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

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## Abstract

**Premise:** The great variation of genome size (C-value) across land plants is linked to various adaptive 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 adaptive features of plants, including their cell division rates (Šimová 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

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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., *Japanobotrychium* 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  $100 \times g$  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  $\mu$ L 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- $\mu$ m 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 [2n]” 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  $\sim 400\text{-mm}^2$  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- $\mu$ m 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)	IC-value $\pm$ SD (pg) <sup>a</sup>	IC-value $\pm$ SD (Gbp) <sup>a,b</sup>	Sample CV mean (%)	Ploidy level <sup>c</sup>	References and voucher (herbarium)
<i>Botrychium minganense</i> Vict.	spore* (n)	25.58 $\pm$ 0.11 <sup>d</sup>	25.02 $\pm$ 0.11 <sup>d</sup>	3.59	4	This study; UVMVT-187865 (VT)
<i>Botrychium minganense</i>	leaf (2n)	26.84 $\pm$ 2.53	26.25 $\pm$ 2.47	—	4	Williams and Waller, 2012
<i>Cheiroglossa palmata</i> (L.) C. Presl	spore* (n)	54.70 $\pm$ 0.04 <sup>e</sup>	53.50 $\pm$ 0.03 <sup>e</sup>	1.58	—	This study; UC-1737361 (UC)
<i>Helminthostachys zeylanica</i> (L.) Hook.	spore* (n)	13.35 $\pm$ 0.05 <sup>f</sup>	13.06 $\pm$ 0.05 <sup>f</sup>	4.30	—	This study; TAIIF-192355 (TAIF)
<i>Helminthostachys zeylanica</i>	leaf (2n)	11.93 $\pm$ 0.03 <sup>f</sup>	11.67 $\pm$ 0.02 <sup>f</sup>	3.93	—	This study; Hung 355 (TNM)
<i>Japanobotrychium lanuginosum</i> (Wall. ex Hook. & Grev.) M. Nishida ex Tagawa	spore* (n)	14.43 $\pm$ 0.02 <sup>f</sup>	14.11 $\pm$ 0.02 <sup>f</sup>	3.61	—	This study; TAIIF-211564 (TAIF)
<i>Ophioderma falcatum</i> O. Deg.	spore* (n)	98.88 $\pm$ 0.77 <sup>g</sup>	96.70 $\pm$ 0.75 <sup>g</sup>	2.52	—	This study; TAIIF-463848 (TAIF)
<i>Ophioglossum petiolatum</i> Hook.	spore* (n)	72.07 $\pm$ 0.27 <sup>e</sup>	70.48 $\pm$ 0.26 <sup>e</sup>	2.93	—	This study; TAIIF-286867 (TAIF)
<i>Ophioglossum petiolatum</i>	leaf (2n)	65.55 $\pm$ 1.93	64.11 $\pm$ 1.89	—	—	Obermayer et al., 2002
<i>Ophioglossum petiolatum</i>	leaf (2n)	55.00	53.79	—	—	Price et al., 1972
<i>Sahashia stricta</i> (Underw.) Li Bing Zhang & Liang Zhang	spore* (n)	6.17 $\pm$ 0.03 <sup>f</sup>	6.03 $\pm$ 0.03 <sup>f</sup>	3.76	—	This study; TAIIF-458136 (TAIF)
<i>Sahashia stricta</i>	leaf (2n)	6.31 $\pm$ 0.04 <sup>e</sup>	6.17 $\pm$ 0.03 <sup>e</sup>	4.47	—	This study; Shinohara 2021080501 (Kagawa University)
<i>Sceptridium biternatum</i> (Savigny) Lyon	spore* (n)	9.72 $\pm$ 0.02 <sup>h</sup>	9.51 $\pm$ 0.01 <sup>h</sup>	2.98	2	This study; DUKE-10003123 (DUKE)
<i>Sceptridium dissectum</i> (Spreng.) Lyon	spore* (n)	9.64 $\pm$ 0.01 <sup>e</sup>	9.43 $\pm$ 0.01 <sup>e</sup>	4.74	2	This study; DUKE-10003210 (DUKE)
<i>Sceptridium dissectum</i>	spore* (n)	9.71 $\pm$ 0.02 <sup>h</sup>	9.50 $\pm$ 0.01 <sup>h</sup>	2.91	2	This study; DUKE-10003151 (DUKE)
<i>Sceptridium dissectum</i>	spore* (n)	9.72 $\pm$ 0.03 <sup>e</sup>	9.51 $\pm$ 0.02 <sup>e</sup>	4.27	2	This study; DUKE-10003147 (DUKE)
<i>Sceptridium multifidum</i> var. <i>robustum</i> (Rupr.) M. Nishida	spore* (n)	9.14 $\pm$ 0.03 <sup>e</sup>	8.94 $\pm$ 0.02 <sup>e</sup>	4.55	2	This study; TNS-01230059 (TNS)
<i>Sceptridium multifidum</i> var. <i>robustum</i>	leaf (2n)	9.80 $\pm$ 0.10	9.58 $\pm$ 0.09	1.78	2	Fujiwara et al., 2021
<i>Sceptridium nipponicum</i> (Makino) Holub	spore* (n)	10.11 $\pm$ 0.01 <sup>e</sup>	9.88 $\pm$ 0.00 <sup>e</sup>	4.85	2	This study; TAIIF-500141 (TAIF)
<i>Sceptridium nipponicum</i>	leaf (2n)	9.77 $\pm$ 0.06 <sup>f</sup>	9.56 $\pm$ 0.06 <sup>f</sup>	4.02	2	This study; TNS-1230001 (TNS)
<i>Sceptridium ternatum</i> (Thunb.) Lyon	spore (n)	10.12 $\pm$ 0.06 <sup>h</sup>	9.90 $\pm$ 0.05 <sup>h</sup>	3.10	2	This study; Lu 18937 (TAIF)
<i>Sceptridium ternatum</i>	leaf (2n)	9.65 $\pm$ 0.05	9.43 $\pm$ 0.04	—	2	Fujiwara et al., 2021
<i>Sceptridium daucifolium</i> (Wall. ex Hook. & Grev.) Lyon	spore (n)	14.55 $\pm$ 0.08 <sup>f</sup>	14.23 $\pm$ 0.07 <sup>f</sup>	3.38	4	This study; Wade 5270 (TAIF)
<i>Sceptridium formosanum</i> (Tagawa) Holub	spore* (n)	13.05 $\pm$ 0.03 <sup>f</sup>	12.76 $\pm$ 0.02 <sup>f</sup>	3.72	4	This study; TAIIF-500159 (TAIF)

(Continues)

TABLE 1 (Continued)

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

Note: CV = coefficient of variation.

<sup>a</sup>IC-value defined by Greilhuber et al. (2005); d-h indicate the internal standard used for each sample.

<sup>b</sup>Calculated as 1 pg = 0.978 Gbp.

<sup>c</sup>Cytology data from Sahashi (1981); Wagner (1993), and Takamiya (1996).

<sup>d</sup>*Secale cereale* L. cv. Dankovske, leaf 2C nuclei = 16.19 pg (Doležel et al., 1998).

<sup>e</sup>*Vicia faba* L. cv. Inovec, leaf 2C nuclei = 26.9 pg (Doležel et al., 1992).

<sup>f</sup>*Nicotiana tabacum* L. cv. Xanthi, leaf 2C nuclei = 10.04 pg (Johnston et al., 1999).

<sup>g</sup>*Haemanthus albigifolius* Jacq., leaf 2C nuclei = 76.0 pg (Zonneveld, 2010).

<sup>h</sup>*Sphaeropteris lepidifera* (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.

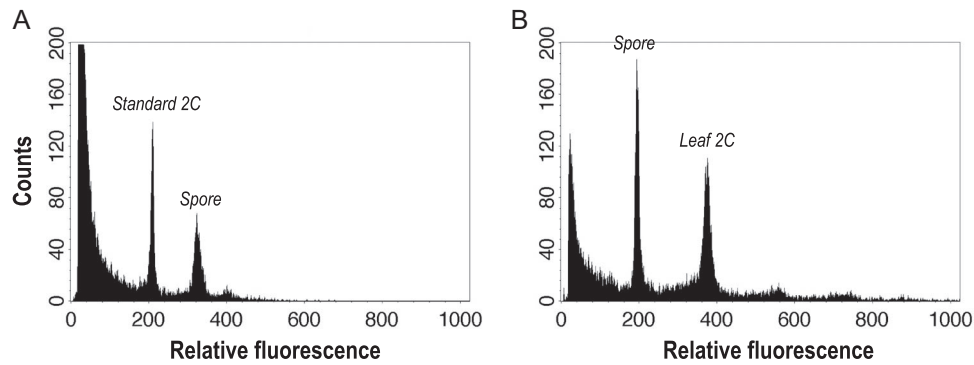
## RESULTS

### Flow cytometry

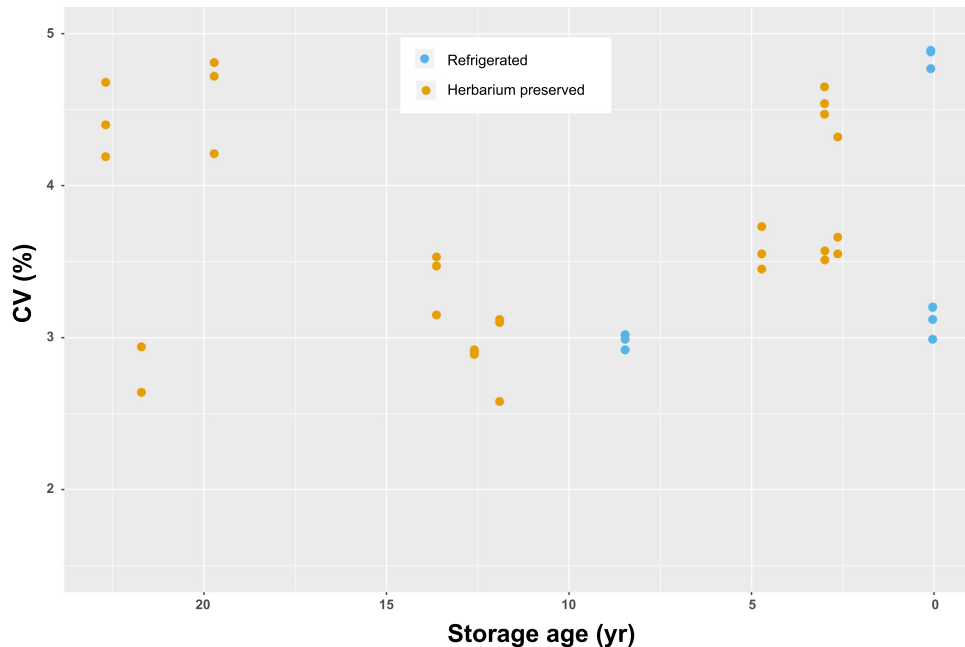
We successfully inferred the genome sizes of all spore and leaf samples, including all tested genera (Figure 1, Table 1), except for seven *Sceptridium* spore samples (Appendix S1) that did not yield a detectable nuclei peak in our FCM analyses. In terms of CV values, we did not find a significant correlation between nuclei quality and the age of the specimen (Figure 2;  $CV(\%) = 3.698 + storage\_age(yr) \times -0.004431$ ,  $F$ -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

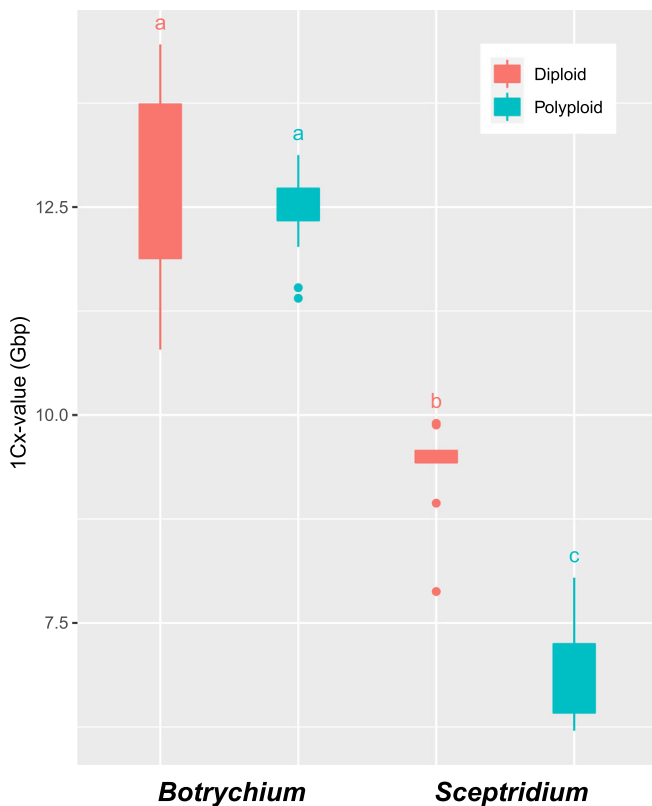
divided by its ploidy level), significant differences had been found between genera and between ploidies (Figure 3).

## 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; Corneille 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  $\mu\text{m}$  in length and 26  $\mu\text{m}$  in width (with a volume of 19,458  $\mu\text{m}^3$  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  $\mu\text{m}^3$ ) 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 $\times$  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 $\times$  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.

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#### 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.

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### 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.

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