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Nuclear DNA C-values in 30 Species Double the Familial Representation in Pteridophytes

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Nuclear DNA C-values and genome size are important biodiversity characters with fundamental biological significance. Yet C-value data for pteridophytes, a diverse group of vascular plants with approx. 9000 extant species, remain scarce. A recent survey by Bennett and Leitch (2001, *Annals of Botany* **87**: 335–345) found that C-values were reported for only 48 pteridophyte species. To improve phylogenetic representation in this group and to check previously reported estimates, C-values for 30 taxa in 17 families were measured using flow cytometry for all but one species. This technique proved generally applicable, but the ease with which C-value data were generated varied greatly between materials. Comparing the new data with those previously published revealed several large discrepancies. After discounting doubtful data, C-values for 62 pteridophyte species remained acceptable for analysis. The present work has increased the number of such species' C-values by 93 %, and more than doubled the number of families represented (from 10 to 21). Analysis shows that pteridophyte C-values vary approx. 450-fold, from 0·16 pg in *Selaginella kraussiana* to 72·7 pg in *Psilotum nudum* var. *gasa*. Superimposing C-value data onto a robust phylogeny of pteridophytes suggests some possible trends in C-value evolution and highlights areas for future work.

Key words: DNA amounts, evolution of C-values, *Equisetum*, ferns, flow cytometry, genome size, Pteridophyte phylogeny, vascular plants.

INTRODUCTION

Nuclear DNA C-values and genome size are important biodiversity characters with fundamental biological significance and many uses (Bennett and Leitch, 1995; Bennett *et al*., 2000). In angiosperms, knowledge of C-values continues to improve. For example, C-values for 691 previously unrepresented species were recently collated by Bennett *et al*. (2000) thus increasing the percentage of species with known C-values from approx. 1 % to 1·4 %. In contrast, C-value data in pteridophytes, which comprise lycophytes (clubmosses and relatives), Equisetophyta (horsetails), Psilotophyta (whisk ferns) and Polypodiophyta (ferns), have remained scarce. At the Angiosperm Genome Size Workshop held at the Royal Botanic Gardens, Kew (RBG Kew) in September 1997, Murray reviewed our knowledge of C-values in non-angiosperm plants and noted that data were only available for approx. 0·4 % of approx. 9000 pteridophyte species, and that these had been difficult to locate (see summary of meeting at http://www.rbgkew.org.uk/cval/conference.html).

A recent literature survey (Bennett and Leitch, 2001) showed that since the 1997 workshop the situation for pteridophytes had changed little: DNA C-values were found for only 48 species, originating from just eight source references. Bennett and Leitch (2001) pooled these data into one reference source and reviewed the origin, reliability and significance of the C-value estimates. A goal of obtaining first C-values for 200 pteridophyte species by 2005 was suggested, with an emphasis on selecting species that maximized systematic and geographic representation of this important vascular plant group. Here we report new work estimating C-values in 30 pteridophyte taxa, which represents the first step towards achieving this goal. Most taxa were selected with the aim of improving phylogenetic representation. In addition, several species listed in Bennett and Leitch (2001) were re-measured using fully verified material from RBG Kew with the aim of checking previous estimates. The current paper also includes C-value estimates for nine pteridophyte taxa given in Grime *et al*. (1988), a source reference not included in Bennett and Leitch (2001).

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MATERIALS AND METHODS

Plant material

Table 1 lists the 30 pteridophyte taxa studied in the present work. Material was obtained from the Living Collections Department at RBG Kew and voucher specimens for all taxa are deposited in the Herbarium (K). Table 1 also gives the verification status of each taxon. Twenty were fully verified but the specific identity of ten has not yet been fully determined. Such verification awaits spore production, and for tissue-cultured material this may not occur for several years. Estimates for these ten taxa are included, as knowledge of first C-values in a known genus is considered

TABLE 1. *The 30 pteridophyte taxa studied in the present work together with RBG Kew identity number (ID no.), identification status (ID status), angiosperm calibration standard used to estimate DNA C-values, spore type, sporangial type and sperm flagella number*

Entry no.	Taxon	Family	ID no.*	ID status†	Standard species‡	Spore type§	Sporangial type¶	Sperm flagella**
Lycopodiophyta								
1a	*Selaginella kraussiana* (Kunze) A.Br. var. *poolteri*	Selaginellaceae	1998–4081	f.v.	*Oryza*	Het	E	B
1b	*Selaginella kraussiana* (Kunze) A.Br. var. *poolteri*	Selaginellaceae	TC3646	a.v.	*Oryza*	Het	E	B
2a	*Selaginella kraussiana* (Kunze) A.Br.	Selaginellaceae	POW	f.v.	*Oryza*	Het	E	B
2b	*Selaginella kraussiana* (Kunze) A.Br.	Selaginellaceae	1998–4013	a.v.	*Oryza*	Het	E	B
Psilotophyta								
3	*Psilotum nudum* (L.) Griseb. var. *gasa*	Psilotaceae	1981–4164	f.v.	*Allium*	Hom	E	M
Equisetophyta								
4	*Equisetum debile* Roxb. ex Vaucher	Equisetaceae	1974–1881	f.v.	*Allium*	Hom	E	M
5	*Equisetum giganteum*††	Equisetaceae	1974–2350	f.v.	*Allium*	Hom	E	M
6	*Equisetum myriochaetum* Schltdl. & Chamb	Equisetaceae	1994–3384	f.v.	*Allium*	Hom	E	M
7	*Equisetum scirpoides* Michx.	Equisetaceae	1934–97905	f.v.	*Allium*	Hom	E	M
8	*Equisetum variegatum* Schleich. ex Weber & Mohr	Equisetaceae	1977–882	f.v.	*Allium*	Hom	E	M
Polypodiophyta								
Basal eusporangiate ferns								
9	*Marattia* sp.	Marattiaceae	1973–20423	a.v.	*Pisum*	Hom	E	M
10	*Ophioglossum gramineum* Willd.	Ophioglossaceae	1981–6838	f.v.	*Allium*	Hom	E	M
11	*Ophioglossum petiolatum* Hook.	Ophioglossaceae	1988–3791	f.v.	*Allium*	Hom	E	M
Basal leptosporangiate ferns								
12	*Todea barbara* (L.) T.Moore	Osmundaceae	1987–188	a.v.	*Pisum*	Hom	L	M
Filmy ferns								
13	*Trichomanes speciosum* Willd.	Hymenophyllaceae	1970–4688	a.v.	*Pisum*	Hom	L	M
Heterosporous water ferns								
14	*Azolla microphylla* Kaulf.	Azollaceae	1986–5976	a.v.	*Oryza*	Het	L	M
Tree ferns								
15	*Cibotium* sp.	Dicksoniaceae	TC 3495	a.v.	*Hordeum*	Hom	L	M
16	*Cibotium hawaiense* Nakai & Ogura	Dicksoniaceae	1970–6171	f.v.	*Petrosel.*	Hom	L	M
17	*Cyathea crinita* (Hook.) Coper.	Cyatheaceae	TC 3492	a.v.	*Pisum*	Hom	L	M
18a	*Dicksonia antarctica* Labill.	Dicksoniaceae	1995–3821	f.v.	*Pisum*	Hom	L	M
18b	*Dicksonia antarctica* Labill.	Dicksoniaceae	TC 3494	a.v.	*Lycopers.*	Hom	L	M
19	*Plagiogyria* sp.	Plagiogyriaceae	TC 3004	a.v.	*Pisum*	Hom	L	M
20	*Plagiogyria matsumureana* Makino	Plagiogyriaceae	1979–5153	f.v.	*Petrosel.*	Hom	L	M
Polypodiaceous ferns								
21	*Cystopteris dickieana* R. Sim.	Aspleniaceae	1959–74801	a.v.	*Pisum*	Hom	L	M
22a	*Cystopteris fragilis* agg. (L.) Bernh.	Aspleniaceae	1983–1683	f.v.	*Pisum*	Hom	L	M
22b	*Cystopteris fragilis* agg. (L.) Bernh.	Aspleniaceae	1982–2706	a.v.	*Pisum*	Hom	L	M
22c	*Cystopteris fragilis* agg. (L.) Bernh.	Aspleniaceae	1987–728	a.v.	*Pisum*	Hom	L	M
23	*Davallia denticulata* (Burm.f.) Mett. ex Kuhn var. *denticulata*	Davalliaceae	1973–5060	f.v.	*Allium*	Hom	L	M
24	*Davallia tyermannii* (T.Moore) T.Moore	Davalliaceae	1990–377	a.v.	*Allium*	Hom	L	M
25	*Dennstaedtia globulifera* (Poir.) Hieron	Dennstaedtiaceae	1963–28204	a.v.	*Allium*	Hom	L	M
26	*Llavea cordifolia* Lag.	Adiantaceae	2000–1743	f.v.	*Pisum*	Hom	L	M
27	*Microlepia speluncae* (L.) T.Moore	Dennstaedtiaceae	1974–17	f.v.	*Allium*	Hom	L	M
28	*Nephrolepis biserrata* (Sw.) Schott	Nephrolepidaceae	1962–57603	f.v.	*Allium*	Hom	L	M
29	*Nephrolepis cordifolia* (L.) C.Presl 'Duffi'	Nephrolepidaceae	1969–17706	f.v.	*Allium*	Hom	L	M
30	*Nephrolepis exsaltata* (L.) Schott	Nephrolepidaceae	1969–17702	f.v.	*Allium*	Hom	L	M

* ID numbers starting with TC were derived from tissue-cultured material.

† Identification information: f.v., fully verified; a.v., awaiting verification.

‡ Calibration standard used: *Oryza* = *Oryza sativa* 'IR36', 4C = 2·02 pg; *Petrosel.* = *Petroselinum crispum* 'Champion Moss Curled', 4C = 9·00 pg; *Lycopers.* = *Lycopersicon esculentum* 'Gardener's delight', 4C = 4·00 pg; *Pisum* = *Pisum sativum* 'Minerva Maple', 4C = 19·46 pg; *Hordeum* = *Hordeum vulgare* 'Sultan', 4C = 22·24 pg; *Allium* = *Allium cepa* 'Ailsa Craig', 4C = 67·00 pg.

§ Hom, Homosporous; Het, heterosporous.

¶ L, Leptosporangiate; E, eusporangiate.

** B, Biflagellate; M, multiflagellate.

useful given the scarcity of C-value data available for pteridophytes.

Chromosome counts

Chromosome counts were obtained from root tip squashes prepared using a standard Feulgen-stained squash technique as previously described in Hanson *et al*. (2001).

Estimation of nuclear DNA C-values in pteridophytes

Flow cytometry. Tissue of the pteridophyte sample and calibration standard was co-chopped in isolation buffer (0·1 M citric acid 1-hydrate, 1 % Triton X-100, pH 2·0), digested with RNase, filtered through a 30 µm nylon tissue and stained with the non-base-specific DNA stain propidium iodide (PI) (50 µg ml^{-1}), as described in Obermayer and Greilhuber (1999). Co-chopping tissue of the standard and unknown sample ensured identical conditions for all material, both during nuclear isolation and in subsequent processing steps. For pteridophytes, sporophytic leaves were used in all cases (normally approx. 0·3–0·4 g of leaf material) except *Dicksonia antarctica* (ID 1995-3821) and *Cibotium hawaiense* (ID 1970-6171), where root tips from the sporophyte plant were used. For angiosperm calibration standards young leaf material was used. Since pteridophytes were found to have a large range of C-values, several different calibration standards were needed since it is recommended that the standard should have a similar C-value to the taxon being investigated. Technical errors in DNA estimation may increase if the C-values for the standard and unknown are too dissimilar (Bennett and Leitch, 1995; Price and Johnston, 1996). The particular standard chosen was determined empirically for each taxon investigated and is given in Table 1. The 4C-values of these calibration standards, used to convert arbitrary units into absolute amounts, were taken from Bennett and Leitch (1995) except for two new calibration standards *Petroselinum crispum* 'Champion Moss Curled' and *Lycopersicon esculentum* 'Gardener's delight', whose 4C-values were determined as follows. For *L. esculentum*, eight plants (30 preparations) were measured against the standard *Zea mays* 'Va35'. With a 4C-value of 10·93 pg for Va35 (Bennett and Leitch, 1995), this gave a 4C amount of 4·0 pg (s.d. = 0·07 pg) for *L. esculentum* 'Gardener's delight', which agrees closely with estimates for two other cultivars also measured using flow cytometry with PI (i.e. 4C = 3·8 pg, Michaelson *et al*., 1991; 4C = 3·9 pg, Dolezel *et al*., 1992). For *Petroselinum crispum*, six plants (27 preparations), were measured against the new calibration standard *L. esculentum* 'Gardener's delight'. This gave a 4C-value of 9·0 pg (s.d. = 0·19 pg) for *P. crispum* 'Champion Moss Curled', which agrees closely with 4C = 8·9 pg given by Yokoya *et al*. (2000).

Samples were analysed on a Partec PA II flow cytometer (Münster, Germany), equipped with a 100 W high pressure mercury lamp, a quartz air objective (50× 0·82 N.A.) and a high-quality red sensitive photo-multiplier. The filter combination used was KG1, BG38, EM520, TK560 (to/from objective), 2 × 3 (diaphragm), TK560 and RG590. The bandpass filter EM520 was from Partec; all other filters were from Schott (Mainz, Germany). As sheath fluid, distilled water was used. The correct functioning of the cytometer was tested on a regular basis using chicken red blood cells. For each pteridophyte taxon, three preparations of unknown and standard material were usually made and each was analysed three times (5000 nuclei per run). Coefficients of variation (CVs) were usually less than 3 %, otherwise the number of preparations was increased. To calculate the absolute DNA amount, first the peak ratio (PR) between the positions of G_1 fluorescence peaks of the pteridophyte sample and calibration standard was calculated as follows: PR = mean channel number of sample/mean channel number of standard. Absolute 2C DNA values (pg) of each pteridophyte species were then calculated according to the following formula: PR × mean 2C-value of calibration standard used.

Feulgen microdensitometry. The DNA C-value for *Trichomanes speciosum* was determined by Feulgen microdensitometry using the methods described in Hanson *et al*. (2001) and *Pisum sativum* 'Minerva Maple' (4C = 19·46 pg) as the calibration standard.

RESULTS AND DISCUSSION

Chromosome counts

No count was obtained for ten species. Counts for four species (i.e. *Equisetum giganteum*, *E. myriochaetum*, *Trichomanes speciosum* and *Dicksonia antartica*) were unobtainable as roots could not be taken without risk of damaging delicate living specimens. For six other taxa, no dividing or countable dividing cell was found in root material, and none was producing sporangia from which meiotic preparations could be made. However, Table 2 gives a count or an estimated chromosome number ($2n$) for the remaining 20 taxa studied. Chromosome numbers ranged from $2n = 30$ in *Selaginella kraussiana* var. *poolteri* to $2n$ = approx. 960 in *Ophioglossum petiolatum*. A survey of Löve *et al*. (1977) and the Indexes to Plant Chromosome Numbers series published by The Missouri Botanical Garden (e.g. http://mobot.mobot.org/W3T/Search/ipcn.html) showed that there were two new species counts: $2n$ = approx. 216 in *Equisetum scirpoides* and $2n$ = approx. 80 in *Davallia tyermannii*. Both agreed with previously published counts for related species.

Four of the chromosome counts represented new ploidy levels. In *Selaginella kraussiana*, all previously published counts were $2n = 20$, which is considered to be diploid. In the present work, counts of $2n = 30$ and 40 were obtained and thus represent triploid and tetraploid cytotypes. In *Ophioglossum gramineum*, $2n$ = approx. 720 was obtained in contrast to a previous report of $2n = 240$ (Khandelwal, 1990). The base number in this genus is controversial. Löve *et al*. (1977) considered it to be $x = 15$, whereas Khandelwal (1990) gave $x = 30$. The present count may thus be 24- or 48-

TABLE 2. *DNA C-values for 30 pteridophyte taxa studied, together with chromosome numbers obtained (2n) in the present work and counts published in Löve* et al. *(1977) or taken from the Indexes to Plant Chromosome Numbers*

Entry no.	Taxon	Family	2*n*	Published 2*n**	DNA amount† 1C (Mbp)‡	1C (pg)	2C(pg)	4C (pg)
Lycopodiophyta								
1a	*Selaginella kraussiana* var. *poolteri*	Selaginellaceae	30	20	230	0·24	0·47 ± 0·004	0·95
1b	*Selaginella kraussiana* var. *poolteri*	Selaginellaceae	–	20	240	0·24	0·48 ± 0·007	0·96
2a	*Selaginella kraussiana*	Selaginellaceae	–	20	160	0·16	0·32 ± 0·003	0·65
2b	*Selaginella kraussiana*	Selaginellaceae	40	20	350	0·36	0·71 ± 0·013	1·42
Psilotophyta								
3	*Psilotum nudum* var. *gasa*	Psilotaceae	–	104, 156, 208	71 210	72·7	145·3 ± 0·58	290·7
Equisetophyta								
4	*Equisetum debile*	Equisetaceae	~216	216	25 700	26·2	52·5 ± 0·24	104·9
5	*Equisetum giganteum*	Equisetaceae	–	216*	25 610	26·1	52·3 ± 0·49	104·5
6	*Equisetum myriochaetum*	Equisetaceae	–	216*	25 140	25·7	51·3 ± 0·40	102·6
7	*Equisetum scirpoides*	Equisetaceae	~216	216*	20 820	21·3	42·5 ± 0·04	85·0
8	*Equisetum variegatum*	Equisetaceae	~216	216	29 740	30·4	60·7 ± 0·19	121·4
Polypodiophyta								
Basal eusporangiate ferns								
9	*Marattia* sp.	Marattiaceae	78	78*, 156*	7510	7·7	15·3 ± 0·49	30·6
10	*Ophioglossum gramineum*	Ophioglossaceae	~720	240	63 480	64·8	129·6 ± 4·38	259·1
11	*Ophioglossum petiolatum*	Ophioglossaceae	~960	960, 1020	64 240	65·6	131·1 ± 3·86	262·2
Basal leptosporangiate ferns								
12	*Todea barbara*	Osmundaceae	44	44	20 590	21·0	42·0 ± 0·34	84·1
Filmy ferns								
13	*Trichomanes speciosum*	Hymenophyllaceae	–	144	10 520	10·7	21·5	42·9 ± 2·98
Heterosporous water ferns								
14	*Azolla microphylla*	Azollaceae	44	44, 66	750	0·8	1·5 ± 0·04	3·1
Tree ferns								
15	*Cibotium* sp.	Dicksoniaceae	–	136*	6960	7·1	14·2 ± 0·59	28·4
16	*Cibotium hawaiense*	Dicksoniaceae	~130	136*	7120	7·3	14·5 ± 0·32	29·1
17	*Cyathea crinita*	Cyatheaceae	–	138	7200	7·4	14·7 ± 0·33	29·4
18a	*Dicksonia antarctica*	Dicksoniaceae	–	130*	11 260	11·5	23·0 ± 0·27	46·0
18b	*Dicksonia antarctica*	Dicksoniaceae	–	130*	11 400	11·6	23·3 ± 0·41	46·5
19	*Plagiogyria* sp.	Plagiogyriaceae	–	130*, 260*	24 950	25·5	50·9 ± 0·33	101·8
20	*Plagiogyria matsumureana*	Plagiogyriaceae	–	130*	12 320	12·6	25·1 ± 0·29	50·3
Polypodiaceous ferns								
21	*Cystopteris dickieana*	Aspleniaceae	168	168	8110	8·3	16·6 ± 0·21	33·1
22a	*Cystopteris fragilis* agg.	Aspleniaceae	~168	168	8120	8·3	16·6 ± 0·13	33·1
22b	*Cystopteris fragilis* agg.	Aspleniaceae	–	168	8160	8·3	16·7 ± 0·30	33·3
22c	*Cystopteris fragilis* agg.	Aspleniaceae	–	168	8180	8·3	16·7 ± 0·06	33·4
23	*Davallia denticulata* var. *denticulata*	Davalliaceae	~80	80	11 290	11·5	23·0 ± 0·24	46·1
24	*Davallia tyermannii*	Davalliaceae	~80	80*	7790	8·0	15·9 ± 0·11	31·8
25	*Dennstaedtia globulifera*	Dennstaedtiaceae	~176	94	12 890	13·2	26·3 ± 0·32	52·6
26	*Llavea cordifolia*	Adiantaceae	–	58	7510	7·7	15·3 ± 0·06	30·7
27	*Microlepia speluncae*	Dennstaedtiaceae	172	86, 172, 258	7780	7·9	15·9 ± 0·12	31·8
28	*Nephrolepis biserrata*	Nephrolepidaceae	~82	82	9330	9·5	19·0 ± 0·20	38·1
29	*Nephrolepis cordifolia* 'Duffi'	Nephrolepidaceae	~82	82	6680	6·8	13·6 ± 0·08	27·3
30	*Nephrolepis exsaltata*	Nephrolepidaceae	82	82	9370	9·6	19·1 ± 0·35	38·2

* Where no chromosome count was published for the species, counts for other species in the same genus are given and marked with an asterisk.

† For all species except *Trichomanes speciosum*, 2C-values were determined by flow cytometry from the G_1 peak of the flow histogram. For *Trichomanes speciosum*, 4C-values were obtained from measurements made on prophase cells using Feulgen microdensitometry. Standard deviation for each DNA amount is given with the original data.

‡ Conversion factor used 1 pg = 980 Mb (Cavalier-Smith, 1985).

ploid depending on which base number is accepted. The count for *Dennstaedtia globulifera* of 2*n* = approx. 176 is around double the previous report of 2*n* = 94 for this species. Since the base number for this genus is unclear due to a range of chromosome numbers reported for different *Dennstaedtia* species, the ploidy level of the count is also unclear. The remaining 14 chromosome counts all agree with previously published counts for the species.

Counts from root tips of the remaining ten species were unobtainable. For four species (i.e. *Equisetum giganteum*, *E. myriochaetum*, *Dicksonia antartica* and *Trichomanes speciosum*), this was because the material was no longer

available or too fragile to allow roots to be harvested. For the remaining six taxa, attempts to obtain counts were unsuccessful as no dividing cells could be found in the root material and none of the plants were producing sporangia from which meiotic preparations could be made.

Estimating pteridophyte DNA C-values by flow cytometry

Flow cytometry is a widely used method of choice for estimating C-values in plants as it can estimate large numbers of cells easily and rapidly (e.g. Galbraith *et al*., 1983; Price and Johnston, 1996). However, it has some limitations (Bennett and Leitch, 1995) and users rarely mention or assess difficulties, although some have been reported (e.g. Marie and Brown, 1993; Yokoya *et al*., 2000). In the present work, all but one of the C-values in Table 2 were estimated by flow cytometry. Prior to this, Bennett and Leitch (2001) listed C-values for 13 species estimated by this technique. These were taken from Redondo *et al*. (1999*a*, *b*), but the authors did not comment on the ease of use or applicability of the technique (i.e. no CVs or flow histograms were given).

In the present work, flow cytometry proved to be capable of producing distinct peaks in flow histograms for species whose C-values spanned the entire range encountered in pteridophytes so far (i.e. 1C = 0·16–72·67 pg, see Fig. 1). However, the ease of obtaining data varied considerably between taxa. Some, including *Equisetum*, *Ophioglossum*, *Psilotum* and some 'polypodiaceous' ferns such as *Llavea*, *Davallia*, *Microlepia*, *Nephrolepis* and *Dennstaedtia*, were relatively easy to measure. Others proved more problematic, giving peaks with unacceptably high CVs, or, in the worst case (i.e. *Trichomanes speciosum*), no peaks at all. To overcome these problems various approaches were taken. In some cases, peaks could be obtained by increasing the amount of leaf material used. For example, in *Plagiogyria matsumureana*, approx. five times the normal amount of leaf material was needed to obtain peaks in the flow histogram. In other cases, problems were overcome by using root instead of leaf material (e.g. *Dicksonia* and *Cibotium*). For *Trichomanes speciosum*, which gave no discernible peaks in the flow histogram, no solution was found in the current study. For this one species a C-value was estimated using Feulgen microdensitometry. However, five other species also proved intractable using flow cytometry and still await C-value estimation by any method.

We conclude that whilst flow cytometry can be used to estimate accurately DNA amounts in most pteridophyte taxa over a wide range of C-values, the ease and speed with which data are generated varies between materials. Further work is needed to optimize the method (e.g. modify buffer composition, alter stain concentration, etc.) to increase the potential of flow cytometry for estimating C-values in a significant minority of pteridophytes.

Survey of DNA C-values in pteridophytes

Table 2 lists C-values for 30 taxa in 17 different pteridophyte families estimated in the present work. In four taxa (i.e. *Selaginella kraussiana* var. *poolteri*, *S. kraussiana*, *Dicksonia antarctica* and *Cystopteris fragilis*), more than one estimate was made using material from different sources. With one exception, C-value estimates for different accessions of each taxon agreed closely (e.g. *Dicksonia antarctica* 1C = 11·5 and 11·6 pg), showing that the technique is consistent and reliable. In *S. kraussiana*, 1C-values of 0·16 and 0·36 pg were reported. Although no chromosome counts could be obtained for the accession with 1C = 0·16 pg, counts of $2n = 30$ and $2n = 40$ were obtained for *S. kraussiana* var. *poolteri* ID 1998-4081 (1C = 0·24 pg) and *S. kraussiana* ID 1998-4013 (1C = 0·36 pg), respectively. Since the base number for *Selaginella* is $x = 10$, the 1C-values of 0·16 and 0·36 pg presumably correspond to diploid and tetraploid cytotypes, respectively. Thus the genome size (i.e. 2C DNA amount divided by ploidy level) for *S. kraussiana* is approx. 0·16 pg.

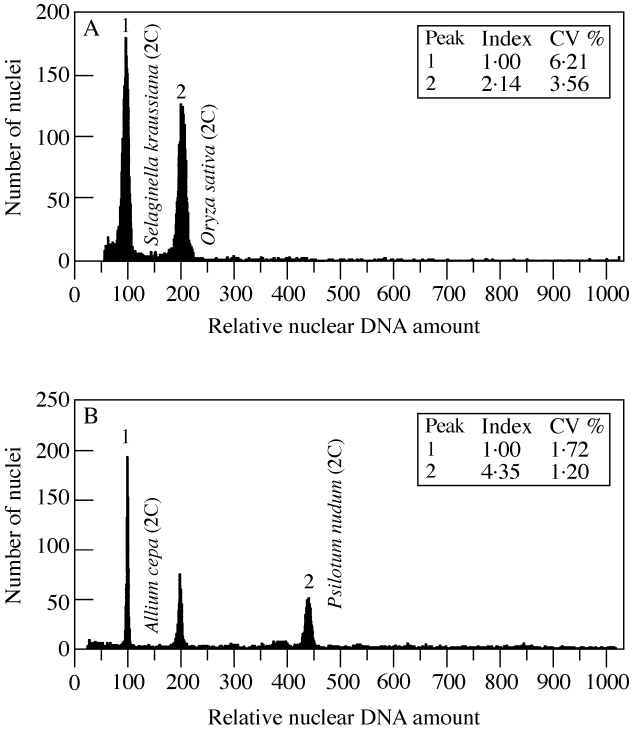

FIG. 1. Flow histograms of fluorescence intensity in nuclei isolated from pteridophytes and the relevant calibration standard after propidium iodide staining. A, *Selaginella kraussiana* run with calibration standard *Oryza sativa.* B, *Psilotum nudum* var. *gasa* run with calibration standard *Allium cepa*.

In the four species with more than one C-value, the estimate considered most reliable (e.g. from fully verified and/or naturally growing material rather than from tissue-cultured samples or material awaiting full verification) was assigned as the prime value (c.f. Bennett and Smith, 1976) and given an 'a' after the entry number in column 1 of Tables 1 and 2. Prime values for these taxa were used in the analysis described below.

Comparing the 30 C-values in Table 2 with those compiled in Bennett and Leitch (2001) revealed some large discrepancies, such that only 24 of the 48 previously compiled estimates are now considered acceptable (see

TABLE 3. *DNA C-values for nine pteridophyte taxa included in Grime* et al. *(1988), together with information on chromosome number, ploidy level, spore type, sporangial type and sperm flagella number*

Taxon	Family	2*n*	Ploidy level	Spore type*	Sporangial type†	Sperm flagella‡	DNA amount 1C (Mbp)§	1C (pg)	2C (pg)	4C (pg)
Equisetophyta										
Equisetum arvense L.	Equisetaceae	216	2	Hom	E	M	13 920	14·2	28·4	56·8
Equisetum fluviatile L.	Equisetaceae	216	2	Hom	E	M	13 230	13·5	27·0	54·0
Equisetum palustre L.	Equisetaceae	216	2	Hom	E	M	12 250	12·5	25·0	50·0
Polypodiophyta										
Leptosporangiate ferns										
Asplenium ruta-muraria L.	Aspleniaceae	144	4	Hom	L	M	5150	5·3	10·5	21·0
Asplenium trichomanes L. ssp. *quadrivalens* D.E.Meyer emend. Lovis	Aspleniaceae	144	4	Hom	L	M	6710	6·9	13·7	27·4
Athyrium filix-femina (L.) Roth	Athyriaceae	80	2	Hom	L	M	2890	3·0	5·9	11·8
Dryopteris dilatata (Hoffm.) A. Gray	Dryopteridaceae	164	4	Hom	L	M	7890	8·1	16·1	32·2
Dryopteris filix-mas (L.) Schott	Dryopteridaceae	164	4	Hom	L	M	8530	8·7	17·4	34·8
Pteridium aquilinum (L.) Kuhn	Dennstaedtiaceae	104	2	Hom	L	M	6270	6·4	12·8	25·6

* Hom, Homosporous; Het, heterosporous.
† L, Leptosporangiate; E, eusporangiate.
‡ B, Biflagellate; M, multiflagellate.
§ Conversion factor used 1 pg = 980 Mb (Cavalier-Smith, 1985).

TABLE 4. *Mean, minimum (min.), and maximum (max.) 1C nuclear DNA amounts for 21 families of pteridophytes for which C-value data are available*

Higher group	Family	Number of species with C-values	1C nuclear DNA amount (pg) Mean	Min.	Max.
Lycopodiophyta	Selaginellaceae	2	0·2	0·16	0·24
Psilotophyta	Psilotaceae	1	72·7	72·7	72·7
Equisetophyta	Equisetaceae	8	21·2	12·5	30·4
Polypodiophyta					
Basal eusporangiate ferns	Ophioglossaceae	2	65·2	64·8	65·6
	Marattiaceae	1	7·7	7·7	7·7
Basal leptosporangiate ferns	Osmundaceae	1	21·0	21·0	21·0
Filmy ferns	Hymenophyllaceae	1	10·7	10·7	10·7
Heterosporous water ferns	Azollaceae	1	0·8	0·8	0·8
Tree ferns	Cyatheaceae	1	7·4	7·4	7·4
	Plagiogyriaceae	2	19·0	12·6	25·5
	Dicksoniaceae	3	8·6	7·1	11·5
Polypodiaceous ferns	Adiantaceae	1	7·7	7·7	7·7
	Aspleniaceae	13	6·7	4·2	8·3
	Athyriaceae	1	3·0	3·0	3·0
	Davalliaceae	2	9·7	8·0	11·5
	Dennstaedtiaceae	4	9·2	6·4	13·2
	Dryopteridaceae	2	8·4	8·1	8·7
	Nephrolepidaceae	3	8·6	6·8	9·6
	Parkeriaceae	1	3·8	3·8	3·8
	Woodsiaceae	1	5·9	5·9	5·9
	Polypodiaceae	11	12·8	7·5	19·7

below). Prior to the present work, reliable C-values were available for 32 species; 24 compiled in Bennett and Leitch (2001) plus eight of the nine species included in Grime *et al.* (1988; see Table 3). The remaining species in Grime *et al.* (*Asplenium trichomanes* ssp. *quadrivalens*, 1C = 6·9 pg) was already included in Bennett and Leitch (2001) who listed a similar 1C-value of 7·7 pg taken from Redondo *et al.* (1999*b*). The present work has thus increased the number of species with C-value estimates acceptable for analysis by 93 %, to give a total of 62 species. It has therefore contributed significantly to the goal of obtaining first C-values for 200 pteridophyte taxa by 2005 (Bennett and Leitch, 2001).

The present data have also contributed towards increasing familial coverage, as first C-values for 11 families not included in Bennett and Leitch (2001) are reported here.

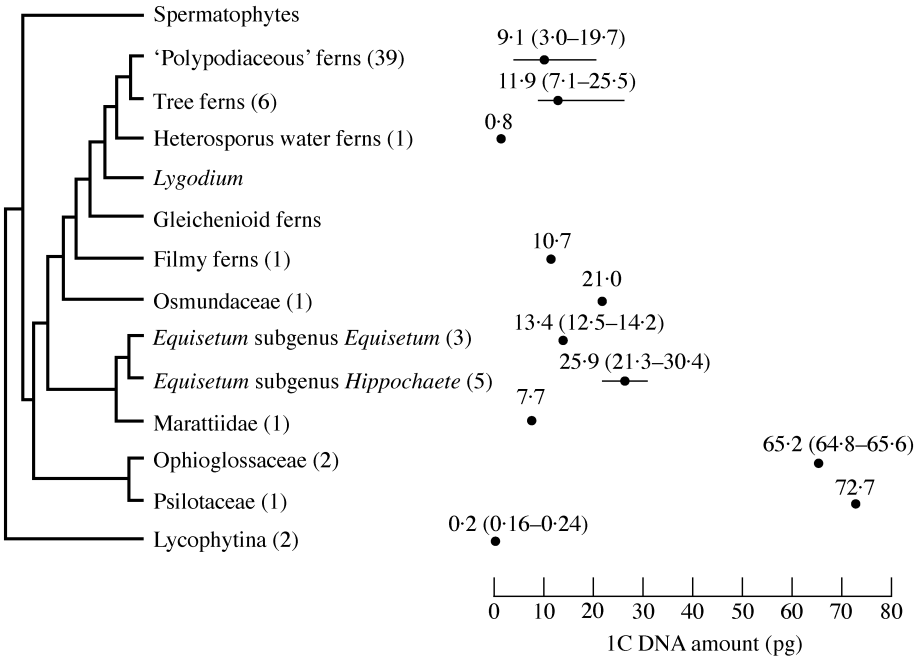

FIG. 2. Pteridophyte phylogeny (left, based on Pryer *et al*., 2001) and C-value data (right) showing the mean and range of C-values in each of the major branches of the phylogeny. The number in parentheses following the branch name gives the number of species with C-value data.

The number of pteridophyte families represented by C-value data has now more than doubled from 10 to 21. Since a total of only approx. 35 pteridophyte families is recognized (e.g. Mabberley, 1997; Wolf *et al*., 1998), the present work has increased familial representation from approx. 29 to 60 %. A summary of the known data and ranges of C-values for all 21 families with C-value data is given in Table 4.

Analysis of the data for 62 species shows that C-values in pteridophytes varied approx. 450-fold, from 0·16 pg in *Selaginella kraussiana* to 72·7 pg in *Psilotum nudum* var. *gasa*. The smallest C-value for fully verified material of *S. kraussiana* (0·16 pg) was noted to be 2·6 times larger than values reported by Bouchard (1976) for five *Selaginella* species (all approx. 0·06 pg). The present work shows that whilst *Selaginella* still has the smallest nuclear DNA amount yet known for any pteridophyte, its C-value is not so small as to approach the theoretical minimum for a plant as previously suggested (Bennett and Leitch, 2001). However, its C-value is similar to estimates reported for the angiosperm *Arabidopsis thaliana* (e.g. 1C = 0·17 pg, Marie and Brown, 1993; 1C = 0·18 pg, Bennett and Smith, 1991), which is widely studied as a model plant species partly due to its small genome size. The highest C-value estimated here was for *Psilotum nudum* var. *gasa* (1C = 72·7 pg), and is the largest known to us for any pteridophyte so far estimated. This large C-value agrees with previous observations that *Psilotum* has some of the largest chromosomes in pteridophytes (4·5–18 μm long; Abraham *et al*., 1962). However, the present value is 35 times greater than a 1C-estimate of approx. 2·0 pg given by Bouchard (1976) for a *Psilotum* species, which raises doubts over the latter value as suggested in Bennett and Leitch (2001). Indeed a further inconsistency was observed between the C-value reported for *Equisetum variegatum* in the present work (30·4 pg) and that given in Bouchard (1976; 1C = 12·15 pg). Whether these C-value discrepancies are taxonomic, methodological or due to some other factor(s) is not known. However, Bennett and Leitch (2001) noted that the paper with data from Bouchard's thesis (Bouchard, 1976) was withdrawn from publication. In view of these concerns and inconsistencies, Bouchard's data were excluded from the analysis presented below and it is suggested that C-value estimates for species from this source should be viewed with caution until independently confirmed.

Phylogenetic survey of C-values in pteridophytes

To interpret the evolutionary significance of the approx. 450-fold range of C-values requires a robust phylogeny of pteridophytes. Recent work combining DNA sequence data of four genes together with morphological data has clarified relationships among the different pteridophyte groups, both to each other and to the other vascular plant group—the seed plants (gymnosperms and angiosperms; Pryer *et al*., 2001). This analysis gave strong support for three clades of extant vascular plants: (1) lycophytes (Lycopodiaceae, Selaginellaceae and Isoetaceae); (2) seed plants (Spermatophytes); and (3) a clade containing the horsetails (Equisetophyta), whisk ferns (Psilotophyta) and all eusporangiate and leptosporangiate ferns (referred to below as the horsetail-fern clade). Pryer *et al*. (2001) also showed unambiguously that the third clade contained the closest living relatives to seed plants.

The species whose C-values were estimated in the present work were selected to improve phylogenetic coverage for pteridophytes, such that a C-value estimate is now available for most of the major pteridophyte clades given in Pryer *et al*. (2001). Given this improved representation of C-values in pteridophytes and the well-supported phylogeny, evaluation of the phylogenetic component of C-value variation can begin. In angiosperms, Leitch *et al*. (1998) superimposed DNA C-values onto a robust phylogenetic tree and concluded that ancestral angiosperms almost certainly had small genomes (defined as 1C ⩽ 3·5 pg; see Leitch *et al*., 1998). Figure 2 superimposes the C-value data for the different pteridophyte groups onto the phylogeny of Pryer *et al*. (2001) in a similar way.

Whilst it is recognized that such an analysis is limited due to the paucity of data, it is useful as it highlights gaps in the data that need to be targeted in future work (see below). In addition, three points of interest are noted: (1) The smallest C-value in the horsetail-fern clade was reported for *Azolla microphylla* (1C = 0·8 pg) in the heterosporous water fern clade. The next largest C-value recorded is 3·8 times larger for *Athyrium filix-femina* (1C = 3·0 pg). Although only one C-value is currently available for this clade, the three families that comprise this group (i.e. Azollaceae, Salviniaceae and Marsileaceae) are typically characterized by possessing low numbers (Klekowski and Baker, 1966) of small chromosomes (e.g. Abraham *et al*., 1962; Loyal, 1972; Nakato, 1996). Low C-values may be typical of this clade and, if so, may be secondarily derived.

(2) The clade comprising two eusporangiate families, Ophioglossaceae and Psilotaceae, is characterized by species with the highest C-values so far encountered in any pteridophyte [i.e. *Ophioglossum gramineum* (1C = 64·8), *O. petiolatum* (1C = 65·6 pg) and *Psilotum nudum* var. *gasa* (1C = 72·7 pg)]. These values are more than double the next largest C-value reported for a pteridophyte (*Equisetum variegatum*, 1C = 30·4 pg). It is hypothesized that the large C-values in this clade represent a derived character. Interestingly, these large C-values have apparently involved two different cytological processes, namely increased ploidy levels and changes in chromosome size. Thus, relative to *Psilotum*, *Ophioglossum* is characterized by possessing numerous, small chromosomes (1·5–4·5 μm long; Abraham *et al*., 1962, and 2*n* up to 1440; Khandelwal, 1990), whereas *Psilotum* is characterized by fewer (2*n* = 104, 156 and 208), but larger chromosomes (4·5–18 μm; Abraham *et al*., 1962).

(3) C-values are available for eight of the 15 recognized *Equisetum* species (see data in Tables 2 and 3), ranging from 12·5 to 30·4 pg. However, their distribution is discontinuous with two size classes, namely 1C = 12·5–14·2 pg and 21·3–30·4 pg. Phylogenetically, *Equisetum* is divided into two distinct branches (Des Marais *et al*., 2001; Pryer *et al*., 2001) which correspond to the morphologically recognized subgenera *Equisetum* and *Hippochaete* (Hauke, 1963, 1978). Interestingly, the two size classes of C-values noted above correspond to the two subgenera. Species in subgenus *Hippochaete* (= *E. debile*, *E. giganteum*, *E. myriochaetum*, *E. scirpoides* and *E. variegatum*) are characterized by C-values (range 21·3–30·4 pg) whose mean (1C = 25·9 pg) is approx. double that of species in subgenus *Equisetum* (= *E. arvense*, *E. fluviatile* and *E. palustre*; mean 1C = 13·4 pg; range 12·5–14·2 pg). The difference in mean C-values was found to be highly significant ($P < 0{\cdot}001$). These two different size classes of C-values also fit Manton's (1950) observations that species in subgenus *Equisetum* characteristically had smaller chromosomes than species in subgenus *Hippochaete*. Recent phylogenetic work by Des Marais *et al*. (2001) showed that the two subgenera are sister groups with the basal group of *Equisetum* represented by *E. bogotense*. The C-value for this species is currently unknown but it will be useful for identifying the direction of C-value evolution within this genus.

CONCLUSIONS AND FUTURE PROSPECTS

The new estimates for 30 species have nearly doubled the number of C-value data available for pteridophytes to 62. However, with approx. 9000 extant species, much remains to be done. In particular, more data are needed to extend taxonomic representation in both the lycophyte and horsetail-fern clades to determine more fully the pattern of C-value evolution in this group of vascular plants. Key gaps that remain unfilled include: (1) representative species from the lycophyte families Lycopodiaceae and Isoetaceae; (2) representative species in the heterosporous water fern families Marsileaceae and Salviniaceae; (3) species in the gleichenioid fern family Gleicheniaceae; and (4) a representative species from the genus *Lygodium*. With increased understanding of the size of the ancestral genome in gymnosperms and angiosperms (Leitch *et al*., 1998, 2001), information on ancestral C-values in the lycophyte and horsetail fern clade will be invaluable for providing a comprehensive view of C-value evolution throughout vascular plants.

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