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Application of DNA Flow Cytometry to Aid Species Delimitation in *Isoetes*

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ABSTRACT The genus *Isoetes* is known for morphological convergence and a relative paucity of useful gross morphological characters for identification. Thus, the chromosome complements of *Isoetes* spp. are key attributes for species delimitation. Like many other plant lineages, polyploidy and reticulate evolution are important drivers of speciation in *Isoetes*. In the southeastern US *Isoetes* flora, polyploidy is common. We used a rapid DNA flow cytometry protocol to generate C values for four diploid, one tetraploid, and one hexaploid *Isoetes* species and conducted preliminary cytological investigations of two communities of *Isoetes*. Our work showed noteworthy variation in diploid *Isoetes* 1C values (1.39 to 3.32), including the smallest 1C value reported for the genus, *Isoetes melanopoda* ssp. *silvatica*. The hexaploid species, *Isoetes microvela* had the highest 1C value (4.21), and the tetraploid species evaluated had an intermediate 1C value (3.37). However, based on the variation in diploid 1C values measured, a simple 2×, 4×, 6× ratio of C values was not apparent for diploids, tetraploids, and hexaploids, respectively. All *Isoetes* taxa measured had significantly different 1C values and homogenous C values were observed in two communities of *Isoetes* (n = 24). These results indicate that DNA flow cytometry may prove a useful tool for routine *Isoetes* species identification, systematic work, and population-level surveys of *Isoetes* ploidy level.

INTRODUCTION The monotypic genus *Isoetes* L. (Isoetaceae), commonly known as quillworts, represents an ancient lycopod lineage. Worldwide, there are more than 192 recognized species of *Isoetes*, and the discovery and description of new quillworts around the world and in the southeastern USA continues at a relatively rapid pace (Troia et al. 2016). The center of *Isoetes* diversity is South America; however, the southeastern USA contains at least 24 recognized species and is considered a quillwort "biodiversity hotspot" (Brunton 2016, Schafran et al. 2016, Troia et al. 2016). *Isoetes* species are notorious for their morphological reduction and convergence; a limited suite of morphological characters are used to delimit species, including spore ornamentation and size, velum coverage, presence of phyllopodia, and ligule morphology (Taylor et al. 1993). When morphology has historically failed to confidently discriminate species, cytological evidence (e.g., Kott and Britton 1980) has aided species circumscription of many new diploid species, polyploids, and sterile hybrids in the southeastern USA (e.g., Brunton et al. 1994; Musselman et al. 1995, 1996; Brunton and Britton 1997, 1998; Rosenthal et al. 2014; Brunton and McNeill 2015; Brunton 2016). Morphological similarity and character intergradation among many *Isoetes* spp. is likely due in large part to the prevalence of polyploidy in *Isoetes*. Troìa et al. (2016) reports that, of the accepted *Isoetes* taxa worldwide with reported chromosome counts, 46.7% are polyploid (*Isoetes* base chromosome complement is 2n = 22).

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In the southeastern US *Isoetes* flora, diploids, sterile triploids, tetraploids, hexaploids, octoploids, and decaploids are present (Taylor et al. 1993, Brunton 2015). Isozymes (Caplen and Werth 2000, Heafner and Bray 2005), and DNA sequence data (Hoot and Taylor 2001, Hoot et al. 2004, Taylor et al. 2004) have helped to identify cryptic diversity and allopolyploid speciation. Chromosome counts using root tip squashes are a lynchpin of *Isoetes* taxonomy; however, that technique requires floating live material in water for many days to collect actively growing root tips and is time and labor intensive. Moreover, for *Isoetes* species with many chromosomes (e.g., *Isoetes tennesseensis* Luebke & Budke, 2n = 88; *Isoetes lacustris* L., 2n = 110) generating and reliably counting a chromosome figure may result in lower accuracy of chromosome counts (Troìa 2001) or may be impractical.

The C value is a measure of DNA quantity in an unreplicated nuclear genome and is equivalent to picograms of DNA in a haploid nucleus (Swift 1950). C values can be correlated to ploidy level and are thus important diagnostic characteristics in groups with multiple ploidy levels (e.g., Talent and Dickenson 2005). DNA flow cytometry using standardized protocols and reference standards have made estimation of plant C values relatively commonplace (Doležel and Bartos 2005, Bennett and Leitch 2012). C values across lycopods and ferns can vary widely from 1C = 0.08 in *Selaginella selaginoides* (Baniaga et al. 2016) to 1C = 72.68 in *Psilotum nudum* (Obermayer et al. 2002) and significant C value variation within lycopod and fern genera is apparent (Obermayer et al. 2002; Bennett and Leitch 2012). Across angiosperms, intraspecific genome size variation has been reported in *Arabidopsis thaliana* (Long et al. 2013) and other species. The rapid analysis allowed by DNA flow cytometry has facilitated cytogeographic studies of taxonomically difficult polyploid complexes in genera, such as *Crataegus* (Talent and Dickenson 2005), *Amelanchier* (Burgess et al. 2014), and *Solidago* (Peirson et al. 2012).

Only two C values are reported for *Isoetes* spanning the range of *Isoetes* ploidy levels. A 1C value of 1.75 has been reported for diploid *Isoetes engelmannii* A. Braun (Wang et al. 2005) and a 1C value of 11.97 for the decaploid *Isoetes lacustris* (Hanson and Leitch 2002). The aim of this study was to measure and compare *Isoetes* C values from a range of ploidy levels from the eastern USA to assess their utility as diagnostic characters and to sample putative mixed ploidy level populations to determine whether polyploidy could be detected at the population level.

MATERIALS AND METHODS

Sample Material

Cultures of *Isoetes echinospora* Durieu, *I. engelmannii*, *Isoetes flaccida* Shuttlew. Ex A. Braun, *Isoetes melanopoda* J. Gay & Durieu ssp. *silvatica* D.F. Brunt. & D.M. Britton, and *Isoetes microvela* D.F. Brunt., and two field populations of *Isoetes* were collected in large soil turf sections and were maintained in the Catawba College (Salisbury, North Carolina) greenhouse. The field populations were collected from a site known as the "River of *Isoetes*," a small unnamed stream in Dinwiddie County, Virginia (36°59′44.0262″N, 77°27′43.8582″W) and a site known as "Uwharrie's trestle," from a small unnamed stream in the Uwharrie Mountains, Stanly County, North Carolina (35°19′19.005″N, 79°56′51.27″W). These sites were selected for community genomic analyses because they were likely to include a mixture of *Isoetes* with multiple ploidy levels. The River of *Isoetes* area is the type locality of the sterile triploid *Isoetes* × *bruntonii* Knepper & Musselman, an apparent cross of the diploid *I. engelmannii* and the tetraploid *I. "hyemalis"* D.F Brunt., and all three taxa occur there (Musselman et al. 1996). Recent field work by the authors at the Uwharrie's trestle site indicated the presence of possible sterile triploids because of occasional observations of plants with malformed and irregular megaspores.

Ploidy Determination

The ploidy level of cultures before DNA flow cytometry was inferred by a combination of methods, including size and ornamentation of megaspores, chromosome squash of root tips (following the protocol of Brunton et al. 1994), and sequencing of the low-copy nuclear LEAFY homologue intron (LFY) (following the protocol of Hoot and Taylor 2001; data not shown here). Direct sequencing of LFY yields one unique sequence from diploids, whereas sequencing and cloning of PCR products yields two unique sequences from tetraploids (Hoot et al. 2004, Taylor et al. 2004). All cultured collections are vouchered at the Catawba College Herbarium

Table 1. *Isoetes* species and voucher information (CATU = Catawba College Herbarium; ODU = Old Dominion University Herbarium), location/source, known ploidy level, calculated 1C value, standard deviation, and replication. Ploidy level of *Isoetes* was inferred through a combination of methods: size and ornamentation of megaspores (M), chromosome squash of root tips (C), and DNA sequencing of the low-copy LFY homologue (S), indicated as superscripts

Isoetes sp. (Voucher No.)	Location/Source	Ploidy Level ($2n$)	1C Value	SD	Replication, (n)
Isoetes echinospora (CATU LM16-1)	St. Lawrence County, New York	22^{M}	3.23	0.08	3
Isoetes engelmannii (ODU & CATU Schafran 68)	Stanly County, North Carolina	$22^{M,C,S}$	1.79	0.03	6
Isoetes flaccida (CATU JBNC-16-16)	Ward's Scientific Supply Company	$22^{M,C,S}$	2.65	0.10	6
Isoetes melanopoda ssp. *silvatica* (CATU JBNC46)	Rowan County, North Carolina	$22^{M,S}$	1.39	0.02	8
Isoetes hyemalis complex (ODU Schafran VA-01-11)	Isle of Wight County, Virginia	$44^{M,C,S}$	3.37	0.06	6
Isoetes microvela (CATU JBNC202)	Brunswick County, North Carolina	$66^{M,C,S}$	4.21	0.07	6

(CATU) or Old Dominion University Herbarium (ODU) (Table 1), and representative vouchers of the Uwharrie's trestle (JBNC16-19) and River of *Isoetes* (LM16-2) plants are deposited at CATU.

Sample Preparation for DNA Flow Cytometry

Microphylls from plants cultured at Catawba College and ODU were collected from living plants at 2–4 hr before analysis. For genome size estimation, the leaves of the standard *Raphanus sativus* L. 'Saxa' (Doležel et al. 1998) were used. Nuclei were extracted from fresh plant material by chopping 3–4 cm of *Isoetes* microphylls and a 0.5-cm^2 leaf section of the standard *R. sativus* 'Saxa', simultaneously, in 500 µl of ice-cold LB01 medium (Doležel et al. 1989) on a chilled watchglass with a surgical razor for 3 min. An additional 500 µl of cold LB01 medium was added to each processed sample, and released nuclei and LB01 media were transferred, unfiltered, to a new microcentrifuge tube. Nuclei were stained with a 1:10 dilution of propidium iodide (PI) stock solution (1 mg/ml) (P21493; Molecular Probes™ FluoroPure™, Thermo Fisher Scientific, Waltham, Massachusetts) and incubated in the dark on ice for 20–30 min before flow cytometry analysis.

DNA Flow Cytometry

Samples were analyzed on BD Accuri™ C6 flow cytometer with laser illumination (20 mV) at 488 nm using BD Accuri™ C6 software (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Fluorescence signals were screened with a FL-2, 580/20 nm-bandpass filter and a FL-3, 670-nm longpass filter. Analysis of unfiltered homogenate was based on light-scatter (SSC-A) vs. fluorescence signals (FSC-A). The threshold for detection of signal events was set to 15,000 for FSC, with a secondary threshold set to 5,000 for FL2-A. Data acquisition time ranged from 1 min to 10 min per sample to collect 10,000 events. The PI-stained nuclei appeared on bivariate plots as a region of concentrated dots clustered on a diagonal line plot. A polygonal gate was drawn to enclose detected nuclei and univariate histograms of PI fluorescence (FL2-A) were constructed and used to determine the mean peak value of PI-stained nuclei for each combined *Isoetes* sp. and *R. sativus* sample (Figure 1).

Genome Size Estimation

For each known plant species and standard, the final C value was calculated using the mean peak values on the FL2-A histogram plots (Figure 1). Standards paired with each species were used to calculate the C value per replicate according to the following equation (Doležel and Bartos, 2005):

$$\begin{aligned} &\textit{Sample } 2C \textit{ DNA content} = \\ &(\textit{Sample } G1 \textit{ peak mean} / \text{Standard } G1 \textit{ peak mean}) \\ &\quad \times \text{Standard } 2C \textit{ DNA content (pg DNA)} \end{aligned}$$

Calculated C values were then averaged from replicate plants to generate the final C value reported for each *Isoetes* species (n = 3–6). Statistical tests were conducted using IBM SPSS Statistics v.23 (IBM Corp., Armonk, New York).

RESULTS The protocol for DNA flow cytometry was relatively straightforward, although a few notes are provided here to facilitate the

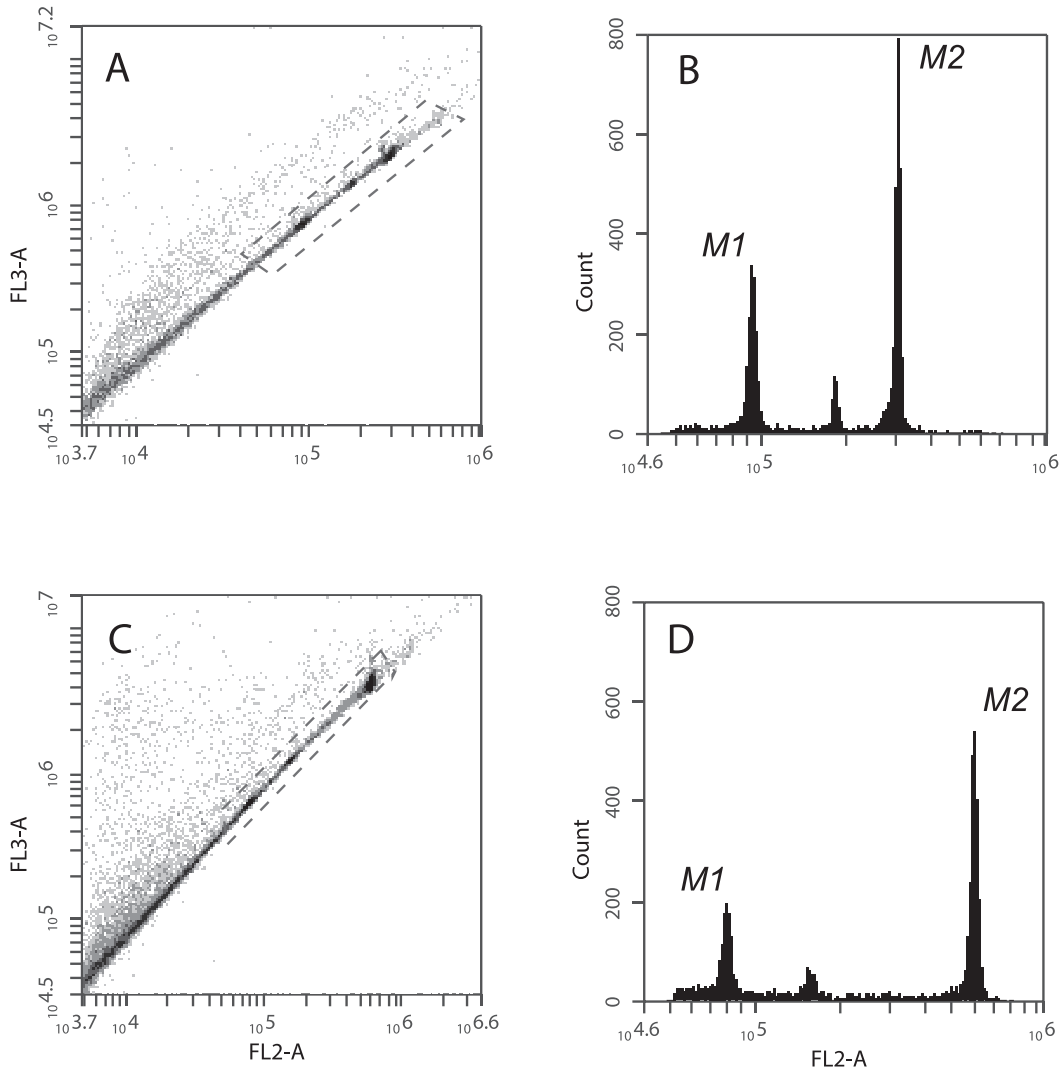

Figure 1. Representative flow cytometer scatterplot diagrams and histograms of events collected from samples of *Raphanus sativus* 'Saxa' as the internal standard and a focal *Isoetes* species stained with propidium iodide (PI). (A and C) The height of the side-scatter peak (FL3-A) and the height of the fluorescence peak (FL2-A) are shown. The dashed polygon represents the gate used to subsample the event population and to generate the histograms in the corresponding B and D. (B and D) The first labeled peak (M1) is the fluorescence signal of *R. sativus* PI-stained nuclei, and the second labeled peak is the PI-stained nuclei signal of *Isoetes* sample (M2). The lower and intervening peak represents endotetraploid nuclei or nuclei in G2 phase of *R. sativus*. (A and B) Flow cytometer data for the diploid *Isoetes engelmannii*. (C and D) Data for the hexaploid *Isoetes microvela*.

use of a standard sample-preparation protocol using LB01 buffer (Doležel et al. 1989, Doležel and Bartos 2005) for *Isoetes*. Because the successful isolation of nuclei can depend on the buffer selected and the tissue chemistry of the target plant (Doležel and Bartos 2005), we also attempted the same *Isoetes* nuclei isolation protocol but with Tris-$MgCl_2$ buffer (Pfosser et al. 1995) in addition to LB01 buffer. In our attempts, Tris-$MgCl_2$ buffer failed to adequately isolate intact *Isoetes* nuclei (data not shown). As suggested in most protocols, tissue quality (bright green fresh growth) is paramount for successful nuclei preparations. Moreover, we

found that it was critical to incubate nuclei and buffer suspensions for 20–30 min on ice and in darkness before analysis on the flow cytometer. Less incubation time resulted in fewer fluorescent signal counts. Finally, we deviated slightly from the standard Doležel et al. (1989) protocol by not using a fine 20–30 μm filter to remove plant fragments from the nuclei and buffer; instead, we carefully pipetted the solutions with a 200-μl pipette tip, thus the homogenate that was run into the flow cytometer was essentially unfiltered.

C value estimates for the diploids *I. echinospora*, *I. engelmannii*, *I. flaccida*, *I. melanopoda* ssp. *silvatica;* the tetraploid *I. hyemalis*; and the hexaploid *I. microvela* are given in Table 1. Diploid *Isoetes* C values ranged from 1C = 1.39–3.32 in four diploid species. The tetraploid *I. hyemalis* and hexaploid *I. microvela* had larger 1C values, 3.37 and 4.21, respectively. All taxa had significantly different 1C values based on analysis of variance ($df = 5$, $F = 1814.17$, $p < 0.001$) and a post hoc Tukey test (all pairwise comparisons, $p < 0.05$). The diploid with the largest genome, *I. echinospora* (1C = 3.32 ± 0.08) and the tetraploid *I. hyemalis* (1C = 3.37 ± 0.06), although similar, had significantly different 1C values.

At both the Uwharrie's Trestle and River of *Isoetes* sites, flow cytometry generated consistent C value estimates for 24 individual plants at each site with little variation, 1C = 1.37 ± 0.02 and 1C = 2.84 ± 0.09, respectively (Figure 2).

DISCUSSION The C values we report here agree with the two existing C values published for *Isoetes*. In fact, the value we report for *I. engelmannii* (1C = 1.79 ± 0.03) is very close to the *I. engelmannii* value (1C = 1.75) reported by Wang et al. (2005). The small variation between the two measurements may be due to different standards used (this study, *R. sativa* 'Saxa', whereas Wang et al. [2005] used *Glycine max*, cultivar not reported), measurement error, or intraspecific variation possible in a wide-ranging species, such as *I. engelmannii*. We report the smallest genome size for any *Isoetes* spp., *I. melanopoda* ssp. *silvatica* (1C = 1.39 ± 0.02). Perhaps the most interesting finding was the wide variation in size in the four diploid *Isoetes* analyzed. We report a 2.3-fold variation in genome size when comparing the diploid with the largest genome, *I. echinospora* (1C = 3.23 ± 0.08), and the diploid with the smallest genome, *I. melanopoda* ssp. *silvatica*. Comparably, Little et al. (2007) measured genome size of seven *Selaginella* species with a chromosome complement of 2n = 20, and the 1C values ranged from 0.088 to 0.16, representing a 1.8-fold variation. The C value variation across diploid *Isoetes* reported here with relatively small standard deviations suggest that C values may hold promise as a diagnostic character for the genus, although intraspecific variation must first be tested, and a more complete diploid taxon sampling must be performed.

The positive, significant relationship between ploidy level and megaspore size is well established in *Isoetes* (e.g., Kott and Britton 1982 and Pereira et al. 2015). Thus, the large genome size for the diploid *I. echinospora* (1C = 3.23 ± 0.08) relative to the other diploid *Isoetes* species measured here (1C = 1.39–2.65) is supported by the relatively large megaspore size of *I. echinospora* ($\bar{x}$ = 480 μm from Kott and Britton [1983]; $\bar{x}$ = 400–550 μm from Taylor et al. [1993]), relative to the smaller megaspore sizes reported for the other diploid *Isoetes* in the study, *I. melanopoda* ssp. *silvatica* ($\bar{x}$ = 410 μm), *I. flaccida* (<450 μm), and *I. engelmanii* ($\bar{x}$ = 460 μm) (Brunton 2015). Based on a review of the salient literature (Kott and Britton 1983, Taylor et al. 1993, Brunton 2015), *I. echinospora* may represent the diploid quillwort with the largest mean megaspore size in the eastern US flora.

The polyploid taxa *I. hyemalis* and *I. microvela* had larger genome sizes than the diploids had. As expected, there was a significant, positive correlation ($y = 0.1x - 0.38$; $r^2 = 0.89$; $p < 0.001$) between ploidy level and C value in the data set of *Isoetes* C values from this study and those from the literature (Figure 3). Multiple parentage scenarios for allopolyploids are likely, and preliminary reports using low-copy nuclear DNA sequencing suggest multiple parentage scenarios in polyploids may be more common than expected. Three distinct parentage scenarios were revealed in four populations of the tetraploid *I. hyemalis* in North Carolina and Virginia (Bolin et al. 2008), as well as in segregates of the allopolyploid *I. riparia* (Schafran, unpubl. data). Thus, many allopolyploid *Isoetes* may occur in complexes with different parentage and confounding and intergrading morphology. DNA flow cytometry may be useful for aiding the circumscription of segregates within allopolyploid complexes, assuming their

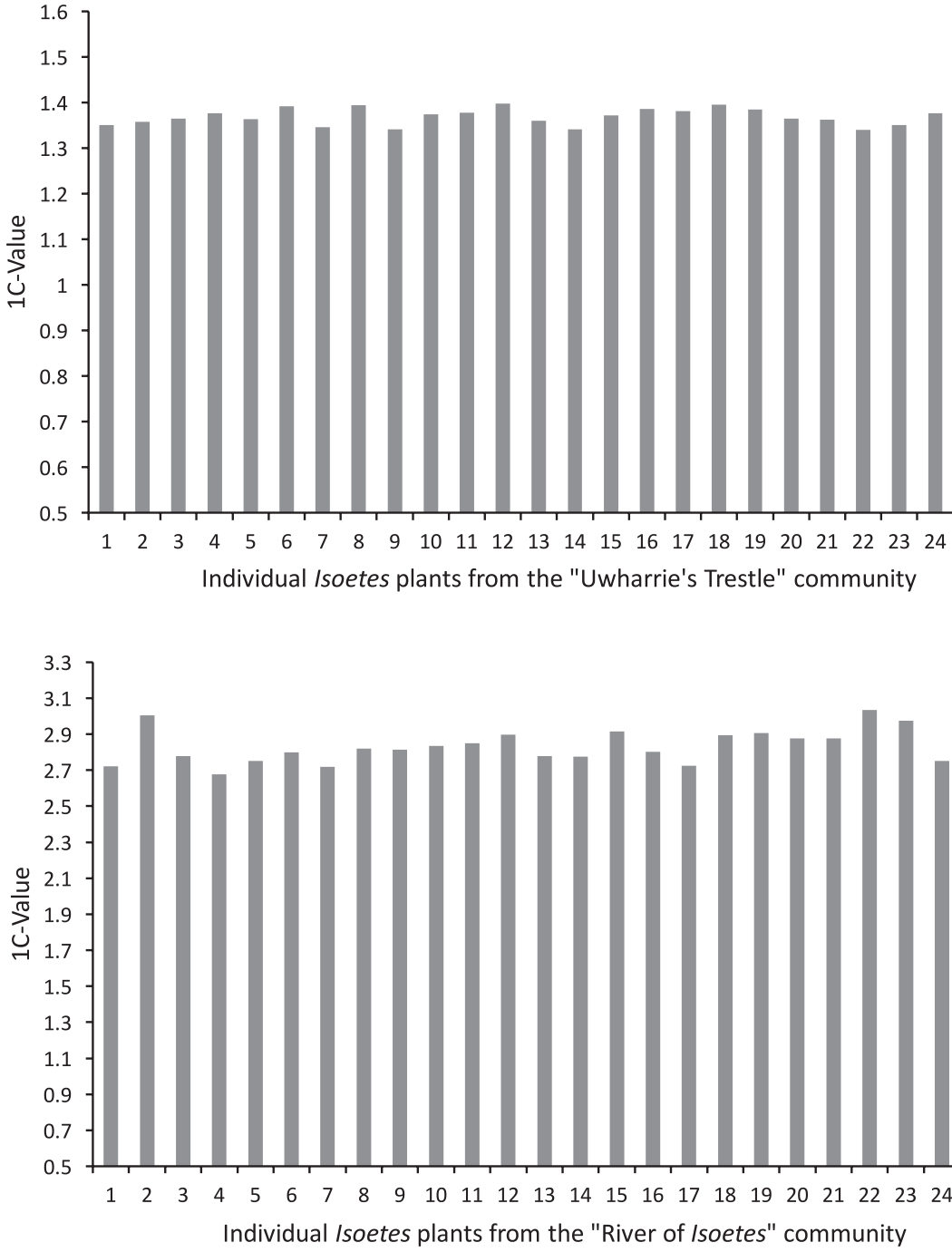

Figure 2. *Isoetes* from two communities were sampled showing the consistency of the C values recorded from the two sites. The randomly sampled, individual *Isoetes* plants, likely represent a single species at each population with a consistent C value (River of *Isoetes* $1C = 2.84 \pm 0.09$; Uwharrie's Trestle $1C = 1.37 \pm 0.018$), despite our assumption that the populations may contain species with mixed ploidy levels.

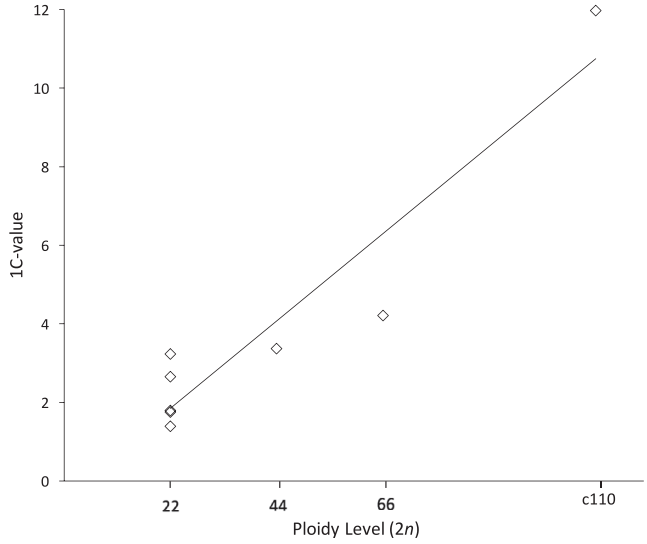

Figure 3. A preliminary correlation of *Isoetes* 1C values and ploidy level ($y = 0.1x - 0.38$; $r^2 = 0.89$; $p < 0.001$). Data are from this study (Table 1) and published values for the diploid *I. engelmannii* (Wang et al. 2005) and the decaploid *I. lacustris* (Hanson and Leitch 2002).

parental genome combinations have different genome sizes.

The preliminary *Isoetes* genome-size analyses of putative *Isoetes* communities demonstrated the promise of DNA flow cytometry for rapidly assessing mixed populations of *Isoetes* and the low variation in C value among individual plants. Critically, the analysis of 24 individual plant samples took approximately 4 hr of bench time, from leaf to data output from the flow cytometer, with two researchers working together. DNA flow cytometry is clearly a quicker procedure than chromosome squashes from root tips. At the Uwharrie's trestle population, C values from 24 individuals suggested a single-chromosome complement at that location ($1C = 1.37 \pm 0.018$). The C value was significantly different (independent samples *t* test, $df = 30$, $t = 3.92$, $p < 0.001$) from the value we measured for *I. melanopoda* ssp. *silvatica* ($1C = 1.39 \pm 0.021$), although the difference between the C values represented only a 1.4% difference. On initial inspection, the prominently tuberculate megaspore ornamentation observed from the Uwharrie's trestle population does not clearly differ from that of *I. melanopoda* ssp. *silvatica* (Brunton and Britton 2006), although further work is required at that site based on LFY sequences that do not fit the concept for *I. melanopoda* ssp. *silvatica* (Schafran, unpubl. data).

At the River of *Isoetes*, three taxa have been reported: *I. engelmannii*, *I. "hyemalis"* complex, and their triploid hybrid *Isoetes* × *bruntonii* (Musselman et al. 1996). From the 24 plants randomly sampled, only one distinct 1C value was resolved with a mean 1C value of 2.84 ± 0.09, suggesting the presence of only one taxon. Interestingly, the megaspores of these plants had dense and prominent ridges and tubercles, megaspore ornamentation corresponding with the tetraploid *I. "hyemalis"* complex, a taxon reported from this site by Musselman et al. (1996). However, a $1C = 2.84$ value for the River of *Isoetes I. "hyemalis"* was significantly different from the $1C = 3.37$ value for *Isoetes "hyemalis"* from Isle of Wight County, Virginia (independent samples *t* test, $df = 27$, $t = 13.5$, $p < 0.001$). This apparent difference in 1C value for two putative populations of the tetraploid *Isoetes "hyemalis"* may indicate different polyploid parentage scenarios and thus genome sizes. However, conclusions must be tempered until the parentage scenarios at these populations are clarified using DNA sequence-based lineage sorting techniques (e.g., Hoot et al. 2004). Still, the plants at River of *Isoetes* collected for this study, may represent the sterile triploid *Isoetes* × *bruntonii*, although that is unlikely because they did not have variable megaspore ornamentation and size attributed to the hybrid (Musselman et

al. 1996). *Isoetes* sequence data from this locality also suggests the presence of *I. melanopoda* ssp. *silvatica* (Schafran, unpubl. data). This area is known as River of *Isoetes* because of the extensive turflike carpet of *Isoetes* that forms in this shallow, braided stream in Dinwiddie County, Virginia. It is conceivable that there is a mélange of diploids present (possibly *I. engelmannii* and *I. melanopoda* ssp. *silvatica*), a sterile triploid (*I.* × *bruntonii*), and one or more members of the tetraploid *I. "hyemalis"* complex, a mixture of taxa that we did not detect despite randomly sampling 24 individuals. Clearly more work is required at this location.

This study demonstrates that DNA flow cytometry shows promise to aid in species delimitation in this notoriously difficult genus. The benefits of DNA flow cytometry are that it is rapid and that microphylls can be collected without destroying the plant for analysis. We recommend that *Isoetes* researchers consider adding C values to their new *Isoetes* species descriptions. We make the following suggestions to *Isoetes* C value estimations to maintain data quality. Multiple lines of evidence (including chromosome squashes, megaspore or other gross morphology, ploidy by sequencing low-copy nuclear genes, such as the LFY homologue) should be presented if possible to infer ploidy level, with vouchers. If possible, reference C values should be taken from plants at the type locality. Only commonly used reference plant material should be used as the internal standard (see Doležel and Bartos, 2005). We consider C value estimations an additional tool in the admittedly limited tool kit of taxonomists working on the many open questions in *Isoetes* taxonomy. As world phylogenies of *Isoetes* become better resolved, a database of reliable C values would facilitate the study of genome-size evolution in this cryptic taxon.

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