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# Cytological study of *Polystichum* (Dryopteridaceae) species from southern South America

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**Abstract.** *Polystichum* is one of the most diverse genera of ferns, with 360–400 species distributed worldwide. South America harbors ~40 species, clustered in three centres of diversity, namely, the Northern and Central Andes Center (NCC), the Brazilian Center (BC) and the Southern South America Center (SSC). To increase our understanding of the systematic relationships within *Polystichum*, mitotic chromosomes and spore features were studied in nine species from Argentina and Chile. All species presented the basic number $x = 41$, with different ploidy levels ($2x$, $4x$ and $8x$). In general, chromosomes were homogeneous in size (average length 2.50–5.75 μm) and mostly subtelocentric; centromeres were inconspicuous and secondary constrictions were frequently observed. All species presented 64 spores per sporangium, suggesting normal sexual reproduction. Significant differences in spore size were found among species and it was positively correlated with ploidy level. A relationship between sum total chromosome length and ploidy level was observed. However, there was also a reduction in single-chromosome length in the polyploids, pointing to genome downsizing. Our results agree with previous records, with diploids being frequent among NCC species and absent among SSC species. In addition to sharing very specific morphological characters, SSC species are cytologically characterised by being polyploids ($4x$ and $8x$). A literature survey covering 116 species of *Polystichum* revealed that Australian and New Zealand *Polystichum* species exhibit similarly high frequencies of polyploidy. In the case of *P. tetragonum* ($2n = 164$), endemic to the Juan Fernandez archipelago, our data suggested that it was originated by transoceanic migration from a South American ancestor, probably also tetraploid.

**Additional keywords:** chromosome number, polyploidy, spore size.

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## Introduction

*Polystichum* Roth. is one of the five most diverse genera of ferns, comprising ±360–400 species distributed worldwide. Its main centre of diversity is in eastern Asia (Li *et al*. 2004). South America, with ~40 species (43% of the American continent species), is an important world centre as well. Its species are clustered in the following three centres of diversity: the Northern and Central Andes Center (NCC) (Exindusiate Andean group of McHenry and Barrington (2014)), with 25 species extending to the Sierras Pampeanas in Central Argentina; the Brazilian Center (BC), with nine species (Condack *et al*. 2010, 2013; Condack 2012); and the Southern South America Center (SSC) (Chilean group of Barrington and Driscoll (2005)), with nine species and infraspecific taxa.

*Polystichum* is considered to be monophyletic. The first world-level phylogenetic hypothesis for the genus was proposed by Little and Barrington (2003). Since then, several molecular phylogenetic and biogeographic studies have contributed to the understanding of *Polystichum* in Asia (Li *et al*. 2008) and Oceania (Perrie *et al*. 2003*a*, 2003*b*). In South America, recent systematic and biogeographic studies have been focussed on Brazilian species (Condack 2012; Condack *et al*. 2013) and NCC species (McHenry and Barrington 2014). For the SSC species, Morero *et al*. (2013) concluded that they constitute a monophyletic group comprising nine endemic taxa, seven of them growing in temperate and temperate–cold Andean forest, plus one species endemic to the South Atlantic Islands (*P. mohrioides*) and one to the Juan Fernandez Islands (*P. tetragonum*).

One of the factors that influence the distribution of *Polystichum* in South America is the ‘arid diagonal’, an important climatic and geomorphologic barrier (Ponce *et al*. 2002) that divides the SSC species from the NCC ones. This geographical separation is accompanied by a clear morphological

differentiation, supporting the hypothesis that the NCC and the SSC species are phylogenetically distant (Morero *et al*. 2013). Most NCC species can be distinguished from the SSC by the former having mostly exindusiate sori, pinnule margin with variously developed spinules and petiole scales with marginal cilia, whereas the latter have peltate indusia, spinules absent to weakly developed along pinnule margin and scales lacking cilia.

Some species that grow in the NCC are also present in the area of the BC (i.e. *P. montevidense* and *P. platyphyllum*) but do not co-occur with SSC species, except for a single site, the Ventania Hills (south-west of Buenos Aires Province, Argentina), where *P. montevidense* (belonging to NCC and BC) and *P. plicatum* (SSC) are found together. This anomaly in the distribution of *Polystichum* in Argentina was noted by de la Sota *et al*. (2004), who considered these hills as true 'continental islands' in the pampas, because they constitute a refuge for species with different provenance and ecological requirements.

From the cytological point of view, chromosome counts provide a key tool in studies of systematics, phylogeny and evolution, and they are especially useful for understanding speciation and hybridisation of ferns (Manton 1950). In *Polystichum*, data have been obtained for an array of species from different parts of the world (Taylor and Lang 1963; Löve *et al*. 1977; Wagner 1979; Barrington 1985*a*, 1985*c*, 1990, 2003; Tsai and Shieh 1985; Khullar *et al*. 1988; Fraser-Jenkins 1997; Roux 2000, 2001, 2004; Tindale and Roy 2002; Perrie *et al*. 2003*b*). However, cytological knowledge of the South American species is meager. Until now, the chromosome numbers of only five taxa from NCC and BC are known (*P. dubium* (Wagner 1980); *P. sodiroi* (Löve *et al*. 1977); *P. orbiculatum* (Barrington 1990); *P. montevidense* (Condack *et al*. 2013) and *P. platyphyllum* (Smith and Mickel 1977; Smith and Foster 1984)). Regarding the SSC group, there is one species so far counted, *P. subintegerrimum* (Jara-Seguel *et al*. 2006). There is also one count of *P. mohrioides* (Löve *et al*. 1977), but this name was misapplied so this plant is not the *P. mohrioides* from South Atlantic Islands.

A high constancy of chromosome number is characteristic of the family Dryopteridaceae; chromosome counts previously published (including *Polystichum* species) indicate that the chromosome basic number is $x = 41$ (Löve *et al*. 1977; Barrington 1985*b*; Ivanova and Piekos-Mirkowa 2003; Lu *et al*. 2006*a*). At the same time, hybridisation and allopolyploidy have played important roles in species diversification and reticulation, being reflected in the high frequency of polyploid species and hybrids (Barrington *et al*. 1986; Barrington 1990; Lin *et al*. 2011).

Spores are another important source of insights into the biology of ferns. In homosporeus species, size (e.g. length), number of spores per sporangium and morphology are frequently related to ploidy level, distinguishing sexual from apomictic species, and differentiating sterile hybrids (Manton 1950; Crane 1953; Barrington *et al*. 1986; Wagner *et al*. 1986; Quintanilla and Escudero 2006). Species with typical sexual reproductive type normally have 64 viable spores per sporangium, whereas most apomictic species produce 32 unreduced spores in some of the sporangia and 64 abortive spores in others (Manton 1950). Allopolyploid sexual hybrids behave like ordinary species, even though they are actually hybrids, and many of these are frequent or common; they are recognised by their spore size, usually larger than that of their diploid parents (Barrington *et al*. 1986; Wagner *et al*. 1986).

Spores of *Polystichum* are monolete, with ellipsoidal to somewhat spheroidal shape; their reported length ranges from 27 to 60 µm. Most species have a complex perispore whose external morphology, as well as the wall structure of the spore, are useful features to differentiate *Polystichum* species (Daigobo 1972; Mitui 1973; Devi 1977; Khullar and Gupta 1978; Morbelli 1980). Also, great variation in shape and size and irregular morphology give a clear indication of the spores being abortive and it is a valuable tool to detecting sterile hybrids (Wagner 1962, 1974; Barrington 1985*b*, 1985*c*, 1990; Cubas and Pardo 1992; Perrie *et al*. 2003*b*; Lin *et al*. 2011). Furthermore, some spore characters are useful for inferring evolutionary relationships. For instance, perispore surfaces that are plain or have papillate folds, present in many Asian polystichums, are considered to be less derived than the cristate or echinate types common in the American species (Tryon and Lugardon 1990).

Considering this background, the aims of this work were (1) to provide new cytological data on seven of the nine *Polystichum* SSC taxa, plus two Argentinian NCC species, (2) to use data on spore morphology and number to explore ploidy level and reproductive biology and (3) to increase our understanding of the systematic relationships of the South American species of *Polystichum.*

## Materials and methods

The study area was southern South America, encompassing species of NCC and SSC present in Argentina and Chile, on both sides of the 'arid diagonal' (Fig. 1*a*). Sporophytes collected from wild populations were cultivated in a greenhouse at the Universidad de Córdoba, Argentina. The geographic origin of these samples is indicated in Fig. 1*b*. The species studied, with collection data, are listed in Table 1. Voucher specimens are deposited at the herbarium of the Botanical Museum of Córdoba (CORD).

### *Mitotic chromosome analysis*

Croziers were cut up into small fragments ~2 mm wide. The fragments were treated in a solution of 2 mM 8-hydroxiquinoline for 1 h at room temperature, followed by 8 h at 14°C, then fixed in an ethanol–glacial acetic acid (3 : 1 v/v) mixture and stored at −20°C. The crozier fragments were rinsed in distilled water four or five times, and then hydrolysed in 3 mL of cellulase 2%-pectinase 20%, for 2 h at 37°C. The hydrolysed cells were washed three or four times to remove residual enzymes. The cells were stained in alcoholic hydrochloric acid–carmine and squashed (as detailed in Guillén and Daviña 2005).

For chromosome analysis, at least 30 metaphases per taxon were photographed with a phase-contrast optic Axiophot microscope (Zeiss). Photomicrographs were used to measure the length for each chromosome, from which average chromosome length for each chromosome and total diploid chromosome length were calculated. At least 30 chromosome slides were observed for each individual. Measurements were undertaken with the free software ImageJ (Rasband 2014).

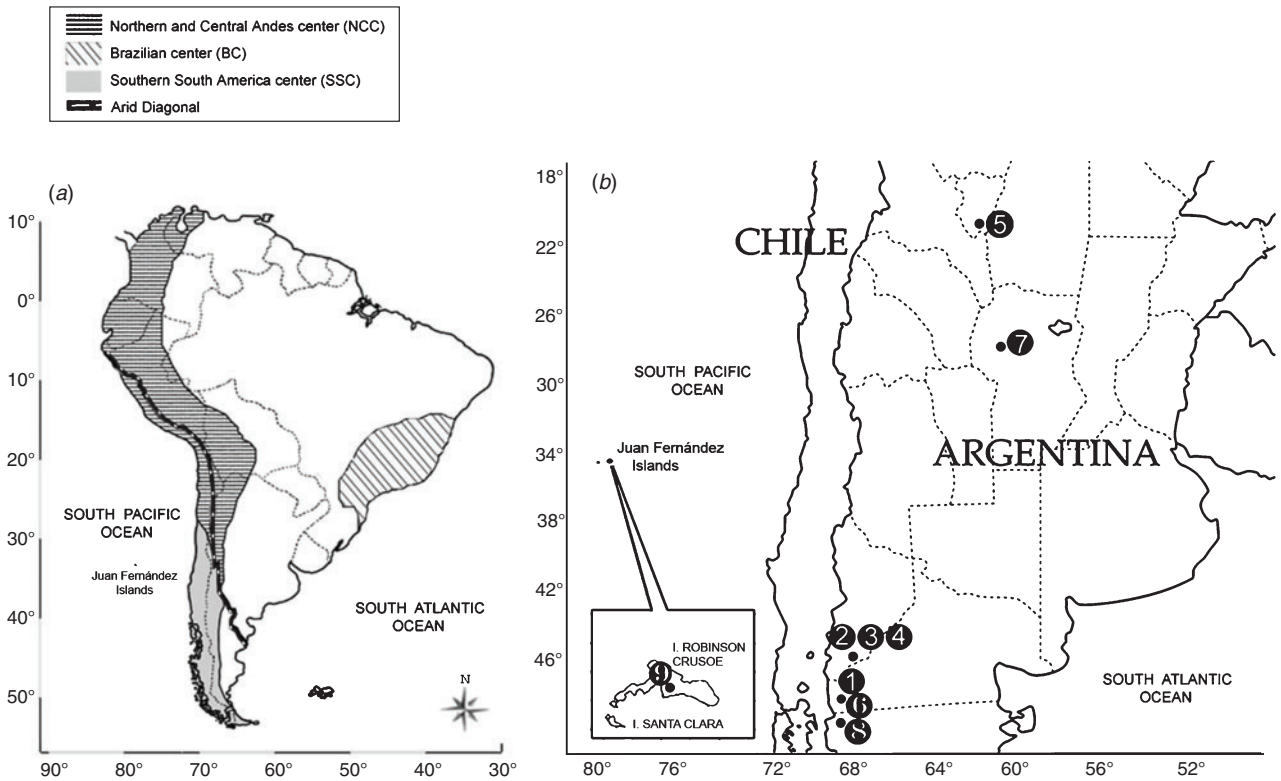


**Fig. 1.** (*a*) Endemism centres of *Polystichum* in South America and arid diagonal position. (*b*) Collection localities for the mitotic samples and location of the Robinson Crusoe Island of the Juan Fernández Archipelago (Chile). References of samples: (1) *P. andinum*; (2) *P. chilense* var. *chilense*; (3) *P. chilense* var. *dusenii*; (4) *P. multifidum* var. *multifidum*; (5) *P. platyphyllum*; (6) *P. plicatum*; (7) *P. pycnolepis*; (8) *P. subintegerrimum*; (9) *P. tetragonum*.

**Table 1.** ***Polystichum*** **taxa investigated with their endemism centres, chromosome counts and measurements, collection data and references of each mitotic figure**

| Taxon | Centre of endemism | Chromosome number | Mean chromosome length (μm) | Total chromosome length (diploid, μm) | Locality | Elevation (m) | Voucher | Figure |
|---|---|---|---|---|---|---|---|---|
| *Polystichum andinum* Phil. | SSC | $2n=4x=164$ | 3.58 | 581.78 | ARGENTINA. Río Negro: Bariloche | 1696 | Morero R. 291, 388 (CORD) | 2*a* |
| *P. chilense* (H.Christ) Diels var. *chilense* | SSC | $2n=4x=164$ | 3.90 | 507.84 | ARGENTINA. Neuquén. Villa La Angostura | 1112 | Morero R. 302, 315 (CORD) | 2*b* |
| *P. chilense* var. *dusenii* (C.Chr.) Looser ex R.Rodr. | SSC | $2n=4x=164$ | 4.10 | 673.00 | ARGENTINA. Neuquén. Villa La Angostura | 1155 | Morero R. 307, 308 (CORD) | 2*c* |
| *P. multifidum* (Mett.) H.Christ var. *multifidum* | SSC | $2n=4x=164$ | 4.62 | 758.15 | ARGENTINA. Neuquén. Villa La Angostura | 1155 | Morero R. 306, (CORD) | 2*d* |
| *P. platyphyllum* (Willd.) C.Presl | NCC | $2n=2x=82$ | 5.34 | 438.08 | ARGENTINA. Tucumán. Cuesta del Clavillo | 1780 | Morero R. 352, (CORD) | 3*b* |
| *P. plicatum* (Poepp. ex Kunze) Hicken | SSC | $2n=4x=164$ | 4.21 | 695.40 | ARGENTINA. Río Negro. S.C. de Bariloche | 1035 | Morero R. 241, 293 (CORD) | 2*e* |
| *P. pycnolepis* (Kunze ex Klotzsch) T. Moore | NCC | $2n=2x=82$ | 5.75 | 440.68 | ARGENTINA.Córdoba. Sierras Grandes | 1889 | Morero R. 341, 319 (CORD) | 3*c* |
| *P. subintegerrimum* (Hook. et Arn.) R.A. Rodr. | SSC | $2n=8x=$ ca.328 | 2.50 | 822.85 | ARGENTINA. Chubut. Lago Puelo | 420 | Morero R. 371, 379 (CORD) | 2*f* |
| *P. tetragonum* Fée | SSC | $2n=4x=164$ | 3.97 | 655.97 | CHILE. Juan Fernández Archipelago. Robinson Crusoe Island | 247 | Morero R. 322, 323 (CORD) | 3*a* |

### Spore and sporangia analysis

For each taxon, number of normal and abortive spores from 10 closed sporangia was counted from two specimens. The spores of these accessions were mounted in Hoyer's medium on glass slides and were photographed with a phase-contrast optic Axiophot microscope, at ×400 magnification. To assess spore size, 30 normal spores from different sporangia were measured. The area and the longest axis for each spore in equatorial view were estimated using the program ImageJ (Rasband 2014). The perispore, which is ornamented in most *Polystichum* species, was excluded from the measurements. For statistical analysis, the median, standard deviation and variance were determined. To compare spore size among species and to test for a correlation between spore size and ploidy level, we performed an ANOVA and a Tukey's test, respectively. We applied logarithmic base-10 transformation of the spore-size variable, so as to obtain a normal distribution of the residuals variable. InfoStat version 1.1 (Di Rienzo *et al*. 2002) was employed in all the analyses involving numerical data.

### Literature survey of chromosome numbers in Polystichum

A summary of worldwide cytological *Polystichum* data was prepared on the basis of available literature (Löve *et al*. 1977; Smith and Mickel 1977; Wagner 1979; Shimura and Ooishi 1980; Wagner 1980; Smith and Foster 1984; Kato and Nakato 1999; Dawson *et al*. 2000; Roux 2000, 2001, 2004; Perrie *et al*. 2003*a*, 2003*b*; Jara-Seguel *et al*. 2006; Lin *et al*. 2011; Condack *et al*. 2013) and an on-line database (Barrington 2006). For each record, the location of the collection site was mapped using DIVA–GIS (Hijmans *et al*. 2004). Then, frequency-distribution analysis was conducted using InfoStat version 1.1 (Di Rienzo *et al*. 2002). In all cases, the mentioned centres of endemism are those of Barrington and Driscoll (2005).

## Results

### Chromosome number and ploidy

Mitotic chromosome numbers for seven SSC taxa and two NCC taxa were obtained (Table 1, Figs 2, 3). With two exceptions (*P. platyphyllum* and *P. subintegerrimum*), these counts are the first chromosome reports. In all cases, the base number was $x = 41$; ploidy level varied. Within the SSC taxa (all indusiate), six were tetraploid ($2n = 4x = 164$) and one, *P. subintegerrimum*, was octoploid ($2n = 8x = \sim 328$). The exindusiate NCC species *P. pycnolepis* and *P. platyphyllum* had a diploid chromosome number ($2n = 2x = 82$).

### Chromosome size and morphology

The sum of chromosome lengths in somatic nuclei ranged from 438.08 µm in *P. platyphyllum* to 822.85 µm in *P. subintegerrimum*, with the highest-ploidy species having the highest values. The two diploid species (*P. platyphyllum* and *P. pycnolepis*) had the shortest lengths.

The average single-chromosome length (in somatic chromosome sets) ranged between 2.50 µm in *P. subintegerrimum* and 5.75 µm in *P. pycnolepis* (Table 1).

In general, the species studied have chromosomes that are homogeneous in size. Centromeres are inconspicuous, although in diploids they are more distinguishable and most chromosomes are subtelocentric (no more than one-third are metacentric); no bimodal karyotypes were found, and secondary constrictions were frequently observed in *P. platyphyllum* and *P. pycnolepis*.

### Spores and sporangia

All species presented 64 spores per sporangium; less than 10% were abortive, except for *P. subintegerrimum* and *P. plicatum* with 29.34% and 28.23% abortive spores, respectively. Significant differences in spore size among species were found ($F = 265.22$, $P < 0.0001$), with *P. pycnolepis* having the lowest mean value (410.32 µm$^2$), followed by *P. platyphyllum* (560.90 µm$^2$). In contrast, the species with the largest area were *P. andinum* (1584.76 µm$^2$) and *P. subintegerrimum* (1306.19 µm$^2$); the remaining species had intermediate spore sizes (649.35–774.23 µm$^2$), comprising values not significantly different from one another (Fig. 4). Significant ($F = 134.37$, $P < 0.0001$) differences in spore size among ploidy levels were found. Spore size was significantly lower for diploid species, intermediate for tetraploid, and higher for the octoploid (Fig. 5).

### Occurrence of polyploidy in Polystichum

Analysis of cytological data allowed us to find the number of records for each ploidy level and estimate the occurrences of ploidy levels in the main centres of endemism of *Polystichum*. We retrieved 130 cytological records for 116 species from the literature. Data showed an array of ploidy levels in the genus ($2x$, $3x$, $4x$, $6x$, $8x$). Of the 11 centres of endemism of Barrington and Driscoll (2005) with cytological data, the eastern Asian region (including the Yunnan and Sino-Japanese centres), which is the area of greatest diversity (Li *et al*. 2008), together with the Himalayan region, had the highest number of cytological records (71). We estimated that 58.46% of the species are polyploids; taxa diploids (41.54%) and tetraploids (47.69%) are the most frequent. The genus has 12 species (most of them from eastern Asia), with at least two cytotypes, being the combination of tetraploid and diploid cytotypes within a single species, the commonest situation (Table 2).

## Discussion

### Basic chromosome number and polyploidy

Our results confirmed the basic chromosome number $x = 41$ already recorded for *Polystichum* (Löve *et al*. 1977; Kato and Nakato 1999; Lu *et al*. 2006*a*, 2006*b*; Xiang *et al*. 2006). Thus, the chromosome number in *Polystichum* follows the trend in which homosporous ferns have chromosome numbers much higher than do heterosporous ones (Klekowski and Baker 1966; Klekowski 1973; Barker and Wolf 2010). Karyological and molecular evidence have documented polyploid origin of the large generic basic numbers in homosporous ferns (Klekowski 1973; Haufler and Soltis 1986; Otto 2007). Even though ferns and another homosporous vascular plants have high ploidy levels, and although they are capable of extreme inbreeding, they behave as true diploids, being predominantly outbreeders (Barker and Wolf 2010). Most evidence suggests that this 'diploidisation' reflects extensive and perhaps repeated episodes of gene silencing, without chromosome loss, after polyploidisation events (Wendel 2000).

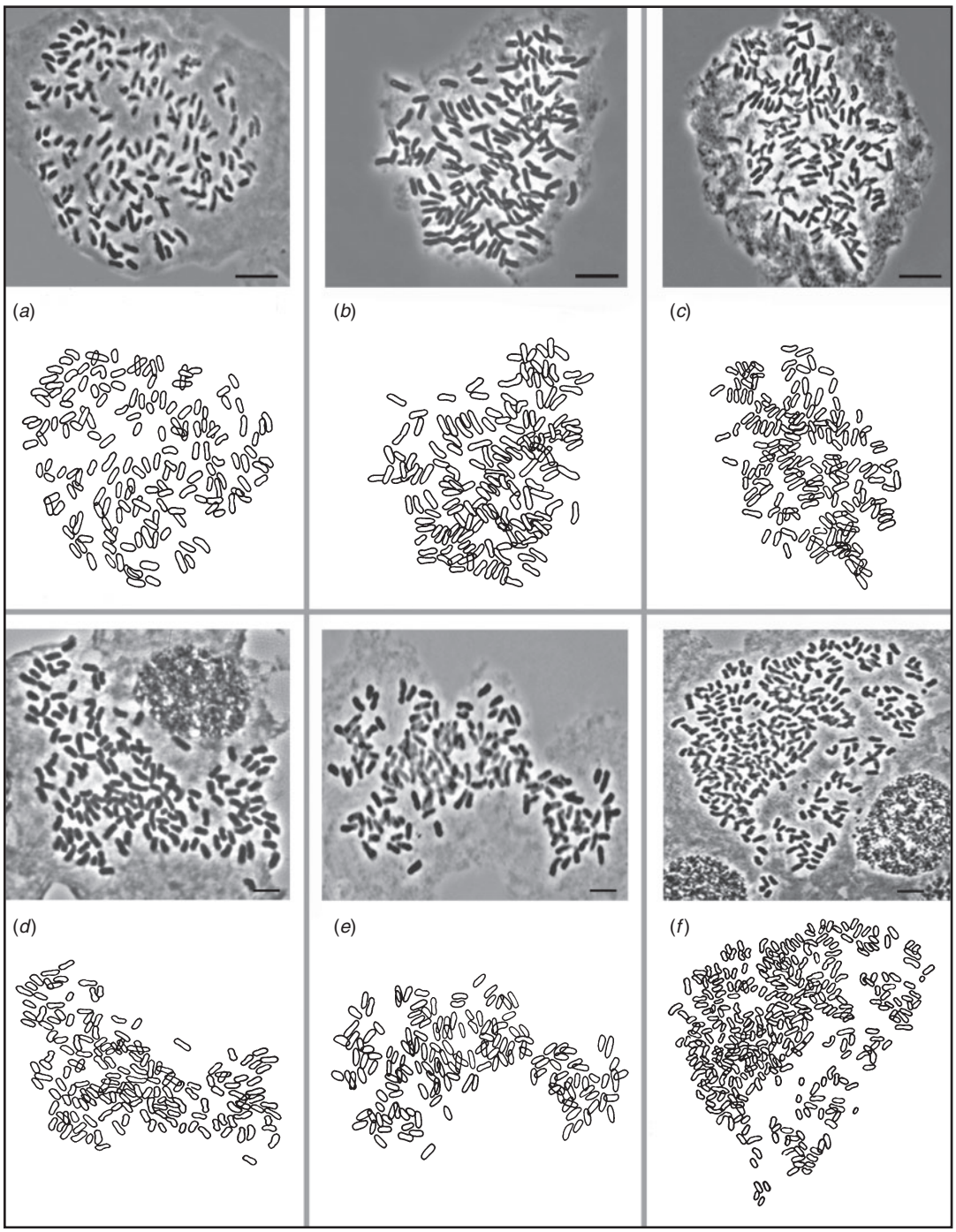


**Fig. 2.** Photograph (top) and explanatory drawing (bottom) of somatic chromosomes at metaphase. (*a*) *Polystichum andinum*, $2n = 164$; (*b*) *P. chilense* var. *chilense*, $2n = 164$; (*c*) *P. chilense* var. *dusenii*, $2n = 164$; (*d*) *P. multifidum*, $2n = 164$; (*e*) *P. plicatum*, $2n = 164$; (*f*) *P. subintegerrimum*, $2n =$ ca. 328. Scale bar = 5 µm.

We recorded an array of ploidy levels, including $2x$, $4x$ and $8x$, in our study set, just as in previous works, on a subset of these species. All the SSC taxa were tetraploid ($2n = 4x = 164$), except for *P. subintegerrimum*, which was octoploid ($2n = 8x = \sim 328$), whereas *P. pycnolepis* and *P. platyphyllum*, both from the NCC, were diploid ($2n = 2x = 82$). Our results for *P. platyphyllum* agree with those of Smith and Mickel (1977), who reported a diploid count for *P. platyphyllum* from México; however Smith and Foster (1984) obtained a tetraploid count from Paraguay for the same species. The Argentinean specimen of *P. subintegerrimum* analysed here is octoploid, as was reported by Jara-Seguel *et al.* (2006) for a Chilean population (Table 1).

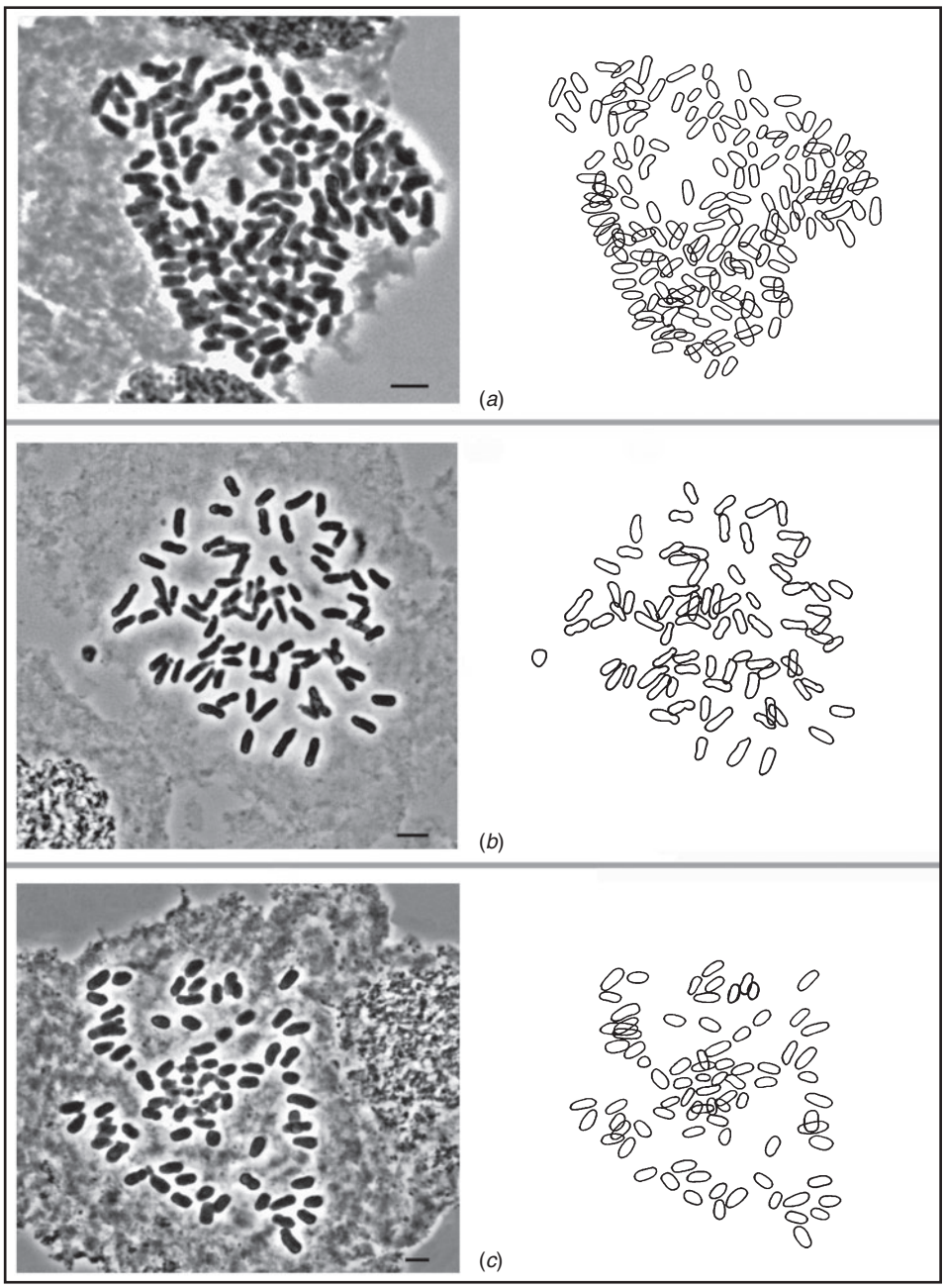


**Fig. 3.** Photograph (left) and explanatory drawing (right) of somatic chromosomes at metaphase. (*a*) *Polystichum tetragonum*, $2n = 164$; (*b*) *P. platyphyllum*, $2n = 82$; (*c*) *P. pycnolepis*, $2n = 82$. Scale bar = 5 µm.

It is evident that polyploidy and hybridisation have played a prominent role in the evolution of *Polystichum* (Löve *et al.* 1977; Barrington 1985*b*), with some species having been identified as allopolyploids (Barrington 1990, 2003) and also more than 80 interspecific hybrids have been reported, most being sterile (Miyamoto and Nakamura 1983; Knobloch 1996). Our cytological survey documents the high frequency of polyploids, with tetraploids being the most frequent. Higher ploidy levels (6*x* and 8*x*) as well as triploids, are rare. In all cases, the triploids share the same centre of endemism with related diploid and tetraploid species (except in the African centre), as is expected if the triploids are hybrids originating diploid–tetraploid contact zones (Table 2).

Apparently, ploidy levels are not randomly distributed, but follow a certain pattern; the eastern Asian region (represented by Yunnan and Sino-Japanese centres) and Himalayan region have a higher proportion of diploids (48.61%) than tetraploids (41.67%); also, similar pattern follows the species from Mayan, Antillean and the NCC. In contrast, there are no diploid records for the African centre, Australian–New Zealand centre and SSC.

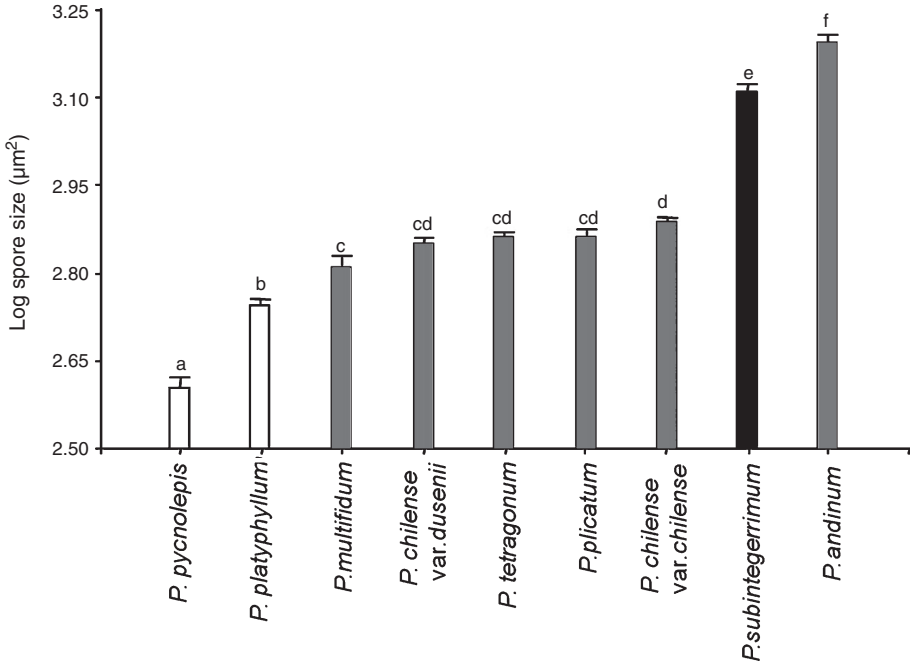


**Fig. 4.** Spore size (base-10 logarithm) of species. Bars with different letters indicate significant differences of spore size among species (ANOVA test, and Tukey a posteriori test, $P < 0.05$). The shade of the bars indicates the ploidy (white = 2*x*, gray = 4*x* and black = 8*x*).

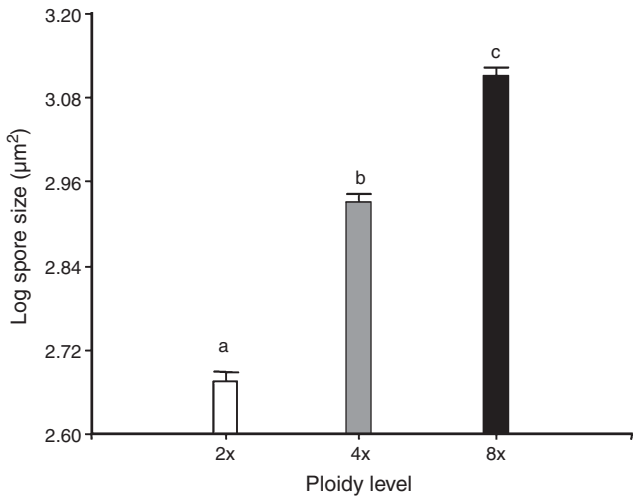


**Fig. 5.** Spore size (base-10 logarithm) and ploidy level of species. The shade of the bars indicates the ploidy (white = 2*x*, gray = 4*x* and black = 8*x*). Different letters over bars indicate significant differences of spore size among ploidy levels (ANOVA test, and Tukey a posteriori test, $P < 0.05$).

The SSC and the Australia–New Zealand centre, besides sharing a historical floristic relationship (Rodriguez 1989; Ponce *et al.* 2002), have a similar pattern of ploidy characterised by having tetraploid species, with only one or two octoploids.

### *Chromosome size*

Within the ferns, there is remarkable variation in the range of chromosome size (0.50–18 µm, Manton 1950; Abraham *et al.* 1962; Marcon *et al.* 2003*a*, 2003*b*, 2005; Guillén and Daviña 2005). Some families, such as Ophioglossaceae and Psilotaceae, are characterised by a high number of chromosomes and a large chromosome size (e.g. *Psilotum nudum* var. *gasa*: 4.5–18 µm, Abraham *et al.* 1962), whereas others, such as *Azolla*, have smaller chromosomes (e.g. *A. pinnata*: 0.5 µm, Stergianou and Fowler 1990). Dryopteridaceae is in the middle of this range (e.g. *Cyrtogonellum inaequalis*: 1.88–3.88 µm, *Lithostegia foeniculacea*: 2.53–5.50 µm, Lu *et al.* 2006*a*; *Dryopteris filix-mas*: 3.60–6.58 µm, Ivanova and Piekos-Mirkowa 2003; *Megalastrum spectabilis* var. *spectabilis*: 2.85 µm, Jara-Seguel *et al.* 2006). Previous information on chromosome sizes in other *Polystichum* species are also similar to our results (e.g. *P. fraxinellum*: 2.25–4.67 µm, Lu *et al.* 2006*a*; *P. igaense*: 4.63 µm, Lin *et al.* 2011; *P. montevidense*: 4.0–6.45 µm, Condack *et al.* 2013).

Our data reflect a direct relationship of an increase in the total chromosome length that accompany the ploidy level, as it was expected. Nevertheless, individual chromosome size decreased with increasing ploidy levels (Table 1). For example, the mean single-chromosome length of the octoploid *P. subintegerrimum* (2.5 µm) is 3.25 µm less than that for diploid *P. pycnolepis* (5.75 µm). Polyploids are supposed to have larger C-values than their diploid progenitors, increasing in direct proportion with ploidy. At the same time, polyploids are expected to have the same mean DNA amount per basic genome (Cx-value) as their diploid progenitors; however, there are examples in which the Cx-value tended to decrease with an increasing ploidy level (Leitch and Bennett 2004; Bennett and Leitch 2011), a phenomenon known as 'genome downsizing'. Because total chromosome length (in haploid nucleus) is positively correlated with C-value (Nagl and Ehrendorfer 1974; Dimitrova and Greilhuber 2000; Garnatje *et al.* 2004; Levin 2002), reduction

**Table 2. Data of ploidy levels based on location of cytological published records for each *Polystichum* endemism centre (proposed by Barrington and Driscoll 2005)**

| Endemism centre (no. of endemic species/no. of records) | No. of records | | | | | No. of species with combinations of cytotypes | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2*x* | 3*x* | 4*x* | 6*x* | 8*x* | 2*x*/4*x* | 2*x*/6*x* | 3*x*/4*x* | 2*x*/3*x*/4*x* |
| North-westAmerica (11/8) | 3 | | 4 | 1 | | 1 | | | |
| Mayan (13/10) | 8 | | 2 | | | | | | |
| Antillean (27/6) | 4 | | 2 | | | 1 | | | |
| Andean or NCC (17/4) | 3 | | 1 | | | 2 | | | |
| Chilean or SSC (7/7) | | | 6 | | 1 | | | | |
| African (11/9) | | 1 | 6 | 1 | 1 | | | 1 | |
| Australia–New Zealand (13/13) | | | 11 | | 2 | | | | |
| Papua (18/1) | 1 | | | | | | | | |
| Himalayan (27/20) | 10 | 1 | 9 | | | | | | |
| Yunnan (43/19) | 8 | 2 | 9 | | | 4 | 1 | 1 | 1 |
| Sino-Japanese (22/33) | 17 | 3 | 12 | 1 | | | | | |
| Total (percentage) | 54 (41.54%) | 7 (5.38%) | 62 (47.69%) | 3 (2.31%) | 4 (3.08%) | 8 | 1 | 2 | 1 |

in a single-chromosome length in polyploid *Polystichum* series can be interpreted as genome downsising. This situation, more prominent in angiosperm families than in ferns and lycophytes (Bennett and Leitch 2001), may have considerable biological significance. Ozkan *et al.* (2003) argued that reduction could be a necessary adaptation for the establishment and stabilisation of the polyploid genome. Furthermore, Leitch *et al.* (2005) suggested that genome downsising might be a widespread biological response to polyploidy, leading to diploidisation of the polyploid genome. Dart *et al.* (2004) indicated that changes in the average DNA content following polyploidisation could be caused by the loss or gain of entire chromosomes or by change in chromosome size. In our case, we did not verify chromosome loss; however, we did observe a reduction of chromosome length in polyploids relative to diploids, probably being related to genome size. C-value measurements are needed to test this assertion.

### *Chromosome morphology*

The morphologic identification of chromosomes was difficult because of the unclear location of centromeres. However, secondary constrictions were frequently observed (especially in *P. platyphyllum* and *P. pycnolepis*), but their number varied among cells within a single individual. This variation may be determined by the transcription level, the number of ribosomal genes, or the state of chromatin condensation (Warburton and Henderson 1979; Von Kalm and Smyth 1984; Medina *et al.* 1986).

### *Chromosomal data, origin and distribution of species*

In our area of study, diploids are absent among SSC species and frequent among the NCC taxa, implying a difference in reproductive biology and history between the two groups. Although only six NCC species have been cytologically studied, three of them are diploids endemic to South America, namely, *P. sodiroi* (Löve *et al.* 1977), *P. pycnolepis* (our results) and *P. montevidense* (Condack *et al.* 2013). The remaining two species, widespread in tropical America, are *P. dubium* (2*x*, Wagner 1980), *P. orbiculatum* (4*x*, Barrington 1990) and *P. platyphyllum*, with two cytotypes (2*x*, Smith and Mickel 1977; 4*x*, Smith and Foster 1984). Thus, more chromosome counts of NCC species are necessary to develop insights into the differences between the NCC and the SSC.

The case of *P. tetragonum* deserves special attention. It is a microendemic species, being the only representative of the genus known from the Juan Fernandez Islands, a small archipelago located in the Pacific Ocean, at ± 670 km west of the Chilean coast. The archipelago is volcanic in origin and consists of two main islands, namely, Masatierra and Masafuera, which are ~4 million years and 1–2 million years old, respectively (Stuessy *et al.* 1984). Evolutionary studies on the Juan Fernandez flora suggest that most of the endemic monotypic genera have originated anagenetically from different continental progenitors (Stuessy *et al.* 1990, 2006). Given the proximity of this archipelago to the mainland and its recent geological origin, the most parsimonious explanation for the origin of *P. tetragonum* is that it originated through transoceanic migration from a continental ancestor. A more complex explanation involving long-distance dispersal across the Pacific Ocean from Australia–New Zealand (situated 8700 km away from the Juan Fernandez Islands) would be unlikely. We suggest that this speciation process would not have involved a new chromosomal duplication event, because *P. tetragonum* is a tetraploid like most of the SSC species.

Even though only 6% of cytologically studied species on the Juan Fernández Archipelago are polyploid, most of the endemic polyploid taxa have apparently evolved at the same ploidy level as their polyploid mainland relatives (Stuessy *et al.* 1990). Moreover, of the 32 taxa (all but one are endemic species), 66% are ancient (pre-colonisation) polyploids and only 6% are recent polyploids (Sanders *et al.* 1983). *Polystichum tetragonum* is included in the first group because there is not a likely diploid progenitor from the continent. The stability of $2n = 164$ might be related to the 'chromosome stasis' hypothesis; surveys of cytological data from oceanic islands have shown little chromosomal change during the evolution of endemic taxa, even though speciation has often been accompanied by remarkable morphological differentiation (e.g. in *Dendroseris*, Crawford *et al.* 1992, 1998). Explanations for such phenomena include low levels of hybridisation because of reproductive isolation, short periods of geological time and selection against

novel cytotypes that might alter the adaptive traits that allowed successful colonisation and radiation (Sanders *et al*. 1983; Carr 1998; Stuessy and Crawford 1998).

### *Spores and sporangia*

The presence of 32 spores per sporangium is indicative of apogamy. This kind of reproduction is particularly important in triploid hybrids (which are often out of balance in meiosis) and it is necessary for their survival, although some species are represented by viable cytotypes as well (Manton 1950). In our case, all species presented 64 spores per sporangium, which suggests that they would be ancient polyploids with regular sexual reproduction (Gastony 1991; Werth and Windham 1991; Soltis and Soltis 1999). Additional studies would be helpful to elucidate whether these species do actually have normal chromosome pairing during meiosis.

Another phenomenon that is indicative of chromosomal abnormalities is spore abortion (Barrington *et al*. 1989; Lin *et al*. 2011). Although most species had ~10% irregular spores, we considered the species to have normal sexual reproduction, because nearly all spore samples from normal species showed at least some proportion of deviant spores, probably owing to genetic determination or environmental conditions (Wagner *et al*. 1986). *Polystichum plicatum* and *P. subintegerrimum* had a high proportion (25–30%) of abortive spores. The first species presented with most spores small and irregular, whereas the last one presented with mainly large roundish spores, suggesting that both taxa may have originated in recent allopolyploidisation events.

Polyploids usually present certain cells and organs larger than diploids (Barrington *et al*. 1986; Otto 2007). Particularly, spore size has been useful in differentiating diploid and polyploid individuals in *Osmunda regalis* (Manton 1950), in *Asplenium trichomanes* (Moran 1982) and also in *Polystichum* species (Barrington *et al*. 1986), where tetraploids consistently had larger exospores than did diploids. We also found significant differences in spore size among ploidy levels. Spore size was significantly smaller for 2*x* individuals, intermediate for 4*x* and higher for 8*x*. Thus, ploidy level is related to spore size, so that the higher the ploidy level, the larger the spore size (Manton 1950; Tryon and Lugardon 1990). However, the tetraploid *P. andinum*, which has the highest values for spore size, does not fit this relationship. In fact, although there is a general trend for spore size to be related to ploidy level, the relationship is not strict; spore size may be influenced not only by ploidy level, but also by reproductive biology and environmental factors (Barrington *et al*. 1986).

At the species level, a previous study (Morbelli 1980) concluded that spore measurements were not useful in distinguishing some *Polystichum* species from Patagonia. However, we found significant differences among most studied taxa; just four taxa presented similar spore size. Thus, within each ploidy level, spore size was useful to single out the species.

The present study is the first approach to understanding the systematic relationships of the southern South American species of *Polystichum* and contributes basic information about the reproductive system and patterns of speciation of these species. However, it is necessary to obtain more complete cytogenetic, molecular and morphological data to get a clearer idea about the evolutionary history of this group.

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