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A COMPARATIVE STUDY OF THE GAMETOPHYTIC GENERATION IN THE *POLYSTICHUM ACULEATUM* GROUP (PTERIDOPHYTA)

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The gametophytic generation of the allotetraploid *Polystichum aculeatum* and its diploid parents, *Polystichum setiferum* and *Polystichum lonchitis*, was studied in order to compare their morphology, gametangial ontogeny, and breeding system. Six populations, two of each species, were selected for spore collection. Germination, gender expression, and antheridiogen experiments were established on agar and soil culture media. Germination percentage in the tetraploid was higher, and the only morphological difference was found in the length of marginal hairs that were also longer in *P. aculeatum*. Gender expression in the allotetraploid was a mixture of the diploids. Differences in gender expression of both diploids, with many male prothalli in *P. lonchitis* and many female ones in *P. setiferum*, may favor the formation of the hybrid that originated the allotetraploid. An antheridiogen system was observed in both *P. aculeatum* and *P. setiferum*, and each species responded to one another's antheridiogen. In contrast, exudates from *P. lonchitis* failed to induce precocious maleness within the species but did induce an antheridiogen response in gametophytes of *P. setiferum*.

Keywords: Polystichum, gender expression, breeding system, gametophyte, antheridiogen.

Introduction

Allopolyploidy is widely recognized as a process of secondary speciation in ferns (Haufler 1996). Many examples have been reported in which a tetraploid species is recognized as having originated from the hybridization of two diploid species and the subsequent duplication of the chromosomes (Manton 1950; Shivas 1969; Walker 1979; Reichstein 1981). Sporophytes of these tetraploids usually show a combination of morphological characters from the diploid parents. These processes have been corroborated in many fern species by the use of cytological techniques modeled after a crossing program for producing artificial hybrids of the polyploid with its supposed parents (Lovis 1977; Cubas 1990; references therein). More recently, isozyme electrophoresis has simplified and accelerated the study of these processes (Gastony 1986; Haufler et al. 1990; Soltis et al. 1990; Werth 1991; Pryer and Haufler 1993).

Nevertheless, although there are several studies that have focused on the gametophytic generation of ferns, including some allotetraploid species, few have focused on a comparative study of the gametophytes of an allotetraploid with those of its diploid parents. Because of the morphological simplicity of fern gametophytes, few differences are found between prothalli of different species, but when differences exist, a combination of them may be found in the gametophytes of the derived allopolyploids (Prada et al. 1995).

However, some comparative studies of the reproductive biology of allotetraploids and their diploid parents have been carried out. These studies have indicated that gametophytes of the polyploids may be more tolerant of intragametophytic selfing than the gametophytes of their diploid parents (Masuyama 1979). The objective of our study was to compare the morphology, gametangial ontogeny, and breeding system in the gametophytic generation of an allotetraploid and its diploid parents; we hypothesize that if the prothalli of the diploids show differences in these features, then the prothalli of the allotetraploid will show a combination of them.

In the Iberian Peninsula, the genus *Polystichum* is represented by three species: two diploids, *Polystichum lonchitis* (L.) Roth. and *Polystichum setiferum* (Forsskal) Woynar, and the allotetraploid derived from them, *Polystichum aculeatum* (L.) Roth. Thus, it was a suitable group to test our hypothesis. *Polystichum lonchitis* is a Holarctic species with a preference for mountain and rocky habitats, especially in its southern populations; *P. setiferum* is a Mediterranean and Macaronesian species that extends northward along Atlantic Europe to Scotland, living in woods and hedges; and *P. aculeatum* is mainly a western Euro-Siberian species found also in North Africa and some of the Macaronesian islands in woods and hedges too.

The morphology, cytology, and ecology of the sporophytes of these species are well known (Manton 1950; Manton and Reichstein 1961; Sleep and Reichstein 1967; Vida and Reichstein 1975; Salvo and Hidalgo 1986; Salvo et al. 1986; Bremer 1995). However, only a few studies about the morphological development of the gametophytes have been published (Nayar et al. 1969; Chandra and Nayar 1970). All *Polystichum* prothalli have unicellular hairs on the margins and on both surfaces. Thus, among the morphological characters, we chose the length and density of marginal hairs because allotetraploids usually show an increase in size of individual cells, as in most polyploids (Stebbins 1950). In addition, we studied the gender expression and the reproductive system of the three species.

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In some fern species, a pheromone, antheridiogen, that stimulates antheridia initiation is produced by maturing female gametophytes, thus promoting outcrossing. We assayed each species for an antheridiogen system and for interspecific sensitivity among each species pair.

Material and Methods

Spores from six populations, two from each species, were used in our experiments. Acronyms are used for the identification of populations throughout the text (table 1).

For all our experiments, gametophytes were grown in 6-cm-diameter petri dishes on either of the following culture media: (1) sterile mineral agar (Dyer 1979) to which Nystatin 100 units mL^{-1} was added to eliminate fungal contaminants (originating from the spore wall) because the spores were not surface sterilized; and (2) a 3 : 1 mixture of commercial potting soil ("Blumenerde," Floragard, Oldenburg, Germany) and sand, autoclaved at 125°C for 20 min. After gametophytes were transplanted, the petri dishes were sealed with parafilm and placed in a growth cabinet at 21°C, 30 μmol photons m^{-2} s^{-2}, and 12 h of light and 12 h of dark.

To study spore germination, spores from several sporophytes from each population were sown on mineral agar. There were two replicate petri dishes for each population. The germination percentages were determined by counting 50 spores from each plate and calculating the mean value of the two replicates. Germination was defined as emergence of the first rhizoid. At 4-d intervals, plates were scored until percentages became stable. The final score was made after 32 d.

The early development of gametophytes was studied on the same agar plates as used for the germination percentages. Ca. 25 prothalli were randomly sampled weekly and were fixed and stained in a mixture of aceto-carmine and chloral-hydrate (Edwards and Miller 1972). Prothalli were rinsed with distilled water, mounted on a slide, and examined under a light microscope. From each of the weekly samples at 10 and 25 wk, 10 gametophytes were randomly selected to measure their hair length and density around the margin of the gametophyte. Marginal hairs were preferred instead of the surface ones to facilitate measuring. Mean length was determined by measuring 10 hairs from each of the 10 gametophytes and calculating the mean value of the 100 hairs. Mean density (number of hairs per perimeter) was determined by counting the number of hairs per millimeter on the same 10 gametophytes as used for the hair length and calculating the mean value. These measures were made on the wings of the prothalli, and hairs in the notch region were ignored because they may not have been fully developed. A hierarchical single-factor ANOVA (Zar 1996) was performed on the results to reveal statistically significant differences in gametophyte hair length and hair density between species and populations. Species were considered a fixed factor, and populations were considered a random factor nested within species. A Tukey test ($P < 0.05$) was performed to compare species means (Zar 1996).

To elucidate patterns of gender expression in gametophytes populations, 50 presexual gametophytes were transferred from agar to soil and planted at a density of 1.8 prothalli cm^{-2}. There were two replicates for each population (i.e., 100 prothalli per population). Every 2 wk, each prothallus was picked off the soil, rinsed with distilled water, mounted on a slide, examined under a light microscope for the presence of antheridia and archegonia, and replanted again. Gametophytes were not appreciably damaged; the soil was loose, not compact, minimizing injury to rhizoids or other parts of the prothalli. Gametophytes were scored as presexual, male, female, or bisexual. These cultures were kept until the production of sporophytes had stabilized (32 wk).

Multispore cultures where all the spores came from the same sporophyte were established on mineral agar to provide gametophytes for this experiment. To assay the isolate potential (Peck et al. 1990; Ranker et al. 1996), presexual gametophytes, ca. 5 wk old, were isolated and grown on soil (same mixture as above) in small plastic boxes (3 × 3 cm). For each popu-

Table 1

Acronyms and Localities of the Populations Where Plants Used to Obtain the Spores Were Collected

Population	Locality	No. plants	Germination (%)
Polystichum lonchitis (L.) Roth.:			
PlBEN	Spain: Huesca province, Benasque, between Hospital and Renclusa, 1900 m, granites; A. Herrero, 7-IV-1996	5	64
PlUSA	United States: unknown locality; spore exchange of the American Fern Society, 1997		66
Polystichum setiferum (Forsskal) Woynar:			
PsSIL	Spain: Pontevedra province, Silleda, confluence of the rivers Toxa and Deza, oak and laurus forest, 185 m; L. G. Quintanilla and B. Pias, 25-XI-1997	8	40
PsPOL	Spain: Burgos province, road between Burgos and Santander, near Polientes, beech forest; C. Prada, S. Pajarón, and E. Pangua, 28-X-1997	9	56
Polystichum aculeatum (L.) Roth:			
PaPAN	Spain: León province, Panderrueda; C. Prada, S. Pajarón, and E. Pangua, 29-X-1997	7	100
PaRIA	Spain: León province, Pedrosa del Rey on the road to Riaño, beech forest; C. Prada, S. Pajarón, and E. Pangua, 29-X-1997	8	100

Table 2

Hierarchical Single-Factor ANOVA for Testing the Differences of Gametophyte Hair Density and Hair Length between the Three *Polystichum* Species

	After 10 wk				After 25 wk			
Source of variation	df	Mean squares	*F*	*P*	df	Mean squares	*F*	*P*
Hair density:								
Species	2	7.197	0.547	0.6275	2	3.267	0.339	0.7369
Populations within species	3	14.483	14.674	<0.0001	3	9.650	5.955	0.0014
Error	54	0.987	…	…	54	1.620	…	…
Hair length:								
Species	2	1793.010	6.982	0.0744	2	2390.510	11.829	0.0378
Populations within species	3	256.813	30.479	<0.0001	3	202.094	6.760	0.0002
Error	594	8.426	…	…	594	29.894	…	…

lation, there were 50 boxes: 25 containing only one prothallus and 25 containing paired prothalli (all 75 prothalli originated from the same plant). Paired gametophytes were planted ca. 1 cm apart. They were watered every week by pouring several drops of tap water on the prothalli to facilitate fertilization and to keep substrate humidity high. Thirty-two weeks after the gametophytes were transplanted, gender expression and the appearance of sporophytes were quantified.

To assess whether the three study species produced an antheridiogen and were sensitive to it, we carried out two experiments. Both experiments involved population PlUSA of *Polystichum lonchitis*, PsSIL of *Polystichum setiferum*, and PaPAN of *Polystichum aculeatum*. First, five female prothalli from a population were placed in the center of a petri dish with agar, and spores from the same population were sown around them. In these experiments, source and target species were the same in each plate. To test whether they were sensitive to the antheridiogen of the other species, we undertook a similar experiment in which the spores sown around the female prothalli were not the same species as those prothalli. Two replicates of each were made. After 8 wk, concentric sampling was made at 0.5 cm, 1.0 cm, and 1.5 cm distant from the central prothalli; 25 prothalli were sampled at each distance. The gender of each gametophyte was assessed. In every target species with every source species, χ^2 analysis was performed to test the dependence of sex expression and distance from the potential antheridiogen source. In order to compare the three antheridiogen sources, a heterogeneity χ^2 (Zar 1996) was made. Two gender expressions were considered for the statistical analysis: males and no males and the sum of presexuals, females, and bisexuals.

All statistics were conducted using SPSS (1999).

Results

Maximum germination percentages 32 d after sowing for each population ranged between 40% and 100% (table 1). Only populations of the allotetraploid reached 100% germination.

In the young prothalli, a terminal unicellular hair appeared either at the end of the filamentous stage or soon after the initiation of the plate formation. Female and bisexual prothalli were typically always cordate and meristic, whereas male prothalli may be meristic or ameristic in all three species and in both multispore and isolate cultures. Gametophyte hair density after 10- or 25-wk culture was not significantly different between the three species (table 2), averaging ca. 8 hairs mm^{-1} (table 3). There was also no significant difference between species in hair length after 10 wk, but there was after 25 wk ($P < 0.05$; table 2), with means of 35.5 μm for *Polystichum setiferum*, 38.6 for *Polystichum lonchitis*, and 42.4 for *Polystichum aculeatum* (table 3). Pairwise comparison showed sig-

Table 3

Summary of Mean Values ± SE for Gametophyte Hair Density and Hair Length of the Three *Polystichum* Species

	10 wk		25 wk	
Species	Mean ± SE	Means of populations studied	Mean ± SE	Means of populations studied
Hair density (hairs mm^{-1}):				
Polystichum lonchitis	8.0 ± 0.3	7.1, 8.9	8.8 ± 0.3	7.9, 9.6
Polystichum aculeatum	8.8 ± 0.3	7.9, 9.6	8.0 ± 0.3	8.0, 8.1
Polystichum setiferum	7.5 ± 0.3	6.7, 8.3	8.8 ± 0.3	7.9, 9.6
Hair length (μm):				
P. lonchitis	26.1 ± 0.2	25.2, 27.0	38.6 ± 0.4	37.8, 39.4
P. aculeatum	32.1 ± 0.2	30.4, 33.8	42.4 ± 0.4	41.6, 43.2
P. setiferum	28.6 ± 0.2	28.2, 29.1	35.5 ± 0.4	34.2, 36.8

Note. Mean values of the populations studied also given. To obtain hair density mean values, 10 gametophytes from each population at each age were used. To obtain hair length mean values, 100 hairs from each population at each age were measured.

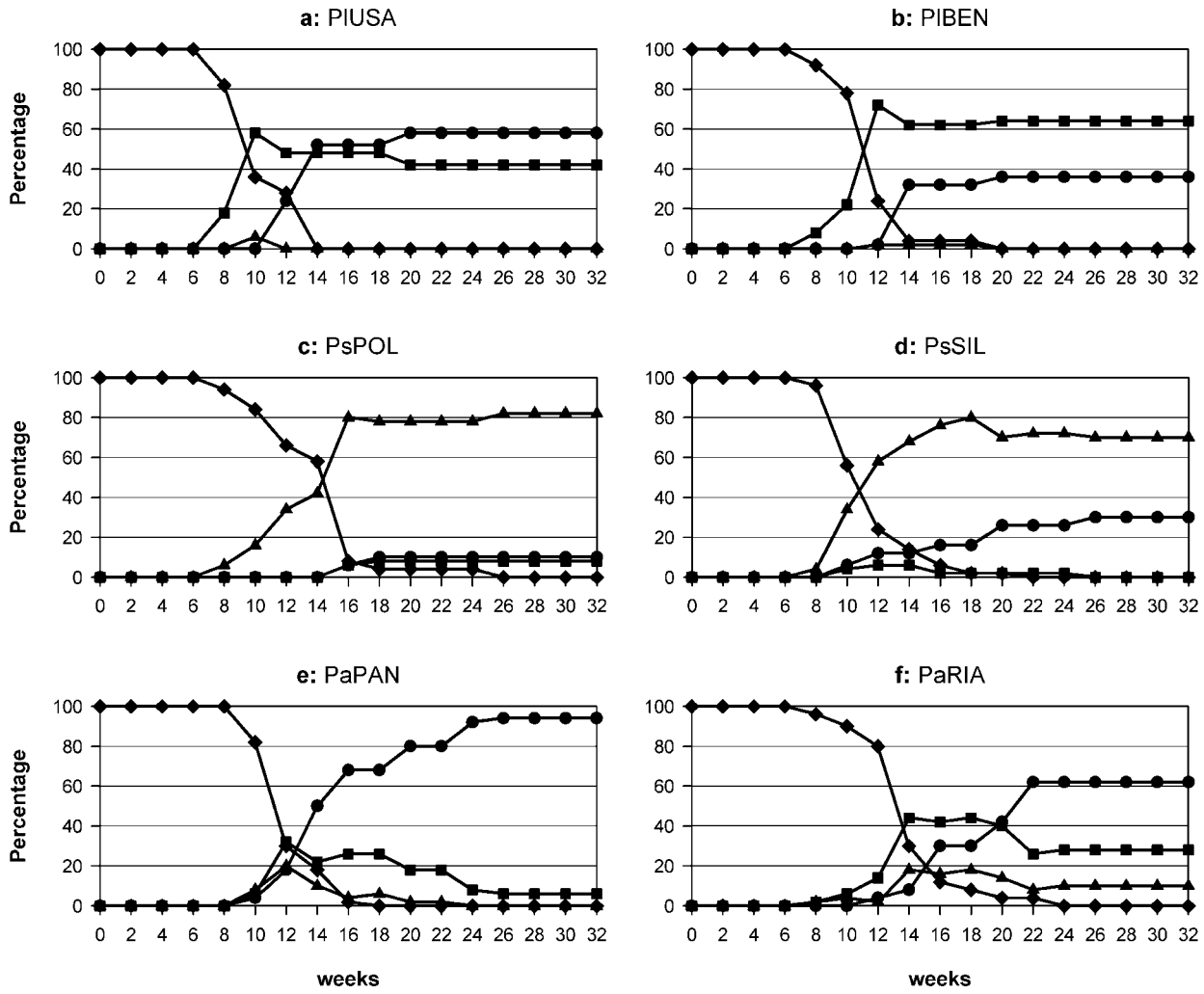

Fig. 1 Gender expression of the six populations studied. *a*, *b*, *Polystichum lonchitis*, populations PlUSA and PlBEN; *c*, *d*, *Polystichum setiferum*, populations PsPOL and PsSIL; *e*, *f*, *Polystichum aculeatum*, populations PaPAN and PaRIA. Diamonds = presexuals; squares = males; triangles = females; circles = bisexuals.

nificant differences between all three means (Tukey test, $P < 0.05$). In the six populations studied, there were highly significant differences in hair density and hair length only within the three species at the two culture times (table 2).

In all soil cultures, sex expression began 8 wk after sowing; however, there were differences in the ontogenetic sequence of gametangia among the three species. Antheridia were the first gametangia developed in both populations of *P. lonchitis* (fig. 1*a*, 1*b*), and after 12 wk bisexual gametophytes were detected. A small percentage in PlBEN population (2%) formed archegonia and then became bisexual. After 14 wk, the system stabilized with male and bisexual prothalli, with 40% males and 60% bisexuals in PlUSA and vice versa in PlBEN (60% males, 40% bisexuals).

In *P. setiferum* (fig. 1*c*, 1*d*), the ontogenetic sequence started with the production of archegonia, and later, but simultaneously, a small proportion of males and bisexuals appeared. Once both populations had become stable, there was a high proportion of females, with 82% in PsPOL and 70% in PsSIL. Male and bisexual prothalli percentages were similar in PsPOL, with 8% males and 10% bisexuals. In PsSIL, the proportion of male prothalli was very low, 2%, throughout most of the culture period, and after 18 wk they formed archegonia and turned into bisexuals.

The gametangial sequence in the allotetraploid *P. aculeatum* was not as clear as in the diploids (fig. 1*e*, 1*f*). All three types of prothalli—males, females, and bisexuals—appeared at the same sampling time. In population PaPAN, the proportion of male and female prothalli decreased during the culture period, while the proportion of bisexual prothalli increased. In PaRIA, a similar pattern developed, although a small percentage of male and female prothalli were always present. When sex expression in the population became stable, 28% males, 10% females, and 62% bisexuals coexisted.

In these multispore cultures, sporophytes formed in all three species, although not in all populations. The number of sporophytes formed in female and bisexual prothalli was different in all three species after 32 wk of culture (table 4).

Table 4

Percentages of Sporophytes in Archegoniate Prothalli in Multispore Cultures after 32 wk

Species/population	Sporophytes	Female prothalli	Bisexual prothalli
Polystichum lonchitis:			
PlUSA	14	0	14
PlBEN	0	0	0
Polystichum setiferum:			
PsSIL	60	34	26
PsPOL	0	0	0
Polystichum aculeatum:			
PaPAN	13	0	13
PaRIA	7	0	7

After 32 wk of culture, the gender of the isolated gametophytes was different according to the species (table 5). All isolated *P. setiferum* gametophytes were female, while all isolated *P. aculeatum* gametophytes were bisexual, and there were *P. lonchitis*–isolated gametophytes of the three sexes. After these weeks of culture, sporophytes appeared in all the bisexual prothalli.

In the paired gametophyte cultures, all possible sex combinations were observed. Sporophytes were formed in several pairs in which antheridiate and archegoniate gametophytes were present, i.e., the only cases in which fertilization was possible (table 6). In both female/bisexual pairs of *P. lonchitis* (PlUSA), sporophytes were formed only in the bisexual gametophyte. In *P. setiferum*, sporophytes appeared on both prothalli in all female/bisexual and bisexual/bisexual couples. In *P. aculeatum*, sporophytes usually appeared on both prothalli of a pair, irrespective of whether they were female/bisexual or bisexual/bisexual. The only exception was in two of the five female/bisexual pairs of population PaPAN, in which only one sporophyte per gametophyte pair was formed, one in a female prothallus and the other in a bisexual one.

In the antheridiogen experiment in which putative source and target species were the same, different results were observed 8 wk after sowing was made. In *P. lonchitis*, all sampled prothalli were presexual at all distances; therefore, results are not shown. In *P. setiferum* (fig. 2*a*), at 0.5 cm of the source, 100% of sampled prothalli were male; this percentage decreased as the distance from the source increased. A similar situation was observed in *P. aculeatum* (fig. 2*b*), but the percentage of male gametophytes was lower. In the experiment in which source and target species were different, different results were also observed after 8 wk. *Polystichum lonchitis* prothalli stayed presexual when *P. setiferum* was used as the source. However, when the source was *P. aculeatum* (fig. 2*c*), 20% of the sampled gametophytes at 0.5 cm were male, while the rest at this distance and at 1.0 and 1.5 cm were presexual. *Polystichum setiferum* showed a different behavior when used as target species and when *P. lonchitis* was used as source (fig. 2*d*). Male prothalli decreased with distance, while the number of presexuals increased. With *P. aculeatum* as source (fig. 2*e*), a high percentage of male prothalli was formed at 0.5 cm, but also a few bisexual and female ones were observed. As distance increased, the percentage of male prothalli decreased, and the proportion of bisexuals increased. In *P. aculeatum*, no effect was observed when the source species was *P. lonchitis*. When the source was *P. setiferum* (fig. 2*f*), 80% of the prothalli at 0.5 cm were male. This percentage decreased with distance, and the percentage of presexuals increased.

In all three species, sex expression depends on the distance from the antheridiogen source (table 7). All the cases in which there is a statistically significant ($P < 0.05$) dependence of the sex expression with the distance from the source result from the decrease of male gametophyte frequency as this distance increased (fig. 2). No male prothalli were formed in three combinations of source and target species—*P. aculeatum*/*P. lonchitis*, *P. lonchitis*/*P. setiferum*, and *P. lonchitis*/*P. lonchitis*—so it was not possible to perform χ^2 tests. The heterogeneity χ^2 to compare the three antheridiogen sources was possible only in *P. aculeatum*, and as a result, their frequencies could be grouped ($\chi^2 = 8.104$; df = 4; $0.05 < P < 0.10$). In this way, the power of the analysis increases and the change in the sex expression with distance to the antheridiogen source is also detected (table 7; $\chi^2 = 13.156$).

Discussion

Our study of spore germination in the different populations showed that there were differences among them. The collection dates were similar, and all of them received the same treatment, except for the sample PlUSA, for which we do not know the storage conditions before arriving to us from the Spore Exchange of the American Fern Society. Thus, the variability in this case depends on the population of origin. Similar dif-

Table 5

Percentages of Male, Female, and Bisexual Prothalli in Isolated Gametophyte Cultures after 32 wk

Species/population	Males	Females	Bisexuals[a]
Polystichum lonchitis:			
PlUSA	40 (10)	16 (4)	44 (11)
PlBEN	76 (19)	16 (4)	8 (2)
Polystichum setiferum:			
PsSIL	0	100 (25)	0
Polystichum aculeatum:			
PaPAN	0	0	100 (25)
PaRIA	0	0	100 (25)

Note. It was not possible to use data of population PsPOL of *P. setiferum* because of contamination. The absolute number of gametophytes of each are shown in parentheses.

[a] Number in parentheses indicates the formation of sporophytes in all the gametophytes.

Table 6

Percentages of Male, Female, and Bisexual Prothalli in Paired Gametophytes Cultures

Species/population	Male/male	Male/female	Female/female	Male/bisexual	Female/bisexual	Bisexual/bisexual
Polystichum lonchitis:						
PlUSA	64 (16)	8 (2)[a]	0	20 (5)[a]	8 (2)[b]	0
PlBEN	80 (20)	4 (1)[a]	0	16 (4)[a]	0	0
Polystichum setiferum:						
PsSIL	18 (4)	9 (2)	45 (10)	5 (1)[a]	18 (4)[c]	5 (1)[c]
Polystichum aculeatum:						
PaRIA	0	0	0	8 (2)[a]	8 (2)[c]	84 (21)[c]
PaPAN	0	4 (1)	0	0	20 (5)[d]	76 (19)[c]

Note. It was not possible to use data of population PsPOL of *P. setiferum* because of contamination. The absolute number of couples of each are shown in parentheses. Footnotes indicate the formation of sporophytes.

[a] Sporophytes in all archegoniate prothalli.

[b] Sporophytes only in the bisexual gametophyte of each pair.

[c] Sporophytes in both gametophytes of each pair.

[d] Three pairs with sporophytes formed in both gametophytes of each pair: one pair with sporophyte on the female gametophyte and the other with gametophyte on the bisexual one.

ferences among populations had also been observed in species of *Asplenium* (Pangua et al. 1994; Prada et al. 1995), *Cryptogramma* (Pangua et al. 1999), *Woodwardia*, *Culcita* (Quintanilla et al. 2000), and many others. The high germination percentage of the tetraploid stands out compared with the diploids. Similar results have been obtained with diploid and tetraploid species of *Dryopteris* (Whittier 1970) and *Polypodium virginianum* (Kott and Peterson 1974). However, Windham et al. (1986) showed that *Pellaea* germination ability was independent of ploidy level using plants from 10 different herbaria of the United States.

The morphological development of the prothalli is of the *Aspidium* type. These results do not differ from those of Nayar et al. (1969) and Chandra and Nayar (1970). No differences were found with previous studies of species of this genus, except for the presence of ameristic male prothalli that was reported previously only in *Polystichum setiferum* (Lindsay 1992) of the three studied species. These reduced and always ameristic male prothalli are common in other species of fern genera such as *Blechnum*, *Athyrium*, *Dryopteris*, *Pteridium* (Lindsay 1992), and *Asplenium* (Pangua et al. 1994), especially when an antheridiogen system exists. Hair density showed no statistically significant differences among species, but hair length was significantly longer in the allotetraploid after 25 wk of culture, as could be expected. As Stebbins (1950) wrote, allotetraploids usually show an increase in size of individual cells as in most polyploids. In fern sporophytes, this shows up when measuring spores and stomata guard cell length (Barrington et al. 1986). Thus, it is not unexpected to see the same effect in the unicellular marginal hairs of these gametophytes. However, in other fern groups, e.g., the *Asplenium adiantum-nigrum* group (Prada et al. 1995), the gametophyte of the allotetraploid has shorter hairs than one of the putative parental diploids. This contradiction can be explained because, in this case, the tetraploid hair length is more or less simply the mean between both diploid parents' hair length, one with shorter hairs and the other with longer ones. Thus, the gametophyte of the allotetraploid also combines features of the gametophytes of the diploid progenitors. Hair size variation with age has also been observed in other taxa such as the subspecies of *Asplenium trichomanes* (Herrero et al. 1993). The time for sexual maturation in laboratory culture under similar conditions is as in other fern taxa, i.e., *Asplenium* (Pangua et al. 1994), and slower than in others, i.e., *Cystopteris* (Pajarón et al. 1996), and no differences were found between the diploids and the allotetraploid.

The ontogenetic sequence of gametangia in *Polystichum lonchitis* shows a bigametophytic system, as defined by Klekowski (1969); the sequence begins with male prothalli, and later some of these became bisexuals. This is the most common sequence in homosporous ferns (Klekowski 1969; Raghavan 1989), as determined from artificial laboratory cultures; there is still very little known about what really happens in natural gametophyte populations. This sequence favors intragametophytic selfing (Klekowski 1969; Lloyd 1974, 1988; Raghavan 1989). In *P. setiferum*, the first gametangia to appear are archegonia, and later some of the presexual prothalli became male and others became bisexual. The high proportion of female prothalli present throughout the culture period favors intergametophytic crossing (Cousens 1979; Lloyd 1980). Lindsay and Dyer (1996) found a different sequence in *P. setiferum* in spore cultures in pots in the field. They found a high proportion of male prothalli, with the remainder being female and bisexual. These differences may probably result from culture density, since we used a density of 1.8 gametophytes cm^{-2} and they sowed at a density of 500 viable spores cm^{-2}; although most of them probably did not germinate and the actual density of gametophytes was difficult to determine accurately, it was higher than in our experiment. Sex expression of the gametophytes of the allotetraploid is different from that of the gametophytes of both diploids, presenting a mixture of both. Male, female, and bisexual prothalli appear more or less simultaneously. Throughout the culture period, males and females became bisexual, although a percentage of each type remained until the end of the experiment. This ontogenetic sequence allows selfing, which is important for polyploid establishment. The variability in gender expression among these closely related species is noteworthy. Ease or difficulty for reproduction imposed by the habitat, rocky versus forest, may be a source of selection over the reproduction system.

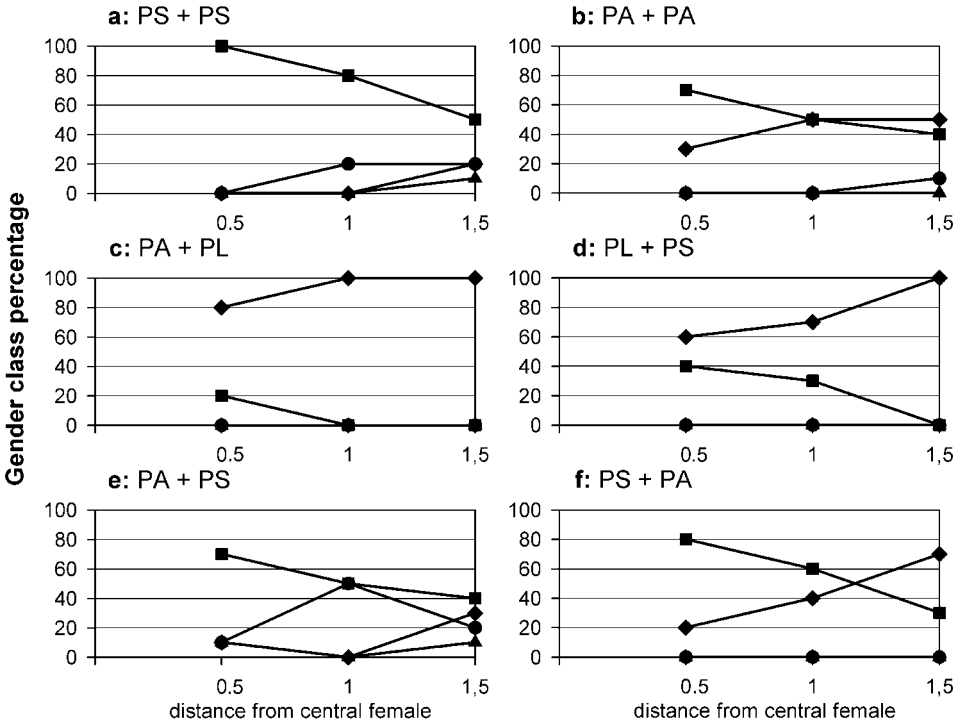

Fig. 2 Percentages of each of the gender classes in the antheridiogen experiments at 0.5, 1.0, and 1.5 cm from the female central prothalli. *a*, *Polystichum setiferum* source and target species; *b*, *Polystichum aculeatum* source and target species; *c*, *P. aculeatum* source species and *Polystichum lonchitis* target species; *d*, *P. lonchitis* source species and *P. setiferum* target species; *e*, *P. aculeatum* source species and *P. setiferum* target species; *f*, *P. setiferum* source species and *P. aculeatum* target species. Diamonds = presexuals; squares = males; triangles = females; circles = bisexuals.

The pattern of sporophyte production in the gametophyte cultures of the diploid species provided some support for our conclusions about the breeding systems. In *P. lonchitis*, all prothalli with sporophytes were bisexual; thus, there is a high probability that these sporophytes have arisen by intragametophytic selfing. However, intergametophytic selfings cannot be absolutely excluded because male gametophytes were present throughout the culture period (32 wk). A similar breeding system was described for *Asplenium ruta-muraria* (Pangua et al. 1994). Studies of genetic variability of populations of this species (Schneller and Holderegger 1996) revealed that those growing on recently constructed walls showed low genetic variability and were probably derived from a single and recent spore colonization event, while those on natural cliffs and old walls showed higher genetic variability. Intragametophytic selfing would be the main breeding system in the former, at least at the beginning of the settlement. The arrival of new spores would increase genetic variability and may promote intergametophytic crossing in the old ones. In our experiments on *P. setiferum*, more than 50% of the sporophytes were formed on female prothalli; thus, intergametophytic fertilization must have occurred. The breeding system in *Polystichum aculeatum* is trigametophytic (Klekowski 1969); male, female, and bisexual prothalli coexist. Sporophytes were only formed on bisexual prothalli as in *P. lonchitis*, but since there were always some male prothalli, intergametophytic crossing cannot be rejected. Capacity for intragametophytic selfing in both *P. lonchitis* and *P. aculeatum* was confirmed by the results of the isolated cultures; 100% of the bisexual prothalli produced sporophytes. In *P. setiferum*, all were female; thus, no sporophytes appeared. This idea is also supported by the paired gametophyte cultures. Most of the sporophytes were formed on bisexual prothalli, but also female prothalli were fertilized in the

Table 7

χ^2 Statistics for Testing the Dependence of Sex Expression and Distance to Antheridiogen Source in the Three *Polystichum* Species

Target species/source species	χ^2	*P*
Polystichum aculeatum:		
Polystichum setiferum	5.273	0.0716
P. aculeatum	5.273	0.0716
Polystichum lonchitis	10.714	0.0047
Pooled species	13.156	0.0014
P. lonchitis:		
P. setiferum	12.018	0.0025
P. aculeatum	Not tested	…
P. lonchitis	Not tested	…
P. setiferum:		
P. setiferum	16.582	0.0003
P. aculeatum	13.961	0.0009
P. lonchitis	Not tested	…

Note. For all tests, df = 2.

diploids, *P. setiferum* and *P. lonchitis*. Although there are still few comparative studies, our results for *P. setiferum* and *P. aculeatum* confirm the general belief that polyploid ferns tend to be inbreeders, while diploid ferns usually behave as outcrossers (Masuyama 1979; Masuyama and Watano 1990; Pajarón et al. 1999). However, this may be less a function of gametophyte ontogeny than an effect of buffering of genetic load by a polyploid genome. The behavior of the diploid *P. lonchitis* is contrary to this hypothesis. *Asplenium trichomanes* subsp. *trichomanes*, also a diploid inhabiting rock crevices, behaves as an inbreeder (Vogel et al. 1999). Perhaps this behavior is imposed by the rocky habitat, in which it may be very difficult for spores to find safe places for germination and development. However, other diploid species behave as outcrossers in more or less similar habitats (Vogel et al. 1999).

An antheridiogen system was evident in two species, *P. setiferum* and *P. aculeatum*. In the diploid *P. lonchitis*, there was no response to its own antheridiogen or to that produced by *P. setiferum*. When sown around *P. aculeatum*'s female gametophytes, the proportion of male prothalli was similar to that obtained in multispore cultures after the same time. These results agree with those of Yatskievich (1993). He found no evidence for the production of an antheridiogen by *P. lonchitis* and showed that gametophytes of this species did not respond to an antheridiogen produced by *Pteridium*. The effect of *P. lonchitis* as source inducing maleness in *P. setiferum* was low, and there was no response at all at 1.5 cm from the source. These results may indicate that a putative or degenerate antheridiogen system exists in *P. lonchitis*. In the *Cystopteris tennesseensis* complex, Haufler and Ranker (1985) found differences in the antheridiogen response of both diploids involved in it. *Cystopteris protrusa*, a forest species like *P. setiferum*, responds to the antheridiogen, while *Cystopteris bulbifera*, a rupicolous species, does not. *Polystichum lonchitis*, although not strictly rupicolous, lives in rock fields and crevices, where, as well as in rupicolous habitats, safe sites for spore germination and gametophyte establishment are limited. This particular ecology may be related to the possible loss of an effective antheridiogen system. The high proportion of male gametophytes obtained in *P. setiferum* when using *P. aculeatum* or *P. setiferum* itself as source contrasted with the results obtained in multispore and isolated cultures where archegonia were the first gametangia to develop, and a high percentage of female prothalli remained throughout the experiments. The allotetraploid *P. aculeatum* has an intermediate response in the antheridiogen experiments as compared with its diploid ancestors. The strongest response was observed with *P. setiferum* as source, but with its own antheridiogen, *P. aculeatum* also showed a high increase of males, especially when compared with the sex expression of multispore cultures in which during the same culture period the prothalli remained presexual.

The existence of an antheridiogen system is believed to be very important in the processes of hybridization and also in the reticulate evolution of many fern groups (Schneller et al. 1990). Both diploids in this study have different sex expression, with protandry in *P. lonchitis* and protogyny in *P. setiferum*, which may favor a particular pattern of parentage and cytoplasmic inheritance in areas where both species are in contact. Similar results were found by Haufler and Ranker (1985) in a complex of North American *Cystopteris* species, in which they demonstrated the influence of the antheridiogen in the hybridization of the diploids.

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