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Ploidy level and genome size variation in the homosporous ferns *Botrychium* s.l. (Ophioglossaceae)

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Abstract Recent cytological and molecular studies have investigated genome size variation and evolution in the homosporous ferns, but representatives of the Ophioglossaceae were largely overlooked, despite their evolutionary singularity. Flow cytometry analysis was performed on 41 individuals of eight species of the genera *Botrychium* (*B.*), *Botrypus*, and *Sceptridium* to estimate their ploidy level variation. In a subset of individuals, we also estimated the absolute genome size and corresponding *C* values. Additionally, a classical chromosome count was made on the recently described species *B. alaskense*. Ploidy level and new genome size records were determined for *Botrychium alaskense*, *B. boreale*, *B. lanceolatum*, *B.* "neolunaria" ined., *B. pinnatum*, *Botrypus virginianus* and *Sceptridium multifidum.* In addition, we confirmed the genome size of *B. matricariifolium*, *B. minganense* and *B. lunaria.* Two of the three major sub-clades of *Botrychium* differ slightly in their averaged homoploid genome size (subclade Lanceolatum, 24.72 ± 0.40 pg; subclade Lunaria, 27.51 ± 0.47 pg). Flow cytometry and chromosome counting confirmed that *B. alaskense* is a tetraploid. A new hexaploid cytotype, putatively formed through an autopolyploidization from the sympatric tetraploid cytotype, was detected in a single individual of *B. boreale*. This is only the second report of hexaploidy in the genus *Botrychium* and our data highlight the potential to find other ploidy levels within other *Botrychium* species. Interestingly, no difference within the monoploid genome sizes was detected between ploidy levels, thus supporting the hypothesis of genome size stability after polyploidization and rejecting the scenario of genome downsizing.

Keywords *Botrychium* · Chromosome · Homosporous ferns · Flow cytometry · Genome size · Polyploidy

Introduction

With the largest genome sizes and highest chromosome number in the plant kingdom, the homosporous ferns, including the Ophioglossaceae family, have a unique evolutionary history with deep divergences from over 300 mya (Pryer et al. 2004; Leitch et al. 2005). Belonging to this fern group, *Botrychium* s.l. (sensu latu, in the broad sense) (or grapeferns) includes five more narrowly circumscribed genera, namely, *Botrychium* (*B.* hereafter) s.s. (sensu strictu, in the narrow sense) (Milde) Clausen, *Botrypus* (L.) Michx, *Japanobotrychium* Masam, *Osmundopteris* (Milde) Clausen, and *Sceptridium* (Lyon) Clausen (Clausen 1936; Kato 1987; Hauk et al. 2003; Shinohara et al. 2013). Representatives of these genera differ mainly in sterile leaf blade size and shape. Their morphological differentiation is further supported by phylogenetic analyses (Hauk et al. 2012; Shinohara et al. 2013; Dauphin et al. 2014). Here, we focus on the cryptic species of *Botrychium* s.s. and its two sister genera *Botrypus* and

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Sceptridium, which have the same base of 45 chromosomes (Paris et al. 1989; Wagner 1993).

The largest genus within *Botrychium* s.l. is *Botrychium* s.s. or the moonworts, which include 30 species (Hauk et al. 2012; Dauphin et al. 2014), of which 28 have known ploidy levels. Twelve species were reported to be diploid ($2n = 2x = 90$), 15 tetraploid ($2n = 4x = 180$) and one species, *B. pseudopinnatum*, hexaploid ($2n = 6x = 270$) (Wagner 1993; Farrar 2011). However, the ploidy levels have not yet been reported for the two species *B. alaskense* and *B. boreale*. Based on these differing ploidy levels, it is obvious that polyploidy has played a key role in the speciation of *Botrychium* s.s. (Wood et al. 2009). Most importantly, the polyploid speciation in *Botrychium* has tightly been associated with interspecific hybridization, as suggested by morphological (Wagner and Lord 1956; Wagner and Grant 2002), karyological (Wagner and Lord 1956; Wagner 1993), allozyme (Hauk and Haufler 1999; Zika and Farrar 2009; Farrar 2011) and molecular phylogenetics data (Hauk et al. 2003; Williams and Waller 2012; Dauphin et al. 2014).

Despite the importance of polyploidization in the evolution of *Botrychium* s.s., records on ploidy level variation within the genus are rather scarce (Table 1), mainly because of methodological limits concerning (1) chromosome counting (up to 270 chromosomes for hexaploid specimens); and (2) problematic long-term cultivation of specimens under experimental conditions because of their dependence on arbuscular mycorrhizae fungi (Kovács et al. 2007; Winther and Friedman 2008). However, the relatively recent introduction of flow cytometry into plant research may resolve the first methodological issue (Doležel et al. 2007). Flow cytometric analyses estimate ploidy levels of samples by measuring an absolute amount of the DNA in isolated nuclei; therefore, when used appropriately, this method might provide an important insight into genome size variation in plants. Absolute genome size is considered an important taxonomic criterion that may help to discriminate closely related taxa and infer their evolutionary histories (Leitch et al. 2005). To date, genome size has been measured in 12 species of *Botrychium* s.l. (Table 1) and consequently, it is still unknown for the majority of species. Given the high chromosome number per nuclei in *Botrychium* s.l., their holoploid genome was considered to be either intermediate or large by Leitch et al. (2005), varying from a $2C$ value $= 22.90 \pm 1.82$ pg in *Botrypus virginianus* to 53.68 ± 5.05 pg in *B. minganense* (Williams and Waller 2012). Importantly, when comparing the holoploid genome sizes in closely related genera of *Botrychium* s.l., Williams and Waller (2012) found that the genome of *Botrypus* ($1Cx = 5.73$ pg) was 2.3 times smaller than that of *Botrychium* s.s. (overall mean $1Cx = 13.36$ pg), which supported their recognition as distinct genera.

The present study aims to assess the ploidy levels and genome size variation from 41 specimens of eight species of *Botrychium*, *Botrypus* and *Sceptridium* collected in the Europe and North America. Using flow cytometry and the classical karyological approach, these data can be used to retrace genome evolution in the Ophioglossaceae which is the plant family with highest chromosome counts observed to date.

Materials and methods

Sampling

Living plants, including their below-ground parts, were dug up together with surrounding soil and co-occurring vegetation in Switzerland, Sweden and the US (Alaska) during the summer of 2012. Plants were then potted and maintained at the botanical gardens of the Université de Neuchâtel and the Université de Fribourg in Switzerland until the analyses were conducted. After analysis, vouchers were deposited in the Herbarium of the Université de Neuchâtel (NEU) (except for the accession BD022C). All accessions sampled are listed in Table 2.

Chromosome counting

In the field, root tips of several individuals of *Botrychium alaskense* were taken, washed with distilled water and pretreated with a bromo-naphthalene solution saturated in the aqueous phase for 3 h. Fixation was performed using 10 mL of fixative solution containing 75 % absolute ethanol, 24 % of glacial acetic acid, five drops of carmine acetic acid and one drop of iron acetate (Sharma and Sharma 1980). The coloration step involved fixing root tips in a porcelain dish filled with carmine acetic acid and 10 ml of iron acetate with gentle heating for 2 min without ebullition. The crushed root tips and young cells in mitosis were viewed using a Leica Leitz microscope. We referred to previous chromosome counting for the remaining species analyzed in this study (Table 1).

Flow cytometry

A Partec CyFlow SL flow cytometer (Partec GmBH, Münster, Germany) equipped with a green 532-nm laser was used to assess ploidy level in 41 accessions of *Botrychium* (Table 3). The samples were prepared from fresh leaf tissue by chopping them with razor blades in 1 ml of general-purpose buffer (0.5 mM spermine, 30 mM sodium citrate, 20 mM 4-morpholine propane sulfonate, 80 mM KCl, 20 mM NaCl, 0.5 % Triton X-100, pH = 7.0) with subsequent incubation for 5 min,

Table 1 Published chromosome counts/DNA-ploidy level estimations of *Botrychium* s.s. with their geographical origins

Taxa	Country	Nb.	Chromosome no. ($2n$)/ploidy level	$2C$ value ± SD (pg) error	References
B. ascendens W.H.Wagner	USA	1	180/4*x*	–	Wagner (1993)
B. campestre W.H.Wagner & D.Farrar	USA	2	90/2*x*	–	Wagner (1993); Wagner and Wagner (1990)
B. crenulatum W.H.Wagner	USA	1	90/2*x*	–	Wagner (1993)
B. echo W.H.Wagner	USA	1	180/4*x*	51.63	Wagner (1993); Williams and Waller (2012)
B. gallicomontanum D.Farrar & Johnson-Groh	USA	1	Unknown/4*x*	–	Wagner (1993)
B. hesperium (Maxon & R.T.Clausen) W.H.Wagner & Lellinger	USA	1	180/4*x*	47.16	Wagner (1993); Williams and Waller (2012)
B. lanceolatum (S.G.Gmelin) Angström ssp. *angustisegmentum* (Pease & A.H.Moore) R.T.Clausen	USA	2	90/2*x*	–	Löve and Löve (1976); Wagner (1993)
B. lanceolatum (S.G.Gmelin) Angström ssp. *lanceolatum* Pease & A.H.Moore	ITA; GRL; USA	4	90/2*x*	29.56 ± 0.95	Fabbri (1963); Löve and Löve (1976); Dalgaard (1989); Wagner (1993); Williams and Waller (2012)
B. lunaria (L.) Swartz	ITA; GEO; GRL; RUS; USA	5	90-60/2*x*	29.44 ± 3.23	Zhukova and Petrovsky (1976); Dalgaard (1989); Wagner (1993); Gagnidze et al. (1998); Peruzzi et al. (2003); Williams and Waller (2012)
B. matricariifolium (Döll) A.Braun	USA	2	180/4*x*	50.82 ± 1.22	Löve and Löve (1976); Wagner (1993); Williams and Waller (2012)
B. michiganense (W.H.Wagner) A.V.Gilmand, D.R.Farrar & P.F.Zika	USA	–	–/4*x*	46.64 ± 2.85	Williams and Waller (2012)
B. minganense M.Victorin	USA	2	180/4*x*	53.68 ± 5.05	Löve and Löve (1976); Wagner (1993); Williams and Waller (2012)
B. montanum W.H.Wagner	USA	1	90/2*x*	28.19	Wagner (1993); Williams and Waller (2012)
B. mormo W.H.Wagner	USA	1	90/2*x*	–	Wagner (1993)
B. pallidum W.H.Wagner	USA	2	90/2*x*	24.05 ± 1.49	Wagner and Wagner (1990); Wagner (1993); Williams and Waller (2012)
B. paradoxum W.H.Wagner	USA	1	180/4*x*	–	Wagner (1993)
B. pedunculosum W.H.Wagner	USA	1	180/4*x*	–	Wagner (1993)
B. pinnatum H.St.John	ITA; USA	2	180/4*x*	–	Fabbri (1963); Wagner (1993)
B. pseudopinnatum W.H.Wagner	USA	2	270/6*x*	–	Wagner and Wagner (1990); Wagner (1993)
B. pumicola (Underw) Coville	USA	1	90/2*x*	–	Wagner (1993)
B. simplex E.Hitchc	USA	1	90/2*x*	22.05	Wagner (1993); Williams and Waller (2012)
B. spathulatum W.H.Wagner	USA	2	180/4*x*	53.07 ± 5.43	Wagner and Wagner (1990); Wagner (1993)
B. watertonense W.H.Wagner	USA	1	180/4*x*	–	Wagner (1993)
Botrypus virginianus (L.) Michaux	USA	1	180/4*x*	22.90 ± 1.82; 20.44 ± 0.22	Wagner (1993); Bainard et al. (2011); Williams and Waller (2012)
Sceptridium multifidum (S.G.Gmelin) M.Nishida ex Tagawa	USA	1	90/2*x*	–	Wagner (1993)

Nb number of localities of each *Botrychium* species that was analyzed for chromosome counts, *GEO* Georgia, *GRL* Greenland, *ITA* Italy, *RUS* Russia, *USA* United States

according to Loureiro et al. (2007). Samples were then filtered through a green Partec filter (40 µm) and stained with a 30-µL mixture of propidium iodide and RNase (ratio 1:1, both of initial concentration of 1 %). *Secale cereale* cv. Dankovské ($2C = 16.19$ pg, Doležel et al. 1998) was used as an internal standard for all species and *Vicia faba*

Table 2 *Botrychium* s.l. samples analyzed using flow cytometry and chromosome squashes

Species	Accession	Country	Locality	Coordinates
Botrychium alaskense W.H.Wagner & J.R.Grant var. *alaskense*	BD047AC	USA	Alaska (Salcha River)	N 64°28′05.3′; W 146°44′58.1″
Botrychium alaskense W.H.Wagner & J.R.Grant var. *alaskense*	BD053BEG	USA	Alaska (320 miles, Richardson Highway)	N 64°25′41.0″; W 146°53′50.3″
Botrychium alaskense W.H.Wagner & J.R.Grant var. *alaskense*	BD068BC	USA	Alaska (322 miles, Richardson Highway)	N 64°26′47.1″; W 146°54′34.4″
Botrychium boreale J.Milde	BD022AD	SWE	Norrbotten province (Siknäs)	N 65°45′30.97″; W 22°40′03.22″
Botrychium boreale J.Milde	BD022C	SWE	Norrbotten province (Siknäs)	N 65°45′30.97″; W 22°40′03.22″
Botrychium lanceolatum (S.G.Gmelin) Ångström ssp. *lanceolatum*	BD022EF	SWE	Norrbotten province (Siknäs)	N 65°45′30.97″; W 22°40′03.22″
Botrychium lanceolatum (S.G.Gmelin) Ångström ssp. *lanceolatum*	BD034C	SWE	Norrbotten province (Pissiniemi)	N 67°34′57″; E 23°33′19″
Botrychium lanceolatum (S.G.Gmelin) Ångström ssp. *lanceolatum*	BD053 K	USA	Alaska (320 miles, Richardson Highway)	N 64°25′41.0″; W 146°53′50.3″
Botrychium lanceolatum (S.G.Gmelin) Ångström ssp. *lanceolatum*	BD063DDX	USA	Alaska (86 miles, Seward Highway)	N 60°54′25.6″; W 149°05′01.1″
Botrychium lunaria (L.) Swartz	BD014A	CH	Graubünden canton (Prontresina, Val Roseg)	N 46°26′00.24″; E 9°51′46.84″
Botrychium lunaria (L.) Swartz	BD016B	CH	Uri canton (Gurtnellen)	N 46°43′47.89″; E 8°36′30.95″
Botrychium lunaria (L.) Swartz	BD035A	SWE	Norrbotten province (Kätkesuando)	N 68°06′59″; E 23°20′19″
Botrychium matricariifolium (Döll) A.Braun	BD016A	CH	Uri canton (Gurtnellen)	N 46°43′47.89″; E 8°36′30.95″
Botrychium minganense M.Victorin	BD063AD	USA	Alaska (86 miles, Seward Highway)	N 60°54′25.6″; W 149°05′01.1″
Botrychium minganense M.Victorin	BD065A	USA	Alaska (88 miles, Seward Highway)	N 60°55′07.9″; W 149°07′50.8″
Botrychium "neolunaria M.Stensvold" ined.	BD058AC	USA	Alaska (8 miles, Chena Hot Springs Road)	N 64°53′03.5″; W 147°21′45.6″
Botrychium "neolunaria M.Stensvold" ined.	BD063LR	USA	Alaska (86 miles, Seward Highway)	N 60°54′25.6″; W 149°05′01.1″
Botrychium pinnatum H.St.John	BD061AI	USA	Alaska (Anchorage, the "Dome")	N 61°10′13.6″; W 149°39′18.6″
Botrychium pinnatum H.St.John	ABI1	USA	Alaska (Dutch harbor)	N 53°53′58″; W 166°33′05″
Botrychium pinnatum H.St.John	L1245316	USA	Washington state (Colville National Forest)	N 48°75′12.05″; W 117°19′67.46″
Botrypus virginianus (L.) Michx.	BD063Q	USA	Alaska (86 miles, Seward Highway)	N 60°54′25.6″; W 149°05′01.1″
Sceptridium multifidum (S.G.Gmelin) M. Nishida ex Tagawa	BD034A	SWE	Norrbotten province (Pissiniemi)	N 67°34′57″; E 23°33′19″

Vouchers were deposited at the Herbarium of the University of Neuchâtel (NEU), Switzerland

USA United States of America, *SWE* Sweden, *CH* Switzerland

ssp. *faba* var. *equine* cv. Inovec (2C = 26.90 pg) (Doležel et al. 1992, 1998) was used only for *Sc. multifidum* and all polyploid species (see Table 3). After initial ploidy level screening, we estimated the absolute genome size in 15 individuals selected from the fittest plants and those that provided enough material for repeated measurements. To isolate and stain nuclei, we used the same protocol as above. Two internal standards were used: *Secale cereale* cv. Dankovské and *Vicia faba* ssp. *faba* var. *equine* cv. Inovec. Holoploid (2*C* value) and monoploid genome size (1*Cx* value) were subsequently calculated according to the method of Greilhuber et al. (2005). Each plant was analyzed once a day on 3 consecutive days to minimize instrumental errors (Doležel et al. 2007), except for four plants (both individuals of *B. lanceolatum* ssp. *lanceolatum* and *B.* "neolunaria" ined.), that were analyzed only twice with *Vicia faba* as the standard. Histograms were accumulated at a flow rate of approximately 10–30 particles per second for a total count of 1500 (ploidy level estimations) and 5000 (absolute genome size estimations) nuclei. Coefficient of variations (CV) of the peaks of internal standards ranged from 1.78 to 6.47 %, with an average value of 3.58 %; and the CV of peaks of measured samples varied between 1.96 and 5.46 %, with an average value of 3.61 %. Measurements exceeding 3 % divergence between independent runs were usually discarded and the sample was re-analyzed. Repeated measurements were averaged to obtained mean 2*C* values per accession.

Table 3 Ploidy level variation in *Botrychium* species analyzed with *Secale cereale* (2*C* value = 16.19 pg) as an internal standard

Species	# plants/# sites	Mean fluorescence ratio	Min–max fluorescence ratio range	SD	Estimated ploidy level
Botrychium lanceolatum ssp. *lanceolatum*	10/4	1.46	1.42–1.50	0.52	2
Botrychium lunaria	3/3	1.69	1.65–1.74	0.75	2
Botrychium "neolunaria" ined.[a]	4/2	1.67	1.56–1.76	1.55	2
Botrychium alaskense	8/3	2.97	2.86–3.05	1.21	4
Botrychium boreale	2/1	3.10	3.04–3.16	1.37	4
Botrychium matricariifolium	1/1	2.38	–	–	4
Botrychium minganense	4/2	2.73	2.67–2.80	1.58	4
Botrychium pinnatum	9/3	2.96	2.88–3.10	1.10	4
Botrychium boreale[b]	1/1	4.55	–	–	6

[a] The North American plants of the *B. lunaria* complex have been proposed as a distinct, yet unpublished, taxon

[b] Analyzed using *Vicia faba* because of the large genome size

Statistical analyses

The estimated genome sizes of this study were averaged per species with 11 published records in Williams and Waller (2012) (Table 1). In total, five diploid and nine tetraploid species of *Botrychium* s.s. were considered in a combined dataset. Monoploid genome size was assessed with the holoploid genome size divided by the ploidy level. Normality assumptions were tested on residuals of a linear model with the Shapiro–Wilk normality test using the package Stats in the R statistical software (R Development Core Team 2015). Overall mean genome size comparison between ploidy levels was performed following the Welch *t* test for both holo- and monoploid genome size. The graphical representation of quantiles was plotted with ploidy level as the grouping factor.

Results

Ploidy level in *Botrychium* s.l.

The ploidy levels for 41 accessions belonging to eight species were inferred by flow cytometry analyses and are listed in Table 3. Among them, three species were confirmed to be diploid, four species were tetraploid and for one species, *B. boreale*, we found both tetra- and hexaploid plants. The hexaploid record is only the second one reported in the genus *Botrychium* (see "Discussion"). Tetraploidy is confirmed here for the first time in both *B. boreale* and *B. alaskense*. In addition to flow cytometric estimations, the tetraploid status of *B. alaskense* was corroborated by a classical chromosome counting ($2n = 4x =$ ca 180), based on $x = 45$ (Wagner 1993) (Fig. 1).

Genome size variation in *Botrychium* s.l.

The absolute genome size values (2*C*) and their derived 1*Cx* values determined for 15 accessions belonging to nine taxa are listed in Table 4. Genome size estimations were fairly similar when two different standards were used (*Secale cereale* and *Vicia faba*), with the maximum divergence (3.54 %) recorded in *B. alaskense* (BD053G). The 2*C* values in diploid *Botrychium* species ranged from 24.72 ± 0.40 to 27.51 ± 0.60 pg (Table 4; Fig. 2a). However, in the closely related diploid *Sceptridium multifidum*, we recorded the smallest genome size with

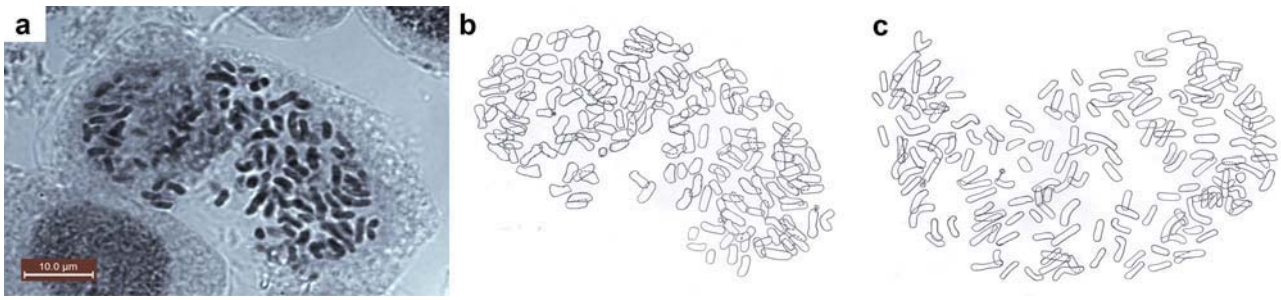

Fig. 1 Mitotic metaphase chromosomes of tetraploid ($2n = 4x = 180$) *Botrychium alaskense* (**a**, **b** plant BD1204805; **c** plant BD04803; Salcha River population, Alaska, USA; N 64°28′05.3″, W 146°44′58.1″). The drawings were made by Philippe Küpfer and Benjamin Dauphin

Table 4 Absolute genome size values of 15 accessions of seven species of the genera *Botrychium*, *Botrypus* and *Sceptridium*, compared with two internal standards (*Secale cereale* and *Vicia faba*)

Species	Accession	Ploidy level (*x*)	2*C* value *Secale* (pg) with (±SD)	1*Cx* value *Secale* (pg)	2*C* value *Vicia* (pg) with (±SD)	1*Cx* value *Vicia* (pg)
Sceptridium multifidum	BD034A	2	NM	NM	16.11 (±0.06)	8.06
Botrypus virginianus	BD063Q	4	NM	NM	18.93 (±0.26)	4.73
Botrychium lanceolatum ssp. *lanceolatum*	BD063T	2	24.62 (±0.37)	12.31	NM	NM
Botrychium lanceolatum ssp. *lanceolatum*	BD063U	2	24.81 (±0.49)	12.41	NM	NM
Mean values for *B. lanceolatum*			24.72 (±0.40)	12.36	–	–
Botrychium "neolunaria" ined.[1]	BD058A	2	27.26 (±0.17)	13.63	NM	NM
Botrychium "neolunaria" ined.[1]	BD058B	2	27.75 (±0.60)	13.87	NM	NM
Mean values for *B.* "neolunaria" ined.[a]			27.51 (±0.47)	13.75	–	–
Botrychium alaskense	BD053E	4	50.77 (±0.49)	12.69	51.96 (±0.32)	12.99
Botrychium alaskense	BD053G	4	50.36 (±1.01)	12.59	52.21 (±0.52)	13.05
Botrychium alaskense	BD053B	4	50.54 (±0.78)	12.63	52.06 (±0.12)	13.01
Botrychium alaskense	BD068B	4	50.91 (±0.60)	12.73	51.04 (±0.10)	12.76
Mean values for *B. alaskense*			50.64 (±0.67)	12.66	51.82 (±0.52)	12.95
Botrychium pinnatum	BD061D	4	50.20 (±0.88)	12.55	50.58 (±0.80)	12.64
Botrychium pinnatum	BD061H	4	49.17 (±0.72)	12.29	50.45 (±0.31)	12.61
Mean values for *B. pinnatum*			49.69 (±0.91)	12.42	50.51 (±0.50)	12.63
Botrychium boreale	BD022A	4	52.70 (±0.47)	13.17	52.63 (±0.16)	13.16
Botrychium boreale	BD022B	4	51.43 (±0.61)	12.86	51.65 (±0.33)	12.91
Botrychium boreale	BD022C	6	79.09 (±4.02)	13.18	76.52 (±1.31)	12.75
Mean values for *B. boreale*			–	13.07	–	12.94
Overall mean values for diploid species of *Botrychium* s.s.			26.11 (±1.52)	13.06	–	–
Overall mean values for tetraploid species of *Botrychium* s.s.			50.80 (±1.14)	12.72	51.49 (±0.81)	12.84

NM not measured

[a] The North American genotype of the *B. lunaria* complex has been proposed as a distinct, yet not described, species

16.11 ± 0.06 pg (Table 4; Fig. 2b). In tetraploids, the 2*C* values measured against *Secale* and *Vicia* standards ranged from 49.69 ± 0.91 to 52.07 ± 1.6 pg and from 50.51 ± 0.50 to 52.14 ± 1.16 pg, respectively. The smallest genome size (2*C* = 18.93 ± 0.26 pg) among tetraploids was recorded in *Botrypus virginianus* (Table 4). The genome size of one hexaploid individual of *B. boreale* (BD022C) was estimated as 79.09 ± 4.02 pg with *Secale*, and 76.52 ± 1.31 pg with *Vicia* as standards, respectively (Table 4; Fig. 2f).

Holo- and monoploid genome size comparison in *Botrychium* s.s.

For the holoploid genome size, median values differed greatly between ploidy levels, with 27.14 pg for diploid and 50.82 pg for tetraploid species, whereas they were very similar between ploidy levels for monoploid genome sizes: 13.57 pg for diploids and 12.71 pg for tetraploids (Fig. 3). Additionally, holoploid genome sizes varied significantly ($p < 0.001$) between ploidy levels, with a 2*C* value mean of 25.98 pg for diploids and 50.65 pg for tetraploids (Fig. 3a). In contrast, monoploid genome sizes were not significantly different between diploids and tetraploids ($p = 0.64$), displaying very similar means of 12.99 and 12.66 pg for diploid and tetraploid species, respectively (Fig. 3b).

Discussion

Ploidy level and genome size variation in *Botrychium* s.l. and related species

Our results confirmed the previously reported ploidy levels of six species (*B. lanceolatum*, *B. lunaria*, *B.* "neolunaria" ined., *B. matricariifolium*, *B. minganense*, and *B. pinnatum*), that were mostly based on exact chromosome counts (Wagner 1955, 1993; Wagner and Lord 1956; Wagner and Wagner 1986, 1990; Stensvold 2008), or more recently, by flow cytometry on specimens obtained exclusively from North America (Bainard et al. 2011; Bai et al. 2012; Williams and Waller 2012). Here, we analyzed specimens from the Alps and Scandinavia, as well as North America.

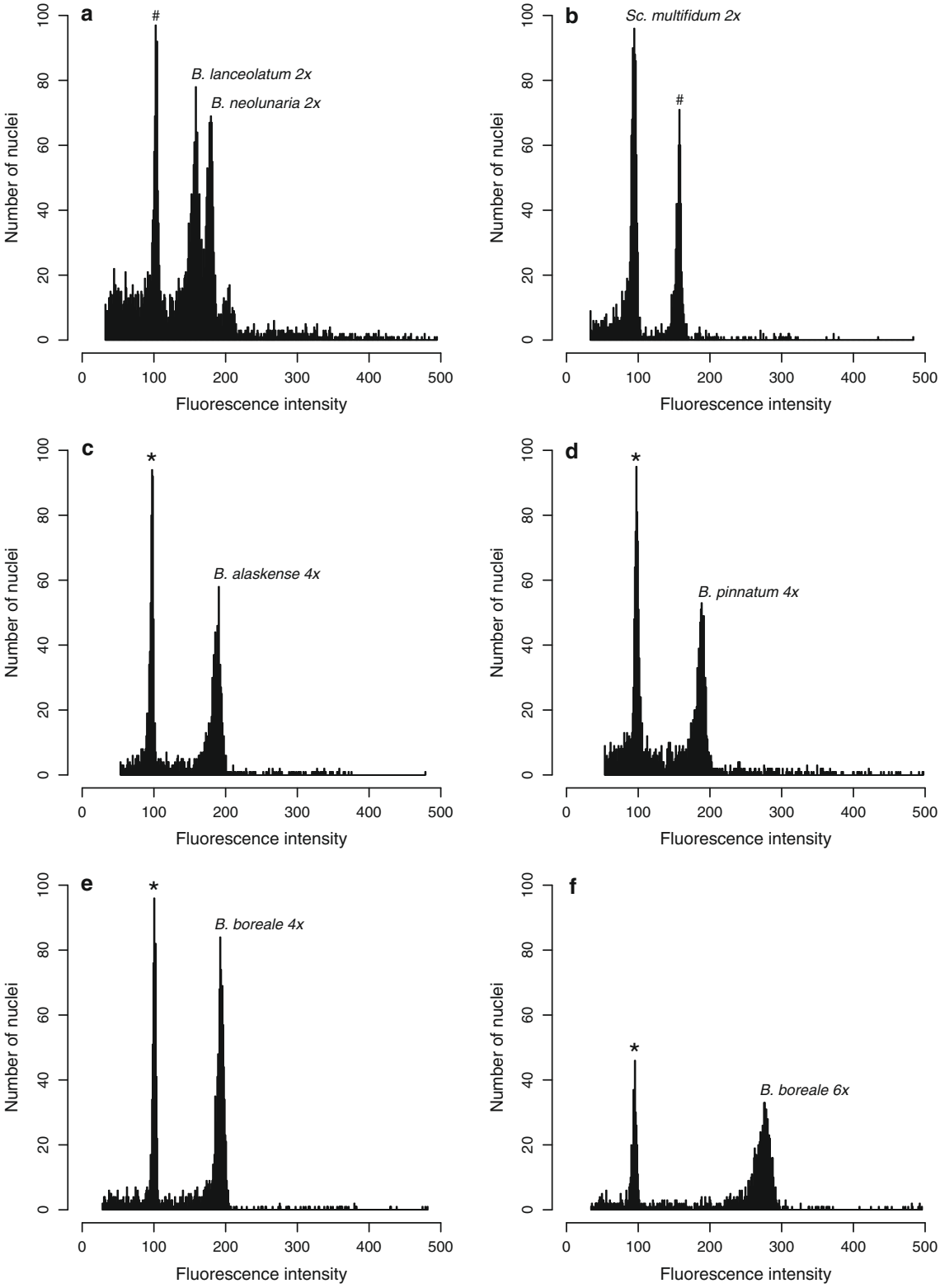

Fig. 2 Histograms of the absolute DNA content of *Botrychium* s.l.: **a** *B. lanceolatum* 2*x* and *B. "*neolunaria*"* 2*x*; **b** *Sc. multifidum* 2*x*; **c** *B. alaskense* 4*x*; **d** *B. pinnatum* 4*x*; **e** *B. boreale* 4*x*; **f** *B. boreale* 6*x*. The two internal standards are symbolized by "*hash symbol*" for *Secale cereale* and "*asterisk*" for *Vicia faba*

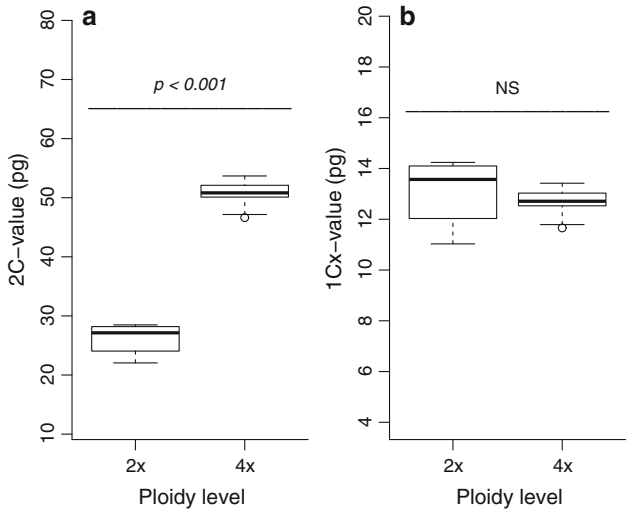

Fig. 3 Averaged $2C$ (**a**) and $1Cx$ (**b**) values of *Botrychium* s.s. per ploidy level, including published genome sizes in Williams and Waller (2012). It includes five diploid and nine tetraploid species. The significance of the statistical test (*t test*) is given for both holoploid and monoploid genome sizes

In addition, we determined the tetraploid level of *B. alaskense* and *B. boreale* for the first time, and found intraspecific variation in the latter taxon, showing both tetra- and hexaploid cytotypes. The hexaploid record is only the second one in the genus *Botrychium*, and the first outside North America. Previously, hexaploidy had only been reported in one population of *B. pseudopinnatum* from the northwest shore of Lake Superior (Ontario, Canada) (Wagner and Wagner 1990).

The absolute genome sizes were assessed for the first time in four species *B. alaskense*, *B. boreale*, *B. pinnatum* and *Sceptridium multifidum* (see Table 4). For three species (*B. lanceolatum*, *B.* "neolunaria" ined. and *Botrypus virginianus*) with previously published genome sizes (Bainard et al. 2011; Williams and Waller 2012), we found very similar values, despite different internal standards, protocols and types of material (fresh vs. dried) used. Exceptions occurred when the genome size of the standard species was too different from that of the species being analyzed, as was the case with *Zea mays* and *Botrypus virginianus* with 22.9 ± 1.8 pg (Williams and Waller 2012; in our study $2C = 18.93 \pm 0.26$ pg), or *B. lanceolatum* with 30.44 ± 0.9 pg (Williams and Waller 2012; in our study $2C = 24.72 \pm 0.40$ pg). Conversely, when *Allium cepa* was used as an internal standard (Bainard et al. 2011), the difference between the reported ($2C = 20.44$ pg) and our genome size estimate ($2C = 18.93 \pm 0.26$ pg) for *Botrypus virginianus* was 7.4 %. Thus, with an appropriate internal standard (i.e. with the genome size value close to that of measured samples), genome size can be estimated on material dried in silica gel, which however exhibits higher coefficient of variation (around 5 %) (Williams and Waller 2012). This method may even be preferable, since *Botrychium* is hardly ever cultivated because of its obligate arbuscular mycorrhizal fungal symbionts (Clausen 1936), in addition to the problem of extracting living specimens in populations of rare or endangered species (Casson et al. 2002).

The newly recognized species *B.* "neolunaria" ined. shows almost identical genome size (±0.02 of the mean fluorescence ratio, Table 3) to *B. lunaria*, supporting their close relationship in the cryptic Lunaria group, which has been corroborated by phylogenetic inferences based on plastid regions, where both taxa are positioned with the allopolyploid *B. yaaxudakeit* (*B. lunaria* × *B.* "neolunaria" ined.) in the Lunaria clade (Stensvold 2008; Dauphin et al. 2014). Furthermore, differences in monoploid genome sizes between *Botrychium* s.s. ($1Cx = 12.83$ pg), *Botrypus* ($1Cx = 4.73$ pg) and *Sceptridium* ($1Cx = 8.06$ pg) were consistent with their phylogenetic placement within distant clades (Hauk et al. 2003).

Absolute genome size variation and its relationships to auto- and allopolyploid events

Based on its morphology *Botrychium boreale* has long been considered a tetraploid species (Hultén 1968; William 1996). We confirmed this hypothesis and even found intraspecific variation in ploidy level. At the Siknäs site (Norrbotten, Sweden), we found one hexaploid individual intermixed (the minimum distance between 4*x* and 6*x* plants was 5 cm) within the typical tetraploid population of *B. boreale*, raising questions about its origin. The following arguments support its autopolyploid origin from co-occurring allotetraploid *B. boreale*. First, the hexaploid plant was morphologically indistinguishable from the co-occurring tetraploids. Second, it had almost exactly 1.5-fold the genome size of tetraploid *B. boreale* plants (1.52- and 1.47-fold with *Secale* and *Vicia*, respectively, as the standards), which suggested a fusion of reduced (2N) and unreduced gametes (4N) (see below). Third, hexaploid and tetraploid plants (BD12022C) from this locality shared the same plastid haplotype (Dauphin et al. 2014). These data suggested that both polyploidization mechanisms (auto- and allo-) could operate in *Botrychium*, although allopolyploidization seems to be more frequent in this genus (Farrar 2011). Nevertheless, because allopolyploidy is more easily detected than autopolyploidy (Soltis et al. 2007), the latter hybridization process could be underestimated in *Botrychium.* As the fusion of reduced and unreduced gametes is the most common pathway of polyploidization (Ramsey and Schemske 1998), we expect that this process (fusion of 2N and 4N gametes) was also involved in the formation of the hexaploid plant. The

hybridogeneous origin of *B. boreale* could have contributed to this pathway by increasing the frequency of unreduced gamete formation, which is 13.5-times higher in allo- ("outcrossing taxa") than in autopolyploid species ("selfing taxa") (Ramsey and Schemske 1998).

Based on allozyme data and morphology, *B. alaskense*, *B. pinnatum* and *B. boreale* have been suggested to be allotetraploids that arose from hybridization between the two diploid species, *B. lanceolatum* and *B. lunaria*/*B.* "neolunaria" ined. (Wagner 1993; Farrar 2011), which diverged ca. 5 mya (Stensvold 2008). The small, but still distinguishable, difference in their genome sizes ($2C = 24.72 \pm 0.40$ pg in *B. lanceolatum*, $2C = 27.51 \pm 0.47$ pg in *B. lunaria*, Fig. 2a), tetraploid *B. boreale* ($2C = 52.07 \pm 0.50$ pg) showed almost an exact addition of both putative genomes. Thus, our genome size data supports the hybrid hypothesis proposed by Wagner (1993) and Farrar (2011). In contrast, the two remaining tetraploids had a slightly lower amount of nuclear DNA than expected under a hybrid scenario and complete additivity: $2C = 50.64$ and $2C = 49.69$ pg for *B. alaskense* and *B. pinnatum*, respectively.

Genome size stability

Despite significant variation between ploidy levels of holoploid genome sizes in *Botrychium* s.s., no difference was detected for monoploid genome size (Fig. 3). Yet, we had hypothesized genomic mechanisms leading to a size reduction in polyploid genomes compared to diploids, as has often been reported in plants (Leitch and Bennett 2004). Interestingly, genome downsizing seems to be ineffective or absent in this genus, which could explain why the genome sizes of *Botrychium* s.l. taxa are among the highest in vascular plants. Our study thus corroborates the pattern found in other groups of ferns (Henry et al. 2014). Accordingly, a low activity of transposable elements (Brandes et al. 1997) combined with a high retention rate of chromosomes (Barker 2013) may lead to an unprecedented conservation in genome sizes of homosporous ferns.

Conclusions

This study reports new ploidy levels and genome size estimates in the genus *Botrychium*. Our data confirm the predominance of polyploid taxa in the genus, and in addition to allopolyploidy, flow cytometric analysis suggests that autopolyploidization may have been involved in the origin of the hexaploid cytotype of *B. boreale*. Applications of efficient flow cytometry methods and more detailed sampling have revealed higher ploidy level variation in the genus than had previously been thought. Intermediate patterns in genome sizes in *B. alaskense*, *B. boreale* and *B. pinnatum* support the hypothesis that their allopolyploid origin arose between two diploid taxa, *B. lanceolatum* and *B. lunaria*/*B.* "neolunaria" ined. Finally, no difference in monoploid genome size was detected between ploidy levels indicating that strong genomic mechanisms must allow the stability of those genomes among the largest land plants.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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