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Research Article

Endopolyploidy in Bryophytes: Widespread in Mosses and Absent in Liverworts

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Endopolyploidy occurs when DNA replication is not followed by mitotic nuclear division, resulting in tissues or organisms with nuclei of varying ploidy levels. Endopolyploidy appears to be a common phenomenon in plants, though the prevalence of endopolyploidy has not been determined in bryophytes (including mosses and liverworts). Forty moss species and six liverwort species were analyzed for the degree of endopolyploidy using flow cytometry. Nuclei were extracted in LB01 buffer and stained with propidium iodide. Of the forty moss species, all exhibited endopolyploid nuclei (mean cycle value = 0.65 ± 0.038) except for the *Sphagnum* mosses (mean cycle value = 0). None of the liverwort species had endopolyploid nuclei (mean cycle value = 0.04 ± 0.014). As bryophytes form a paraphyletic grade leading to the tracheophytes, understanding the prevalence and role of endopolyploidy in this group is important.

1. Introduction

Polysomaty is the occurrence of nuclei of varying ploidy levels in the same individual, often associated with different cell or tissue types. This condition of nuclei of varying ploidy levels, known as endopolyploidy, is a result of endoreduplication, which occurs when DNA replication is not followed by mitosis. The mechanisms behind endoreduplication are suggested to involve changes in the activity of cyclin-dependent kinases, which affect the normal transition of the cell cycle [1]. There is, however, a lack of knowledge and understanding regarding the extent, role, and control of endopolyploidy in plants [2].

Various hypotheses have been suggested to explain the importance of endopolyploidy, including growth, development, and stress response [1, 3–5]. One suggested role of endopolyploidy relates directly to the "Nucleotypic Theory," which states that DNA content directly impacts cell volume and other phenotypic traits, which in turn affects various aspects of organism form and function [6, 7]. Barow and Meister [8] and Jovtchev et al. [9] have produced evidence to support this hypothesis, finding that endopolyploidy can allow plants with small genomes to have increased nuclear and cell volume to assist in growth and development. In turn, endopolyploidy is correlated with life history strategy and phylogenetic affiliation [8] and is influenced by various environmental factors including temperature [10, 11], light [12], drought [13], and salinity [14].

Among land plants, endopolyploidy is common in angiosperms but appears to be rare in gymnosperms and ferns [15]. According to a summary completed in 2007, out of thirty explored angiosperm families, nineteen families contain species that predominantly exhibit endopolyploidy [16]. Endopolyploidy occurs in various algal groups [17–19], but in gymnosperms, endopolyploidy is scarce [8, 20], and in ferns there are only isolated references [21–23].

In bryophytes (broadly referring to mosses, liverworts and hornworts), the frequency of endopolyploidy is not known, though some studies present data on specific species or specifically targeted tissues. These studies include the presence of endopolyploidy in polytrichaceous mosses including food-conducting cells [24] and mucilaginous hairs and parenchyma [25], and endopolyploid caulonema in *Funaria hygrometrica* [26, 27]. Endopolyploidy has also been

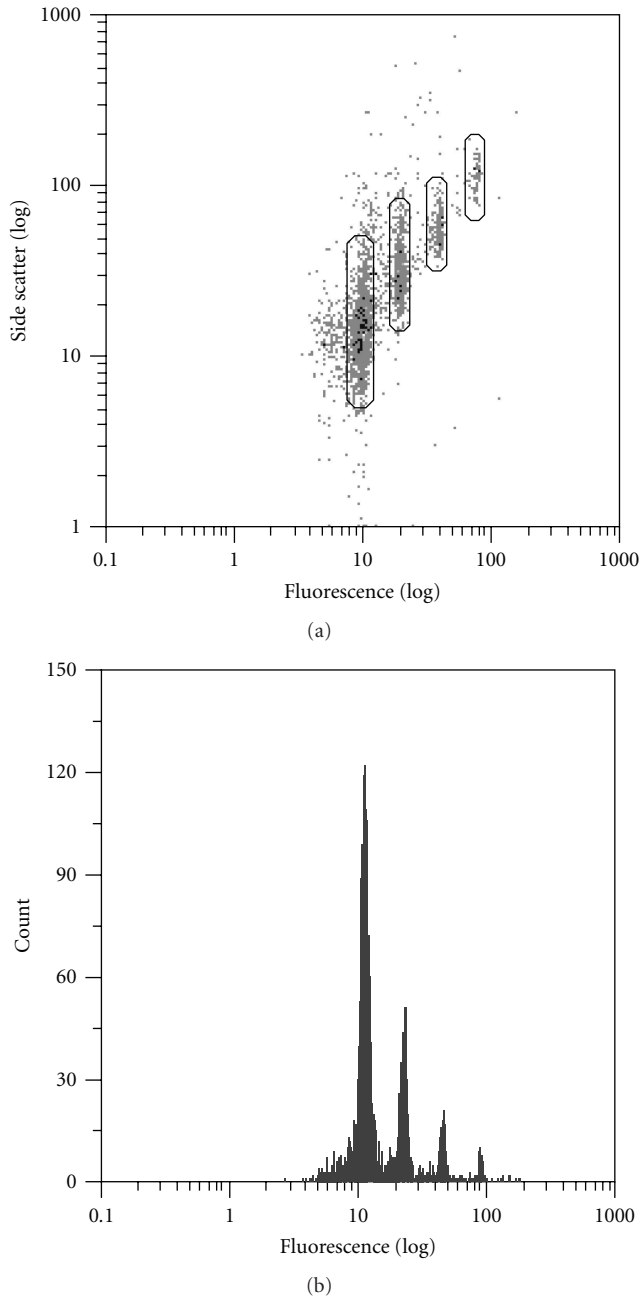

Figure 1: Determination of endopolyploidy in *Rhytidiadelphus triquetrus* using flow cytometry. (a) Scattergram of side scatter versus fluorescence with polygon gates. (b) Histogram of counts versus fluorescence.

observed in moss rhizoids and food conducting tissues [28]. The use of flow cytometry to observe endopolyploidy in bryophytes has been referred to anecdotally [29]. Additionally, endopolyploidy has been explored in the model moss, *Physcomitrella patens*, which has a unique case of exhibiting two distinct ploidy levels in different tissues. Chloronema cells were found to have predominantly 2C or G2 nuclei, while the caulonema nuclei were 1C [30]. In an initial assessment of the *P. patens* genome, there were so few nuclei in the 1C phase; the 2C peak was mistakenly identified as the 1C peak [31]. Treatment of *P. patens* with auxin resulted in an increase in 1C nuclei and also an increase in 4C nuclei [32]. Older caulonema cells also had a higher degree of endopolyploidy [33].

As bryophytes represent the earliest plants to inhabit terrestrial ecosystems [34], the role of endopolyploidy in this group of organisms is relevant in order to increase our understanding of the evolution of endopolyploidy. Bryophytes have small genome sizes [35] and exhibit unique life history strategies [36, 37] as well as habitat specificity [38, 39]. Flow cytometry provides an efficient way to observe endopolyploidy over a range of specimens. These factors make bryophytes ideal organisms to explore the prevalence, role, and biological significance of endopolyploidy. The objective of the present study is to provide the first survey of the prevalence of endopolyploidy in bryophytes.

2. Materials and Methods

Bryophyte specimens were collected in Ontario, Canada, in the summer of 2009. Forty moss species representing seventeen families and six liverwort species from five families were collected (see Table 1). Voucher specimens are deposited in the Biodiversity Institute of Ontario Herbarium (OAC/BIO), University of Guelph. From each population, three independent replicates were analyzed on separate days using flow cytometry, except for three of the liverwort species, where there was insufficient tissue. The samples were composed of green shoots, which included both stem and leaf material. General methodology followed Galbraith et al. [40] and Doležel et al. [41] and was refined according to Bainard et al. [42]. Approximately 10mg of air-dried bryophyte tissue was chopped in 1.2 ml cold LB01 buffer and the resulting solution was filtered through a 30 μm mesh. The nuclei were stained with 150 $\mu g\,ml^{-1}$ propidium iodide (Sigma) in the presence of 0.5 $\mu g\,ml^{-1}$ RNase A (Sigma). Samples were incubated on ice for 20 minutes. For each sample, at least 1000 nuclei were analyzed.

Flow cytometric analysis was completed on a Partec CyFlow SL (Partec GmbH, Münster, Germany) equipped with a blue solid-state laser tuned at 20 mW and operating at 488 nm. Before each use, the instrument was calibrated using 3 μm calibration beads (Partec, Münster, Germany). The parameters recorded for each bryophyte sample included fluorescence intensity at 630 nm measured on a log scale, forward scatter and side scatter. These parameters were observed alone and in combined scattergrams including: fluorescence versus side scatter and fluorescence versus forward scatter.

To determine the degree of endopolyploidy, the number of nuclei (n) in each ploidy level was counted, using FloMax Software by Partec (Version 2.52, 2007). Due to the interference of debris particles, polygon gates were drawn around the nuclei of interest on the fluorescence versus side scatter scattergram to determine the number of nuclei in each peak (Figure 1). To quantify the degree of endopolyploidy, the cycle value was calculated, which is a measure of the number

Table 1: Degree of endopolyploidization of forty moss species and six liverwort species. Mean cycle value and mean *C*-level results are based on three replicates except where noted.

Family	Species	Mean Cycle Value ± Standard Error	Mean *C*-level ± Standard Error
Mosses			
Sphagnaceae	*Sphagnum angustifolium*	0.00 ± 0.000	1.00 ± 0.000
	Sphagnum recurvum	0.00 ± 0.000	1.00 ± 0.000
Polytrichaceae	*Polytrichum commune*	0.33 ± 0.055	1.39 ± 0.068
	Polytrichum juniperum	0.60 ± 0.060	1.80 ± 0.090
Fissidentaceae	*Fissidens taxifolius*	0.40 ± 0.039	1.48 ± 0.056
Dicranaceae	*Dicranum condensatum*	0.87 ± 0.072	2.17 ± 0.148
	Dicranum flagellare	0.50 ± 0.015	1.62 ± 0.022
	Dicranum fuscescens	0.57 ± 0.087	1.75 ± 0.125
	Dicranum montanum	0.52 ± 0.029	1.63 ± 0.041
	Dicranum polysetum	1.11 ± 0.030	2.34 ± 0.049
	Dicranum scoparium	0.98 ± 0.048	2.17 ± 0.101
	Trematodon ambigus	0.51 ± 0.055	1.62 ± 0.063
Ditrichaceae	*Ceratodon purpureus*	0.51 ± 0.060	1.73 ± 0.067
Orthotrichaceae	*Orthotrichum speciosum*	0.29 ± 0.071	1.40 ± 0.114
Hedwigiaceae	*Hedwigia ciliata*	0.34 ± 0.030	1.51 ± 0.056
Aulacomniaceae	*Aulacomnium androgynum*	0.64 ± 0.076	1.91 ± 0.092
Mniaceae	*Plagiomnium drummondii*	1.37 ± 0.054	2.91 ± 0.105
	Plagiomnium medium	1.21 ± 0.152	2.81 ± 0.353
	Pohlia whalenbergia	1.13 ± 0.125	3.33 ± 0.282
Hylocomiaceae	*Hylocomnium splendens*	0.53 ± 0.165	1.71 ± 0.253
	Pleurozium schreberi	0.35 ± 0.079	1.42 ± 0.098
	Rhytidiadelphus triquetrus	0.52 ± 0.036	1.75 ± 0.069
Leskeaceae	*Haplocladium microphyllum*	0.41 ± 0.086	1.55 ± 0.117
Thuidiaceae	*Thuidium delicatulum*	0.91 ± 0.119	2.30 ± 0.184
	Thuidium minulatum	0.41 ± 0.025	1.52 ± 0.032
Campyliaceae	*Campylium chrysophyllum*	0.64 ± 0.057	1.77 ± 0.077
Brachytheciaceae	*Brachythecium acuminatum*	0.54 ± 0.045	1.65 ± 0.049
	Brachythecium salebrosum	0.14 ± 0.018	1.16 ± 0.023
	Brachythecium velutinum	0.51 ± 0.071	1.69 ± 0.116
	Eurhynchium pulchellum	0.27 ± 0.044	1.36 ± 0.066
Plagiotheciaceae	*Plagiothecium denticulatum*	1.05 ± 0.160	2.51 ± 0.282
	Plagiothecium laetum	1.70 ± 0.062	4.01 ± 0.243
Climaciaceae	*Climacium dendroides*	1.48 ± 0.030	3.40 ± 0.086
Hypnaceae	*Callicladium halandianum*	0.69 ± 0.187	1.89 ± 0.246
	Hypnum curvifolium	1.29 ± 0.107	3.03 ± 0.226
	Hypnum lindbergii	0.78 ± 0.236	2.11 ± 0.290
	Hypnum pallescens	0.96 ± 0.078	2.77 ± 0.160
	Hypnum recurvatum	0.34 ± 0.104	1.46 ± 0.144
	Ptilium crista-castrensis	0.27 ± 0.016	1.42 ± 0.018
	Pylaisiella polyantha	0.37 ± 0.054	1.43 ± 0.072
	Mean	**0.65 ± 0.038**	**1.94 ± 0.065**
Liverworts			
Ptilidiaceae	*Ptilidium pulcherrimum*	0.00 ± 0.000	1.00 ± 0.000
Geocalycaceae	*Lophocolea heterophylla*	0.09*	1.06*
Calypogeiaceae	*Calypogeia integristipula*	0.12 ± 0.016	1.12 ± 0.016
Jungermanniaceae	*Barbilophozia barbata*	0.01 ± 0.008	1.08 ± 0.008
	Lophozia heterocolpos	0.06*	1.06*
Radulaceae	*Radula complanata*	0.02** ± 0.008	1.02** ± 0.008
	Mean	**0.043 ± 0.014**	**1.04 ± 0.014**

* Value based on one replicate.
** Mean based on two replicates.

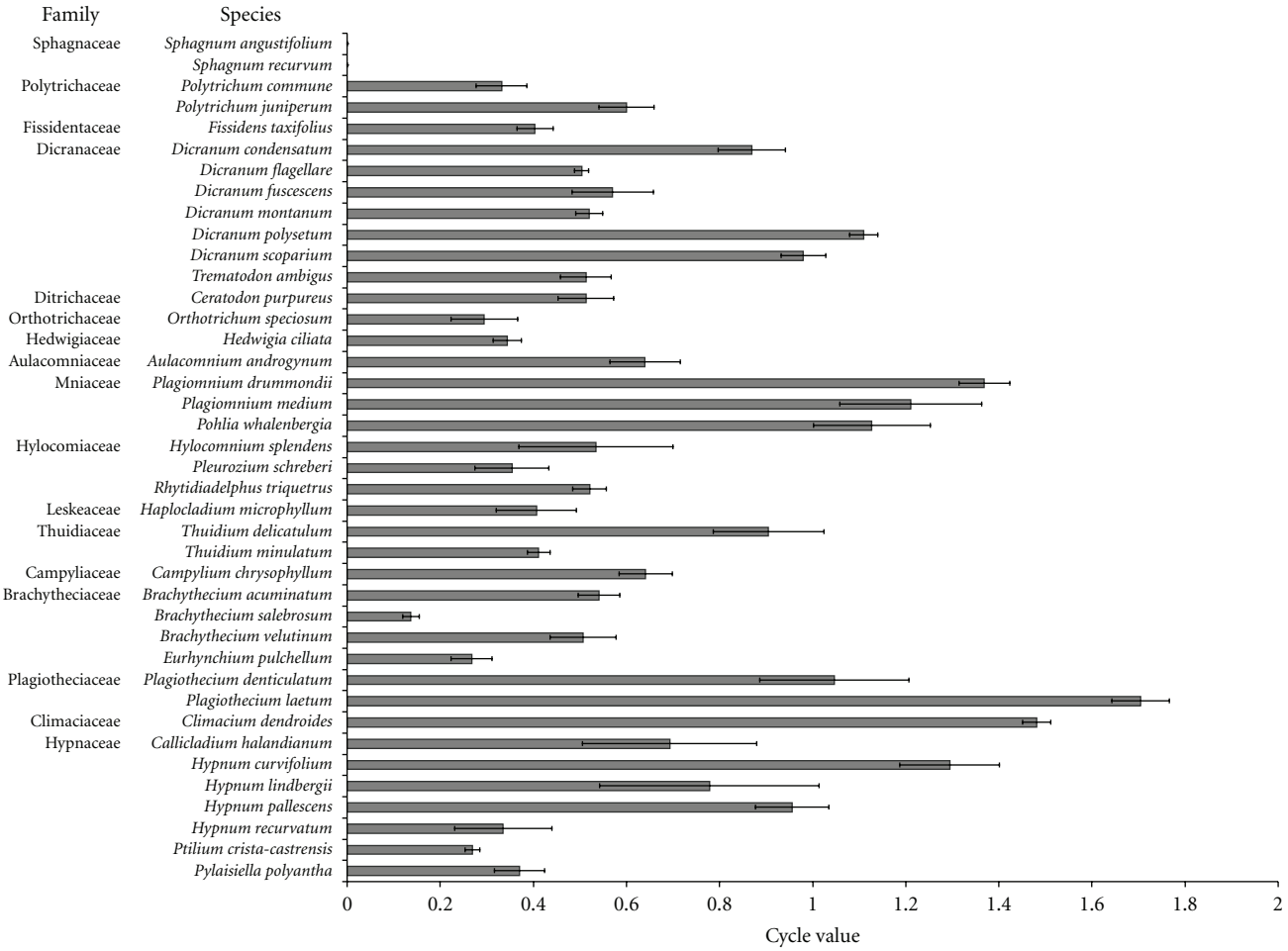

FIGURE 2: Mean cycle value of 40 moss species, determined using flow cytometry. Error bars represent standard error of the mean ($n = 3$).

of endoreduplication cycles per nucleus that occurred in the nuclei measured [8]. As bryophytes are haplophasic, the first endopolyploid level is the 2C level, which corresponds to one endoreduplication cycle. This is calculated according to the following [16]:

$$\text{Cycle value} = \frac{(0 \times n_{1c} + 1 \times n_{2c} + 2 \times n_{4c} + 3 \times n_{8c} + 4 \times n_{16c} \cdots)}{(n_{1c} + n_{2c} + n_{4c} + n_{8c} + n_{16c} \cdots)}. \quad (1)$$

Additionally, the mean C-level was calculated, which is a measure of the mean ploidy level of the nuclei measured [10, 43]. This is calculated using the following [16]:

$$\text{Mean } C\text{-level} = \frac{(1 \times n_{1c} + 2 \times n_{2c} + 4 \times n_{4c} + 8 \times n_{8c} + 16 \times n_{16c} \cdots)}{(n_{1c} + n_{2c} + n_{4c} + n_{8c} + n_{16c} \cdots)}. \quad (2)$$

It should be noted that small amounts of nonendopolyploid nuclei can contribute to the number of nuclei in the different ploidy levels. For example, nuclei that were in the G2 phase of the cell cycle would have a 2C ploidy level, and not necessarily be endoreduplicated nuclei. As well, nuclei can occasionally stick together (forming doublets) and contribute to higher ploidy levels. However, it is expected that in most cases the relative amount of G2 and doublet nuclei will be negligible [16]. Additionally, species with a cycle value less that 0.1 are not considered to be endopolyploid [8, 9].

3. Results

All moss species measured in this study had distinctly endopolyploid nuclei, with the exception of the *Sphagnum* mosses (Table 1 and Figure 2). Examples of the flow cytometry results are shown in Figure 3. The average cycle value over all mosses was 0.65 ± 0.038 and the mean C-level was 1.94 ± 0.065. Other than the *Sphagnum* species, all mosses had 1C, 2C and 4C nuclei present, and several also had 8C and 16C nuclei. The bryophyte with the highest degree of endopolyploidy was *Plagiothecium laetum*, with a mean cycle value of 1.71 and a mean C-level of 4.01 (see Figure 3).

In contrast, the liverworts we sampled had almost no endopolyploid nuclei (Table 1). The mean cycle value for

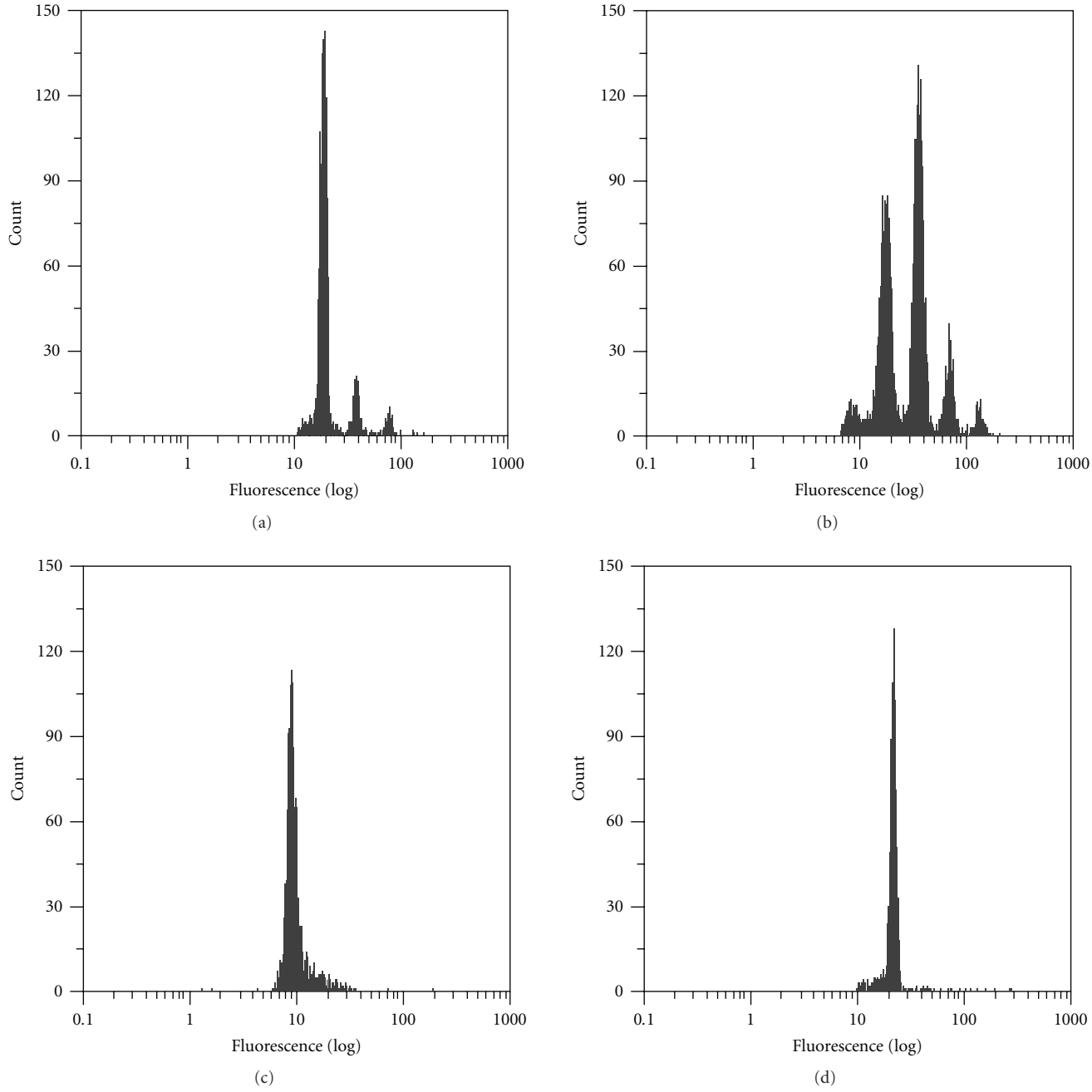

Figure 3: Examples of fluorescence histograms for several species. (a) *Brachythecium salebrosum*, showing a low degree of endopolyploidization (cycle value = 0.14 ± 0.018). (b) *Plagiothecium laetum,* exhibiting the highest degree of endopolyploidization (cycle value = 1.70 ± 0.062). (c) *Sphagnum recurvum*, exhibiting no endopolyploidization. (d) *Barbilophozia barbata* (liverwort), exhibiting no endopolyploidization.

the liverworts was 0.04 ± 0.014 and the mean *C*-level was 1.04 ± 0.014. Only *Calypogeja integristipula* had a cycle value over 0.1, and this was most likely due to the presence of diploid sporophytes in the population, which were difficult to remove at the time of processing. All other species had cycle values below 0.1, which indicates that if there were nuclei in a second peak, they were likely G2 or doublet nuclei.

4. Discussion

Endopolyploidy appears to be widespread in mosses, and absent in liverworts. The species coverage in the current study is not large enough to make conclusions regarding the phylogenetic affiliation of endopolyploidy in bryophytes, however general comments can be made. Some of the moss families analyzed appeared to have a higher incidence of

endopolyploidy than others, such as the Mniacea. Other families showed considerable variation between species, such as the Dicranaceae and Hypnaceae. The lack of endopolyploidy in the Sphagnaceae could be attributed to the unique occurrence of a large proportion of dead cells (large hyaline cells) to small, green, living cells (chlorophyllose cells) within the leaves [44]. Greater species coverage will allow a more comprehensive view of the prevalence of endopolyploidy in relation to taxonomy.

It is interesting that the liverworts sampled are lacking in endopolyploid nuclei, even though they are closely related to the mosses. Although liverworts have similar life history strategies to mosses, there are considerable biological differences that include: short-lived sporophytes that wither away not long after releasing spores; single-celled rhizoids; the lack of clearly differentiated stem and leaves in thallose species; the presence of deeply lobed or segmented leaves arranged in three ranks; and the presence of oil bodies in at least some of their cells, which are absent from most other bryophytes and from all vascular plants [45]. The disparity in the degree of endopolyploidization between mosses and liverworts could be related to these morphological and biological differences.

From a phylogenetic perspective, as liverworts are sister to all land plants [46] and appear to have a low occurrence of endopolyploid nuclei, endopolyploidy is likely a derived trait. Additionally, the lack of endopolyploidy in Sphagnaceae suggests that the trait evolved after this divergence in bryophytes. Endopolyploidy has likely evolved independently in various groups, as angiosperm families also have varying degrees of endopolyploidy. Future research should involve a broader species coverage across land plants to better understand the phylogenetic implications of endopolyploidy.

As the biological significance of endopolyploidy is just beginning to be explored, there is a considerable amount still to be discovered in relation to bryophyte morphology and environment. It is necessary to determine the cells and tissues responsible for the varying DNA contents, in order to understand the biological role that endopolyploidy plays in bryophyte form and function. Additionally, the environmental impact on endopolyploidization will be especially relevant as bryophytes exhibit habitat specificity. We are currently conducting a more comprehensive survey of the prevalence of endopolyploidy in hepatics, and exploring hypotheses concerning the relative frequency of endopolyploidy (particularly in mosses) in a group of plants that are sister to tracheophytes [46].

As genome size and endopolyploidy appears to be correlated [8], the small genome sizes of mosses [35] and the high degree of endopolyploidy in this group seem to fit this trend. However, this relationship should be explored further, and determination of genome size for the bryophyte species mentioned here is already underway by our research group. Understanding genome size in relation to endopolyploidy and relating DNA content to cell size and function in bryophytes will continue to elucidate the biological significance of endopolyploidy.

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