng.) Lyon	spore* (*n*)	9.64 ± 0.01[e]	9.43 ± 0.01[e]	4.74	2	This study; DUKE-10003210 (DUKE)
Sceptridium dissectum	spore* (*n*)	9.71 ± 0.02[h]	9.50 ± 0.01[h]	2.91	2	This study; DUKE-10003151 (DUKE)
Sceptridium dissectum	spore* (*n*)	9.72 ± 0.03[e]	9.51 ± 0.02[e]	4.27	2	This study; DUKE-10003147 (DUKE)
Sceptridium multifidum var. *robustum* (Rupr.) M. Nishida	spore* (*n*)	9.14 ± 0.03[e]	8.94 ± 0.02[e]	4.55	2	This study; TNS-01230059 (TNS)
Sceptridium multifidum var. *robustum*	leaf (2*n*)	9.80 ± 0.10	9.58 ± 0.09	1.78	2	Fujiwara et al., 2021
Sceptridium nipponicum (Makino) Holub	spore* (*n*)	10.11 ± 0.01[e]	9.88 ± 0.00[e]	4.85	2	This study; TAIF-500141 (TAIF)
Sceptridium nipponicum	leaf (2*n*)	9.77 ± 0.06[f]	9.56 ± 0.06[f]	4.02	2	This study; TNS-1230001 (TNS)
Sceptridium ternatum (Thunb.) Lyon	spore (*n*)	10.12 ± 0.06[h]	9.90 ± 0.05[h]	3.10	2	This study; *Lu 18937* (TAIF)
Sceptridium ternatum	leaf (2*n*)	9.65 ± 0.05	9.43 ± 0.04	—	2	Fujiwara et al., 2021
Sceptridium daucifolium (Wall. ex Hook. & Grev.) Lyon	spore (*n*)	14.55 ± 0.08[f]	14.23 ± 0.07[f]	3.38	4	This study; *Wade 5270* (TAIF)
Sceptridium formosanum (Tagawa) Holub	spore* (*n*)	13.05 ± 0.03[f]	12.76 ± 0.02[f]	3.72	4	This study; TAIF-500159 (TAIF)

(Continues)

TABLE 1 (Continued)

Species	Organ (generation)	1C-value ± SD (pg)[a]	1C-value ± SD (Gbp)[a,b]	Sample CV mean (%)	Ploidy level[c]	References and voucher (herbarium)
Sceptridium formosanum	spore (*n*)	13.16 ± 0.02[d]	12.87 ± 0.02[d]	2.74	4	This study; *Lu 31527* (TAIF)
Sceptridium formosanum	leaf (2*n*)	12.69 ± 0.03[d]	12.41 ± 0.03[d]	3.02	4	This study; *Lu 31527* (TAIF)
Sceptridium japonicum (Prantl) Lyon	spore* (*n*)	20.47 ± 0.06[e]	20.02 ± 0.05[e]	2.93	6	This study; TAIF-500146 (TAIF)
Sceptridium japonicum	spore* (*n*)	24.67 ± 0.16[d]	24.13 ± 0.16[d]	3.58	6	This study; TNS-762894 (TNS)
Sceptridium japonicum	leaf (2*n*)	20.28 ± 0.04[e]	19.83 ± 0.04[e]	3.05	6	This study; TNS-1107873 (TNS)
Sceptridium javanicum Sahashi	spore* (*n*)	31.25 ± 0.04[e]	30.56 ± 0.03[e]	3.53	8	This study; TNS-9509028 (TNS)

Note: CV = coefficient of variation.

[a] 1C-value defined by Greilhuber et al. (2005); d–h indicate the internal standard used for each sample.

[b] Calculated as 1 pg = 0.978 Gbp.

[c] Cytology data from Sahashi (1981); Wagner (1993), and Takamiya (1996).

[d] *Secale cereale* L. cv. Dankovske, leaf 2C nuclei = 16.19 pg (Doležel et al., 1998).

[e] *Vicia faba* L. cv. Inovec, leaf 2C nuclei = 26.9 pg (Doležel et al., 1992).

[f] *Nicotiana tabacum* L. cv. Xanthi, leaf 2C nuclei = 10.04 pg (Johnston et al., 1999).

[g] *Haemanthus albiflos* Jacq., leaf 2C nuclei = 76. 0 pg (Zonneveld, 2010).

[h] *Sphaeropteris lepifera* (Hook.) R. M. Tryon, spore 1C nuclei = 6.7 pg (Kuo et al., 2017; Tang et al., unpublished manuscript).

*Spore samples from herbarium specimens.

(i.e., bead-vortexing vs. chopping) to acquire nuclei. These separate preparations have been defined as pseudo-internal standardization (sensu Temsch et al., 2021). Nonetheless, the staining properties of the nuclei per se were unlikely to be changed by the different mechanical pre-treatments. The extractions of a sample and an internal standard were first mixed prior to PI staining; therefore, all the nuclei in the reaction were ensured to be chemically stained under the same conditions in the buffer, so the resulting genome size estimates generated using our approach are expected to be very similar to those generated using a true internal standardization.

RESULTS

Flow cytometry

We successfully inferred the genome sizes of all spore and leaf samples, including all tested genera (Figure 1, Table 1), except for seven *Sceptridium* spore samples (Appendix S1) that did not yield a detectable nuclei peak in our FCM analyses. In terms of CV values, we did not find a significant correlation between nuclei quality and the age of the specimen (Figure 2; $\mathrm{CV}(\%) = 3.698 + \mathrm{storage\_age(yr)} \times -0.004431$, *F*-statistic: $P = 0.797$). Notably, herbarium spores preserved for up to 26 years were still viable for generating FCM data satisfying standards for C-value publication, regardless of genus or the herbarium of origin (Table 1, Appendix S1). The oldest sample was a 1992 collection of *Botrychium minganense* from the VT herbarium (Figure 1A). Voucher data and inferred C-values are summarized in Table 1.

Genome size comparisons

As defined by Greilhuber et al. (2005), the 1C-value of spores, which belong to the haploid gametophytic generation, is determined directly from the DNA content of their nuclei, regardless of reproductive mode. For the leaf samples, the 1C-value corresponds to half of the estimated DNA content from the sporophytic nuclei. The 1C-value inferred from the spore samples is very similar to that from the leaf tissue of the same species (Table 1). These results imply that the genome sizes inferred from herbarium spores are reliable, reflecting the haploid genome sizes expected for spores of sexually reproducing taxa (Table 1, Figure 1B). However, many cases show slightly higher 1C-values based on spores than on sporophytic leaf tissues (Table 1, Figure 1B), and, for *Sceptridium formosanum* (Tagawa) Holub, from both spore and leaf samples from the same individual (Table 1). Higher C-value estimates were previously reported from fern spores (Chen et al., 2017, 2019; Kuo et al., 2017). This is presumably due to the different degrees of chromatin condensation that might affect the binding of the DNA stain (Hare and Johnston, 2012). Interestingly, when comparing their 1Cx-value (i.e., monoploid genome size; nuclear DNA content

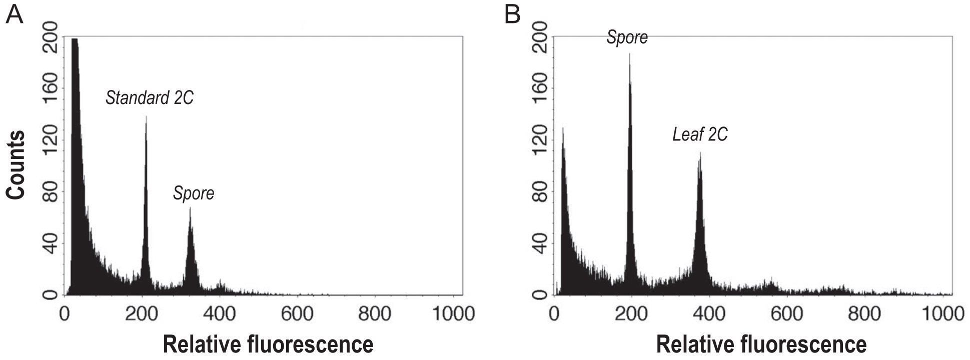

FIGURE 1 Flow cytometric results of (A) a *Botrychium minganense* spore collection (UVMVT-187865 [VT]) from a 26-year-old specimen, and (B) spore and leaf nuclei of *Sceptridium formosanum* (*Lu 31527* [TAIF])

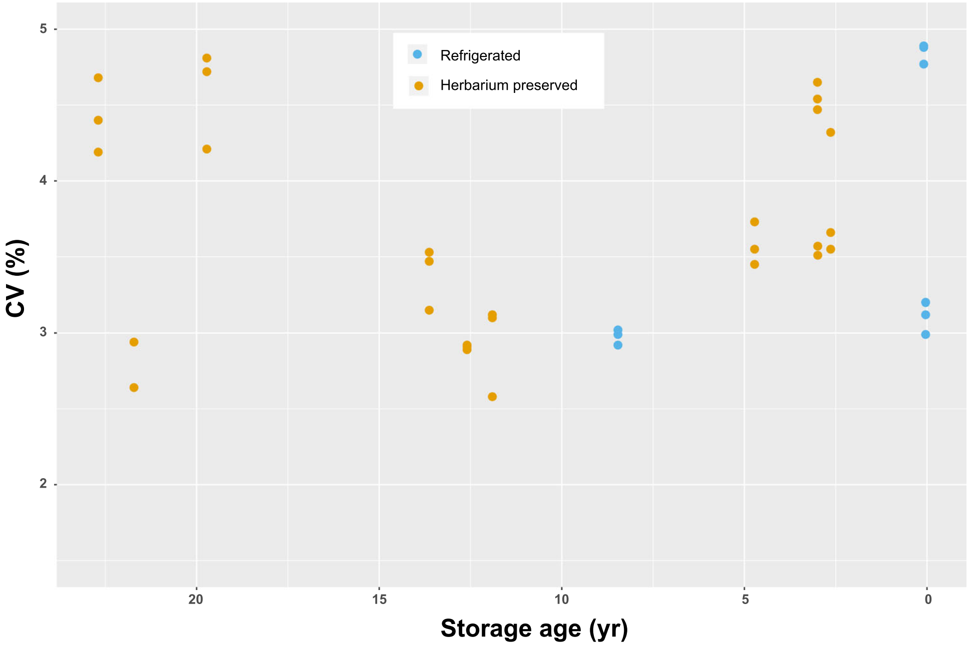

FIGURE 2 Coefficient of variation (CV) of *Sceptridium* spore nuclei from collections of varying ages

divided by its ploidy level), significant differences had been found between genera and between ploidies (Figure 3).

DISCUSSION

Herbarium collections and quality of their spore nuclei

Our study is the first to infer genome sizes from herbarium collections of ferns, and shows that Ophioglossaceae spores collected from specimens up to 26 years old are likely to provide high-quality C-value data (Figure 1A). Based on our small sample size, the quality of herbarium spore nuclei was as high as those of refrigerated and fresh spores (Figure 2). Although a comprehensive sampling was not conducted in every herbarium, we did not see evidence that different storage conditions between herbaria affected the spore nuclei quality of these Ophioglossaceae specimens. We recovered data from >14-year-old collections from four of the five herbaria surveyed here (Appendix S1). Nonetheless, taxon-specific properties might play an important role. So far, we have also succeeded in using herbarium spore collections from other eusporangiate ferns and lycophytes (e.g., *Angiopteris* Hoffm., *Huperzia* Bernh., and *Palhinhaea* Franco & Vasc.; data not shown), but failed in our attempts to measure those of leptosporangiate ferns (Tang et al., unpublished manuscript), in which spore nuclei were apparently highly degraded and therefore difficult to detect with an unambiguous peak in a FCM histogram. Thickened multicellular eusporangia may be better at protecting spores

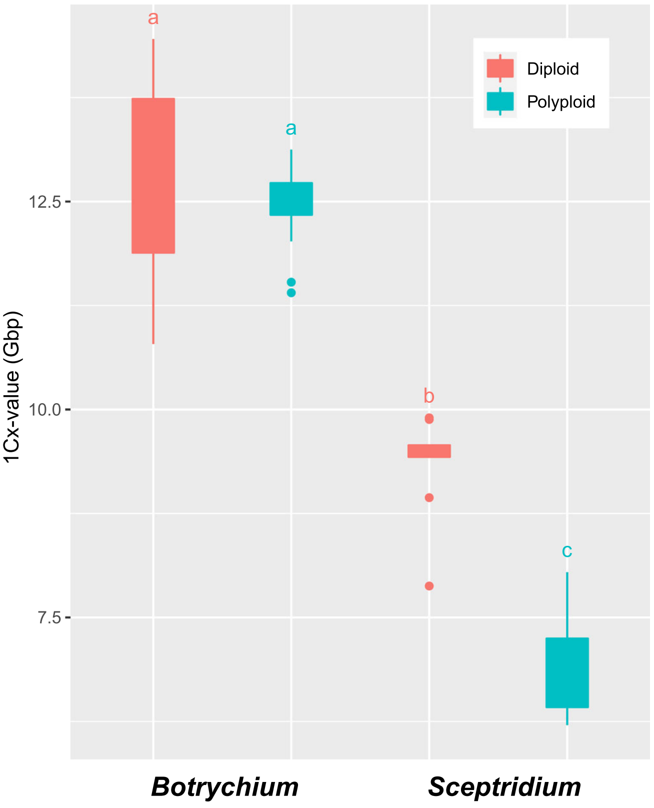

FIGURE 3 1Cx-values of diploids and polyploids in *Botrychium* and *Sceptridium*. The genome size data are from Williams and Waller (2012), Dauphin et al. (2016), Fujiwara et al. (2021), and the present study. The lowercase letters on each bar indicate significant differences as determined using Tukey's honestly significant difference test ($P < 0.05$)

than thin-walled leptosporangia. Notably, the rigid sporocarps of *Marsilea* L. ferns have been shown to preserve spores with high viability nearly one century after the specimens were collected (Johnson, 1985).

Comparing approaches to investigate fern genome sizes

The spore sizes of ferns are widely used to infer ploidy (e.g., Barrington et al., 1986, 2020; Shinohara et al., 2006; Perrie et al., 2010; Chang et al., 2013; Kuo et al., 2016; Patel et al., 2018; and references therein). Plants with two or more times the DNA content in their nuclei than other congeneric species tend to have corresponding larger cell sizes (Comai, 2005; Corneillie et al., 2019), including the sizes of their unicellular spores. Despite this, the correlation between spore size and genome size is evidently not universal across fern lineages (Barrington et al., 2020); for instance, *Tmesipteris elongata* P. A. Dang. has a minimum spore size of 55 μm in length and 26 μm in width (with a volume of 19,458 μm^3 sensu Barrington et al., 2020), and a spore genome size of 74.84 pg (Perrie et al., 2010; Clark et al., 2016). By contrast, *Polystichum imbricans* subsp. *curtum* (Ewan) D. H. Wagner has a similar spore size (volume of 20,399 μm^3) but its haploid genome was only 6.58 pg (Barrington et al., 2020).

Standard FCM-based approaches to estimate genome sizes in ferns and other plants typically rely on fresh materials, particularly leaf tissues, which can be difficult to source. Our recent success using spores for FCM experiments provides a much more convenient way of utilizing "dry" refrigerated materials instead (Kuo et al., 2017). Here, we further demonstrate that the use of herbarium spores of some ferns is promising too. Increasingly, next-generation sequencing (NGS) is being applied to estimate genome sizes by analyzing the *k*-mer frequency or the read mapping of single-copy genes from sequences generated at 10–50× genomic coverage (e.g., Pflug et al., 2020; Pfenninger et al., 2021). However, genome sizes estimated using such NGS approaches can vary greatly depending on the analytic models, and are usually underestimated (Pflug et al., 2020). Seemingly, the genomic characteristics of an organism, including heterozygosity, recent history of polyploidy, and genome size, strongly affect the estimation accuracy of these sequence-based approaches (Pflug et al., 2020). Although such an approach is applicable to archival herbarium specimens, it remains very costly to use for target plants with a large genome size (e.g., most ferns). For example, obtaining 10–50× coverage of Illumina data (i.e., 89–447 Gbp) for diploid *Sceptridium* would cost at least US$900 per sample. Using FCM to estimate genome size thus remains the most cost-effective approach for such samples. The expense of FCM experiments requires no more than one-fifteenth of the cost of NGS, based on a typical one-hour rental fee of a cytometer, and potentially much less than that.

Implication of reproductive modes and genome downsizing

Compared with sexual reproduction, apomixis is less common among ferns, reported in about 10% of species (reviewed in Liu et al., 2012; Grusz, 2016). The vast majority of reports of apomixis are from the Polypodiales, although a few reports also exist from Hymenophyllales. Our current understanding of the prevalence of apomixis across fern diversity, however, seems biased due to difficulties in previous cytological studies (Kuo et al., 2017). One important application of an accurate spore genome size inference is to confirm the reproductive mode of a given fern species (Kuo et al., 2017). For several of the species studied here, we analyzed and compared their genome sizes from both spores and conspecific sporophytic leaf tissues, which we expect to reflect 1C and 2C genome sizes, respectively, in sexually reproducing individuals. For apomicts, we would anticipate similar genome sizes for both gametophytic and sporophytic tissues. In our comparisons of nine species (Table 1), we found no evidence of apomixis for the surveyed taxa, nor of a facultative production of unreduced spores. Given the small size of our sample with both sporophytic and gametophytic data from the same individuals, further FCM work is needed for a conclusive determination of the reproductive modes of these fern species. Previous population genetic studies have shown that Ophioglossaceae species are primarily sexually reproducing (Watano and Sahashi, 1992; Chung et al., 2013; Dauphin et al., 2020; Williams, 2021; and references therein). One cytological report implies apomictic reproduction in an *Ophioglossum* species (Tindale and Roy, 2002; Brownsey and Perrie, 2015), but this requires further confirmation by comparing its spore and leaf genome sizes.

Evidence of genome downsizing is pronounced in *Sceptridium*, in which polyploids have 1Cx-values (i.e., monoploid genome sizes) that are significantly lower than the 1Cx-values of congeneric diploids (Figure 3). This contradicts previous findings that fern species tend to undergo little or no genome downsizing after polyploidization (Nakazato et al., 2008; Henry et al., 2014; Dauphin et al., 2016); for example, the sister genus of *Sceptridium* sensu lato, *Botrychium*, has 1Cx-values that are not significantly different between diploid and polyploid species (Figure 3). Moreover, although both genera share the same basic chromosome number ($x = 45$; Wagner, 1993; Takamiya, 1996), the 1Cx genome sizes of both diploid and polyploid *Botrychium* species are significantly higher than those of *Sceptridium*. This divergent pattern in these sister lineages provides an excellent opportunity for further research on the evolution of genome size and composition.

ACKNOWLEDGMENTS

We thank Pi-Fong Lu (Taiwan Society of Plant Systematics), Cheng-Wei Chen (Biodiversity Research Center, Academia Sinica), and all herbaria curators and staff for their assistance with sample collections; Yao-Moan Huang (Taiwan Forestry Research Institute) for providing refrigerated *S. ternatum* spores; and Fay-Wei Li and two anonymous reviewers for comments on the manuscript. This work was supported by the Higher Education Sprout Project from the Ministry of Education, Taiwan; the Ministry of Science and Technology, Taiwan (grant no. 109-2621-B-007-001-MY3 to L.-Y.K.); and a Japan Society for the Promotion of Science KAKENHI grant (no. 20K06803 to A.E.).

AUTHOR CONTRIBUTIONS

L.-Y.K. and T.-T.K. initiated the idea using Ophioglossaceae herbarium spores as materials. L.-Y.K. and S.K.T. performed the FCM experiments and analyses. L.-Y.K., T.-T.K., A.E., S.F., W.S., and B.D. collected samples. L.-Y.K., T.-T.K., and M.-C.H. cultivated the genome size standards. L.-Y.K. and S.F. prepared the first draft of this manuscript. All authors approved the final version of the manuscript.

ORCID

Li-Yaung Kuo http://orcid.org/0000-0002-3388-3757
Sheng Kai Tang http://orcid.org/0000-0003-3399-2643
Tzu-Tong Kao http://orcid.org/0000-0003-1372-7324
Atsushi Ebihara http://orcid.org/0000-0002-7380-3598
Susan Fawcett http://orcid.org/0000-0001-5021-1779
Benjamin Dauphin http://orcid.org/0000-0003-0982-4252

REFERENCES

Barrington, D. S., C. A. Paris, and T. A. Ranker. 1986. Systematic inferences from spore and stomate size in the ferns. *American Fern Journal* 76: 149–159.

Barrington, D. S., N. R. Patel, and M. W. Southgate. 2020. Inferring the impacts of evolutionary history and ecological constraints on spore size and shape in the ferns. *Applications in Plant Sciences* 8: e11339.

Brownsey, P. J., and L. R. Perrie. 2015. Ophioglossaceae. *In* I. Breitwieser, P. B. Heenan, and A. D. Wilton [eds.], Flora of New Zealand: Ferns and Lycophytes. Fascicle 14. Manaaki Whenua Press, Lincoln, New Zealand.

Chang, Y.-F., J. Li, S.-G. Lu, and H. Schneider. 2013. Species diversity and reticulate evolution in the *Asplenium normale* complex (Aspleniaceae) in China and adjacent areas. *Taxon* 62: 673–687.

Chen, C.-W., M. Sundue, L.-Y. Kuo, W.-C. Teng, and Y.-M. Huang. 2017. Phylogenetic analyses place the monotypic *Dryopolystichum* within Lomariopsidaceae. *PhytoKeys* 78: 83–107.

Chen, C.-W., L.-Y. Kuo, Y.-H. Huang, T.-C. Hsu, M. T. Dang, H. T. Luu, C.-W. Li, and Y.-M. Huang. 2019. A new species and a new record of *Stegnogramma* (Thelypteridaceae; Polypodiales) from southern Vietnam. *Systematic Botany* 44: 768–774.

Chung, M. Y., J. López-Pujol, J. M. Chung, M. O. Moon, and M. G. Chung. 2013. Genetic diversity in the homosporous fern *Ophioglossum vulgatum* (Ophioglossaceae) from South Korea: Inference of mating system and population history. *Journal of Heredity* 104: 263–272.

Clark, J., O. Hidalgo, J. Pellicer, H. Liu, J. Marquardt, Y. Robert, M. Christenhusz, et al. 2016. Genome evolution of ferns: Evidence for relative stasis of genome size across the fern phylogeny. *New Phytologist* 210: 1072–1082.

Comai, L. 2005. The advantages and disadvantages of being polyploid. *Nature Reviews Genetics* 6: 836–846.

Corneillie, S., N. DeStorme, R. VanAcker, J. U. Fangel, M. DeBruyne, R. DeRycke, D. Geelen, et al. 2019. Polyploidy affects plant growth and alters cell wall composition. *Plant Physiology* 179: 74–87.

Dauphin, B., J. Grant, and P. Mráz. 2016. Ploidy level and genome size variation in the homosporous ferns *Botrychium* s.l. (Ophioglossaceae). *Plant Systematics and Evolution* 302: 575–584.

Dauphin, B., J. R. Grant, and D. R. Farrar. 2020. Outcrossing mating system of the early-divergent moonwort fern (*Botrychium lunaria*, Ophioglossaceae) revealed in the European alps. *International Journal of Plant Sciences* 181: 926–936.

Doležel, J., S. Sgorbati, and S. Lucretti. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiologia Plantarum* 85: 625–631.

Doležel, J., J. Greilhuber, S. Lucretti, A. Meister, M. A. Lysák, L. Nardi, and R. Obermayer. 1998. Plant genome size estimation by flow cytometry: Inter-laboratory comparison. *Annals of Botany* 82: 17–26.

Doležel, J., J. Greilhuber, and J. Suda. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244.

Faizullah, L., J. A. Morton, E. I. Hersch-Green, A. M. Walczyk, A. R. Leitch, and I. J. Leitch. 2021. Exploring environmental selection on genome size in angiosperms. *Trends in Plant Science* 26: 1039–1049.

Fujiwara, T., H. Liu, E. I. Meza-Torres, R. E. Morero, A. J. Vega, Z. Liang, A. Ebihara, et al. 2021. Evolution of genome space occupation in ferns: Linking genome diversity and species richness. *Annals of Botany*. https://doi.org/10.1093/aob/mcab094

Greilhuber, J., J. Doležel, M. A. Lysák, and M. D. Bennett. 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Annals of Botany* 95: 255–260.

Grusz, A. L. 2016. A current perspective on apomixis in ferns. *Journal of Systematics and Evolution* 54: 656–665.

Guignard, M. S., R. A. Nichols, R. J. Knell, A. Macdonald, C. A. Romila, M. Trimmer, I. J. Leitch, and A. R. Leitch. 2016. Genome size and ploidy influence angiosperm species' biomass under nitrogen and phosphorus limitation. *New Phytologist* 210: 1195–1206.

Hare, E. E., and J. S. Johnston. 2012. Genome size determination using flow cytometry of propidium iodide-stained nuclei. *In* V. Orgogozo and M. Rockman [eds.], Molecular methods for evolutionary genetics. Methods in Molecular Biology (Methods and Protocols), vol. 772, 3–12. Humana Press, Totowa, New Jersey, USA.

Henry, T. A., J. D. Bainard, and S. G. Newmaster. 2014. Genome size evolution in Ontario ferns (Polypodiidae): Evolutionary correlations with cell size, spore size, and habitat type and an absence of genome downsizing. *Genome* 57: 555–566.

Johnson, D. M. 1985. New records for longevity of *Marsilea* sporocarps. *American Fern Journal* 75: 30–31.

Johnston, J., M. Bennett, A. Rayburn, D. Galbraith, and H. Price. 1999. Reference standards for determination of DNA content of plant nuclei. *American Journal of Botany* 86: 609–613.

Kamentsky, L. A., M. R. Melamed, and H. Derman. 1965. Spectrophotometer: New instrument for ultrarapid cell analysis. *Science* 150: 630–631.

Kuo, L.-Y., A. Ebihara, W. Shinohara, G. Rouhan, K. R. Wood, C.-N. Wang, and W.-L. Chiou. 2016. Historical biogeography of the fern genus *Deparia* (Athyriaceae) and its relation with polyploidy. *Molecular Phylogenetics and Evolution* 104: 123–134.

Kuo, L.-Y., and Y.-M. Huang. 2017. Determining genome size from spores of seedless vascular plants. *Bio-protocol* 7: e2322.

Kuo, L.-Y., Y.-J. Huang, J. Chang, W.-L. Chiou, and Y.-M. Huang. 2017. Evaluating the spore genome sizes of ferns and lycophytes: A flow cytometry approach. *New Phytologist* 213: 1974–1983.

Kuo, L., and F.-W. Li. 2019. A roadmap for fern genome sequencing. *American Fern Journal* 109: 212–223.

Landis, J. B., D. E. Soltis, Z. Li, H. E. Marx, M. S. Barker, D. C. Tank, and P. S. Soltis. 2018. Impact of whole-genome duplication events on diversification rates in angiosperms. *American Journal of Botany* 105: 348–363.

Leitch, I. J., and A. R. Leitch. 2013. Genome size diversity and evolution in land plants. *In* J. Greilhuber, J. Doležel, and J. Wendel [eds.], Plant genome diversity, vol. 2, 307–322. Springer, Berlin, Germany.

Liu, H.-M., R. J. Dyer, Z.-Y. Guo, Z. Meng, J.-H. Li, and H. Schneider. 2012. The evolutionary dynamics of apomixis in ferns: A case study from polystichoid ferns. *Journal of Botany* 2012: 510478.

Mei, W., M. G. Stetter, D. J. Gates, M. C. Stitzer, and J. Ross-Ibarra. 2018. Adaptation in plant genomes: Bigger is different. *American Journal of Botany* 105: 16–19.

Morgan, H. D., and M. Westoby. 2005. The relationship between nuclear DNA content and leaf strategy in seed plants. *Annals of Botany* 96: 1321–1330.

Nakazato, T., M. S. Barker, L. H. Rieseberg, and G. J. Gastony. 2008. Evolution of the nuclear genome of ferns and lycophytes. *In* T. A. Ranker and C. H. Haufler [eds.], Biology and evolution of ferns and lycophytes, 175–200. Cambridge University Press, New York, USA.

Obermayer, R., I. Leitch, L. Hanson, and M. Bennett. 2002. Nuclear DNA C-values in 30 species double the familial representation in pteridophytes. *Annals of Botany* 90: 209–217.

Patel, N., C. Li, L. Zhang, and D. S. Barrington. 2018. Biodiversity and apomixis: Insights from the East-Asian holly ferns in *Polystichum* section *Xiphopolystichum*. *Molecular Phylogenetics and Evolution* 127: 345–355.

Perrie, L. R., P. J. Brownsey, and J. D. Lovis. 2010. *Tmesipteris horomaka*, a new octoploid species from Banks Peninsula. *New Zealand Journal of Botany* 48: 15–29.

Pfenninger, M., P. Schönnenbeck, and T. Schell. 2021. Precise estimation of genome size from NGS data. *BioRxiv* 444645 [Preprint]. Published 18 May 2021 [accessed 1 June 2021]. https://doi.org/10.1101/2021.05.18.444645

Pflug, J. M., V. R. Holmes, C. Burrus, J. S. Johnston, and D. R. Maddison. 2020. Measuring genome sizes using read-depth, k-mers, and flow cytometry: Methodological comparisons in beetles (Coleoptera). *G3: Genes, Genomes, Genetics* 10: 3047–3060.

PPG I. 2016. A community-derived classification for extant lycophytes and ferns. *Journal of Systematics and Evolution* 54: 563–603.

Price, H. J., R. W. Levis, L. W. Coggins, and A. H. Sparrow. 1972. High DNA content of *Sprekelia formosissima* Herbert (Amaryllidaceae) and *Ophioglossum petiolatum* Hook. (Ophioglossaceae). *Experimental Cell Research* 73: 187–191.

R Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website: http://www.R-project.org/ [accessed 1 June 2021].

Roberts, A. V. 2007. The use of bead beating to prepare suspensions of nuclei for flow cytometry from fresh leaves, herbarium leaves, petals and pollen. *Cytometry Part A* 71: 1039–1044.

Sahashi, N. 1981. Morphological and taxonomical studies on Ophioglossales in Japan and the adjacent regions (6). Examination of *Sceptridium daucifolium* (1). *Journal of Japanese Botany* 56: 339–347.

Shinohara, W., T.-W. Hsu, S.-J. Moore, and N. Murakami. 2006. Genetic analysis of the newly found diploid cytotype of *Deparia petersenii* (Woodsiaceae: Pteridophyta): Evidence for multiple origins of the tetraploid. *International Journal of Plant Sciences* 167: 299–309.

Šímová, I., and T. Herben. 2012. Geometrical constraints in the scaling relationships between genome size, cell size and cell cycle length in herbaceous plants. *Proceedings of the Royal Society B: Biological Sciences* 279: 867–875.

Šmarda, P. 2006. DNA ploidy levels and intraspecific DNA content variability in Romanian fescues (*Festuca*, Poaceae) measured in fresh and herbarium material. *Folia Geobotanica* 41: 417–432.

Takamiya, M. 1996. Index to chromosomes of Japanese Pteridophyta (1910–1996). Japan Pteridological Society, Tokyo.

te Beest, M., J. J. LeRoux, D. M. Richardson, A. K. Brysting, J. Suda, M. Kubesová, and P. Pysek. 2012. The more the better? The role of polyploidy in facilitating plant invasions. *Annals of Botany* 109: 19–45.

Temsch, E. M., P. Koutecký, T. Urfus, P. Šmarda, and J. Doležel. 2021. Reference standards for flow cytometric estimation of absolute nuclear DNA content in plants. *Cytometry Part A*. https://doi.org/10.1002/cyto.a.24495

Thiers, B. 2021 (continuously updated). Index Herbariorum. Website: http://sweetgum.nybg.org/science/ih/ [accessed 26 October 2021].

Tindale, M., and S. Roy. 2002. A cytotaxonomic survey of the Pteridophyta of Australia. *Australian Systematic Botany* 15: 839–937.

Viruel, J., M. Conejero, O. Hidalgo, L. Pokorny, R. F. Powell, F. Forest, M. B. Kantar, et al. 2019. A target capture-based method to estimate ploidy from herbarium specimens. *Frontiers in Plant Science* 10: 937.

Wagner, F. S. 1993. Chromosomes of North American grape ferns and moonworts (Ophioglossaceae: *Botrychium*). *University of Michigan Herbarium* 19: 83–92.

Watano, Y., and N. Sahashi. 1992. Predominant inbreeding and its genetic consequences in a homosporous fern genus, *Sceptridium* (Ophioglossaceae). *Systematic Botany* 17: 486–502.

Williams, E. W. 2021. Population genetics of species in the genera *Botrychium* and *Botrypus* (Ophioglossaceae). *American Fern Journal* 111: 129–146.

Williams, E. W., and D. M. Waller. 2012. Phylogenetic placement of species within the genus *Botrychium* s.s. (Ophioglossaceae) on the basis of plastid sequences, amplified fragment length polymorphisms, and flow cytometry. *International Journal of Plant Sciences* 173: 516–531.

Windham, M. D., P. G. Wolf, and T. A. Ranker. 1986. Factors affecting prolonged spore viability in herbarium collections of three species of *Pellaea*. *American Fern Journal* 76: 141–148.

Zhang, L., X. Fan, S. Petchsri, L. Zhou, R. Pollawatn, X. Zhang, X. Zhou, et al. 2020. Evolutionary relationships of the ancient fern lineage the adder's tongues (Ophioglossaceae) with description of *Sahashia* gen. nov. *Cladistics* 36: 380–393.

Zonneveld, B. J. M. 2010. New record holders for maximum genome size in eudicots and monocots. *Journal of Botany* 2010: 527357.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. Spore sampling for the flow cytometric experiments in this study. All experiments were performed in 2018.

How to cite this article: Kuo, L.-Y., S. K. Tang, T.-T. Kao, A. Ebihara, S. Fawcett, M.-C. Hsiao, W. Shinohara, and B. Dauphin. 2021. A dormant resource for genome size estimation in ferns: C-value inference of the Ophioglossaceae using herbarium specimen spores. *Applications in Plant Sciences* 9(11-12): e11452. https://doi.org/10.1002/aps3.11452