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Comparative study of the sympatric ferns *Culcita macrocarpa* and *Woodwardia radicans:* Sexual phenotype

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Abstract

The sexual phenotypes of 1152 gametophytes from four populations of *Culcita macrocarpa* and *Woodwardia radicans* were monitored over a 1-year period. Gametophytes were maintained under three experimental conditions: (1) isolated, (2) pairs from the same sporophyte, or (3) pairs from different sporophytes. The frequencies of the sexual phenotypes did not vary significantly among these three conditions, and although there were some quantitative differences between populations, the sexual-phenotype sequences observed were species-specific. Gametophytes of *C. macrocarpa* were first male and then hermaphrodite: this sequence, together with the absence of antheridiogens, favours intragametophytic selfing. Natural populations of *C. macrocarpa* are presumably androdioecious. Gametophytes of *W. radicans* were first female and then hermaphrodite: this sequence and antheridiogen activity favour intergametophytic and even xenogamous mating. Despite these laboratory findings, populations of *W. radicans* are probably trioecious (because of the effects of antheridiogen). Few sporophytes of *W. radicans* were obtained in the present study, and none of *C. macrocarpa*: this is attributable to limiting illumination or substrate.

Keywords: Antheridiogen; Breeding system; Homosporous fern; Dichogamy; Sexual phenotype; Spain

Introduction

The gametophytes of homosporous ferns are potentially bisexual, making possible all breeding systems described in embryophytes (Cruden and Lloyd, 1995; Klekowski, 1979; Lloyd, 1974). Automixis (i.e. intragametophytic selfing) is the most extreme form of endogamy, since the sporophytes produced are completely homozygous; this system allows new populations to be established from a single spore. Autogamy (i.e. intergametophytic selfing) occurs between gametophytes derived from two different meiotic cells of a single sporophyte; heterozygosity is markedly reduced, but theoretically not entirely absent as in automixis. Xenogamy (intergametophytic crossing) occurs between gametophytes derived from different sporophytes. Between xenogamy and the two types of endogamy are mixed systems in which selfing and crossing have varying importances.

For a long time it was thought that automixis was the predominant system in natural populations of homosporous ferns (Klekowski, 1979; Klekowski and Baker, 1966). This view is inconsistent with numerous studies

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based on electrophoretic methods, which allow testing for random mating, and even estimation of rates of automixis and autogamy (e.g. Haufler, 1987; Ritland et al., 1990; Soltis and Soltis, 1992). In addition, in experimental cultures, relatively few species show high frequencies of automixis (see Lott et al., 2003, and references therein).

There are various mechanisms by which the gametophytes of homosporous ferns may avoid automixis. For example, the female and male gametangia may mature at different times. In addition, in ferns of various families it has been shown that gametophytes with archegonia produce pheromones that induce nearby immature gametophytes to produce antheridia (e.g. Yamane, 1998). Consequently, these compounds, together known as antheridiogens, stimulate intergametophytic reproduction.

Culcita macrocarpa C. Presl is the only European member of the family Dicksoniaceae, while *Woodwardia radicans* (L.) Sm. is one of only two European members of the family Blechnaceae, together with *Blechnum spicant* (L.) Roth. The principal populations of both ferns are located in northern coastal regions of Spain, and in the Azores, Madeira and Canary Islands. The ideal habitat for these species is mature riparian woodland on north-facing slopes of enclosed valleys, close to the coast (Amigo and Norman, 1995). Both species typically show strong clonal growth leading to patch formation. In many locations in which both species are present, their patches may be contiguous.

In addition to overlap in distribution and ecology, *C. macrocarpa* and *W. radicans* show great similarity in various life history characteristics, despite their phylogenetic remoteness. Both species have very large fronds, often over 2 m long, which persist throughout the winter. The period during which frond expansion occurs is the same in both species (unpublished own data). Spore release likewise occurs during the same period. Finally, the spores of both species are highly sensitive to desiccation (Quintanilla et al., 2002).

The present study investigated whether *C. macrocarpa* and *W. radicans* have adopted similar sex expression strategies. The gametophyte of *C. macrocarpa* has been studied previously by Stokey (1930), Rezende-Pinto (1943) and Mukherjee and Sen (1986). Although these studies described the sequence of appearance of gametangia, their central focus was on morphology. In contrast, there have been studies of the reproductive system in *W. radicans* (Klekowski, 1969b), but no studies of gametophyte morphology. Furthermore, it is difficult to compare the findings of these studies, because of differences in the culture methods used.

In the present study, we cultured isolated and paired gametophytes of *C. macrocarpa* and *W. radicans*. Our specific aims were (1) to compare the sequence of appearance of the gametangia of each species, (2) to determine whether there is among-population variation in sex expression, and (3) to assess whether gametangia sequence can be affected by antheridiogens. We also utilize this laboratory information and some field data to infer the likely breeding systems and population sex structure in nature.

Materials and methods

Plant material

We selected two populations of *C. macrocarpa* and two populations of *W. radicans* in northwest Spain (Table 1), the northernmost limit of the ranges of these species. We have reported data on the germination of spores from these populations in a previous study (Quintanilla et al., 2000); Table 1 maintains the acronyms used in this study. In addition, the two populations in the Eume watershed (C3 and W5) were included in a previous study comparing different methods of spore conservation (Quintanilla et al., 2002). From each population we collected spores from four individuals assumed to be genetically distinct: specifically, we collected spores from four ramets each separated by at least 10 m. All spores were collected in March 2000.

Experimental conditions

Spores of each individual were sown onto a 5.5-cm-diameter Petri dish with mineral agar (see Dyer 1979, p. 282). To minimize the risk of fungal contamination, the culture medium contained nystatin (100 U/ml). The dishes were sealed with Parafilm (American National

Table 1. Populations from which spores were obtained

Species	Acronym	Location (river valley)	Latitude	Altitude (m a.s.l.)
Culcita macrocarpa	C2	Seixo	43°41′N	280
	C3	Eume	43°24′N	90
Woodwardia radicans	W5	Eume	43°24′N	80
	W6	Xubia	43°30′N	250

Can, Chicago, IL, USA), and maintained in a temperature-controlled room at $20 \pm 2\,^{\circ}C$ with a 16-h light period (daylight fluorescent tubes, photon irradiance 30–45 $\mu mol/m^2/s$ in the 400–700 nm region).

Seven weeks after sowing, we transplanted gametophytes (still presexual at that time) to transparent plastic trays divided into 25 square cells with 20-mm sides, each cell containing 3 ml of culture medium identical to that in the Petri dishes. Gametophytes were randomly assigned to three experimental conditions: (1) *isolated* (each cell containing a single gametophyte); (2) *same-sporophyte pairs* (each cell containing two gametophytes from a single sporophyte); and (3) *different-sporophyte pairs* (each cell containing two gametophytes from two sporophytes of the same population). All three conditions permit automixis, while condition 2 additionally permits autogamy and condition 3 xenogamy. In each condition we used 24 gametophytes from each sporophyte, giving a total of 576 gametophytes for each species. The four sporophytes of every population taken two at a time made six combinations for condition 3 possible. We selected 12 pairs of gametophytes from four of these combinations.

The trays were then sealed with Parafilm and placed on a matt black surface to simulate natural lighting (Dyer, 1979). The trays were then maintained for 44 weeks in a temperature-controlled room under the same light and temperature conditions as for gametophyte obtention. For each gametophyte we determined sexual phenotype (presexual, female, male, hermaphrodite, or dead) on the basis of the presence of mature gametangia, and sporophyte production 7, 10, 13, 17, 21, 26, 31, 36, 41, 46 and 51 weeks after sowing. After these determinations, we eliminated algae, bacteria and fungi growing on the medium in some cells (despite the fungicide), and added 3–5 droplets of distilled water to each cell. We also changed the position of the trays to minimize possible effects of environmental heterogeneity within the room. After 21 and 36 weeks, we transplanted the gametophytes to new trays containing fresh medium.

Statistical analysis

For each species and each of the three experimental conditions, we analysed the relationship between gametophyte sexual phenotype and population using χ^2 tests; subsequently, these relationships were compared among conditions using χ^2 heterogeneity tests (Zar, 1999). The non-significant result in all heterogeneity tests (see the next section) justified grouping the three conditions, allowing analysis of the pooled data with a new χ^2 test with greater statistical power. These analyses were performed independently for data obtained at 17, 21, 26 and 31 weeks. Sexual phenotypes with expected frequencies of less than 5% (after grouping the three conditions) were not included in the analysis (Zar, 1999).

Considering the 384 paired gametophytes (conditions 2 and 3) of each species, we also analysed whether the nature of the first sexual phenotype transition (i.e. presexual to next phenotype) depends on the sexual phenotype of the other gametophyte of the pair (as determined at the preceding monitoring date; thus for example for a sexual phenotype transition in week 21, we considered the sexual phenotype of the other gametophyte as recorded in week 17). This analysis was also done with χ^2 tests; the first transition was categorized as presexual-to-male, or presexual-to-non-male (i.e. female, hermaphrodite, dead, or no-transition); sexual phenotype of the other gametophyte was defined as archegoniate (i.e. female or hermaphrodite), or non-archegoniate (i.e. presexual or male).

In χ^2 tests with only one degree of freedom, Yates' correction was applied (Zar, 1999). All statistical analyses were performed with the program SPSS for Windows (SPSS, 1999).

Results

Both in *C. macrocarpa* and *W. radicans*, and on all four analysis dates, sexual phenotype frequencies were homogeneous among the three experimental conditions ($P \geqslant 0.05$ in all χ^2 heterogeneity tests; Table 2). This allowed the frequencies to be grouped for joint analysis with better power. The results of these joint analyses indicate that in the period under comparison (17–31 weeks after sowing), the sexual phenotype of gametophytes was dependent on population of origin, except in week 21 for *C. macrocarpa* and week 17 for *W. radicans* (Table 2). The differences between populations of *C. macrocarpa* are due to the fact that sexual phenotype transitions occurred sooner in population C3 than in population C2 (Fig. 1). The sequence was similar in the two populations: almost all gametophytes initially produced antheridia, while less than 50% subsequently became hermaphrodite. However, both the peaks of these two periods and the onset of massive gametophyte mortality took place several weeks earlier in population C3. In *W. radicans*, there was no comparable between-population difference in timing (Fig. 2): gametophytes were initially female, with peak frequency in week 13, and subsequently became hermaphrodite, with peak frequency in week 26. The proportion of females becoming hermaphrodite was higher in population W5 (~80%) than in W6 (~50%).

Summing the frequencies of each sexual phenotype in each species (i.e. overall frequency in both populations and all three conditions), the frequency of gametophytes with gametangia peaked at 21 weeks in *C. macrocarpa*,

Table 2. χ^2 statistics for analysis of relationships between sexual phenotype and population

	17 weeks		21 weeks		26 weeks		31 weeks	
	df	χ^2	df	χ^2	df	χ^2	df	χ^2
Culcita macrocarpa								
Condition 1	2	14.731***	2	1.231^{NS}	3	12.855**	3	24.161***
Condition 2	2	18.216***	2	2.823^{NS}	3	11.551**	3	12.339**
Condition 3	2	14.650***	2	1.713^{NS}	3	0.318^{NS}	3	8.715*
Among-condition heterogeneity	4	6.881^{NS}	4	3.166^{NS}	6	11.308^{NS}	6	4.364^{NS}
Pooled conditions	2	40.716***	2	2.601^{NS}	3	13.416**	3	40.851***
Woodwardia radicans								
Condition 1	1	0.004^{NS}	1	0.042^{NS}	2	16.279***	2	32.801***
Condition 2	1	2.706^{NS}	1	4.043*	2	4.744^{NS}	2	6.851*
Condition 3	1	0.004^{NS}	1	2.726^{NS}	2	25.414***	2	13.499**
Among-condition heterogeneity	2	2.102^{NS}	2	2.046^{NS}	4	7.961^{NS}	4	8.485^{NS}
Pooled conditions	1	1.046^{NS}	1	5.574*	2	38.476***	2	44.666***

NS, not significant ($P \geqslant 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

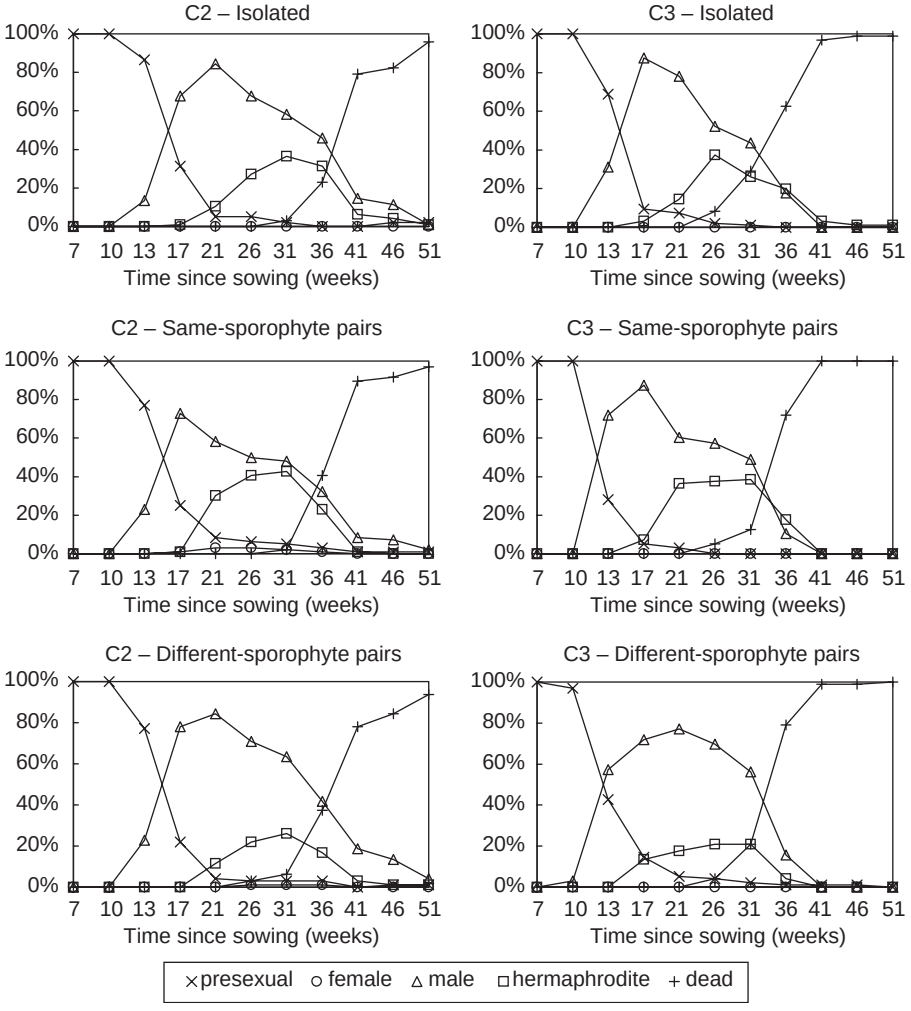

Fig. 1. Relative frequencies of sexual phenotypes of gametophytes from the two populations of *Culcita macrocarpa* in each of the three experimental conditions, over the 51-week culture period.

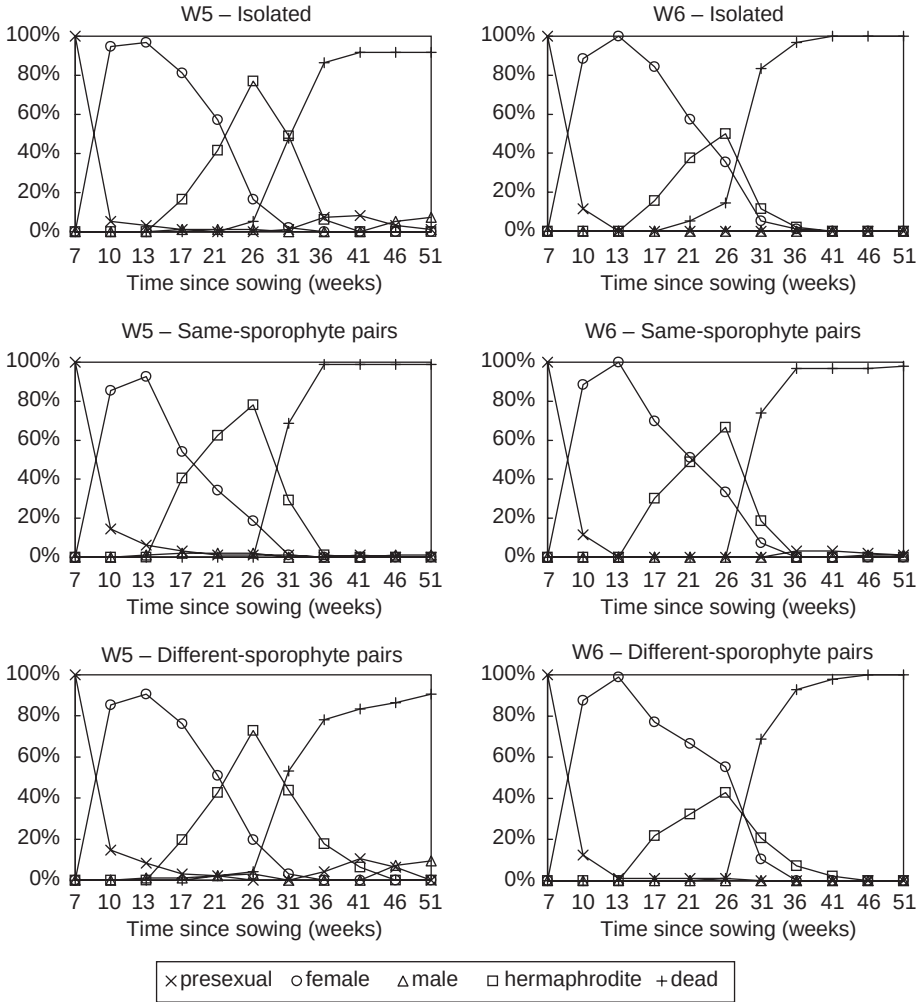

Fig. 2. Relative frequencies of sexual phenotypes of gametophytes from the two populations of *Woodwardia radicans* in each of the three experimental conditions, over the 51-week culture period.

and at 17 weeks in *W. radicans*. At these dates, female, male and hermaphrodite gametophytes together accounted for 94% of the total in *C. macrocarpa* and 99% of the total in *W. radicans*.

During culture we did not detect any *C. macrocarpa* sporophytes and only four *W. radicans* sporophytes, in all cases in experimental condition 3 (automixis and xenogamy). Specifically, two gametophytes of population W6 produced a sporophyte in week 21, while two gametophytes of population W5 produced a sporophyte in week 26.

The sexual phenotype of the neighboring gametophyte significantly affected the nature of first transition, in both *C. macrocarpa* ($\chi^2 = 54.153$, 1 df, $P < 0.001$) and *W. radicans* ($\chi^2 = 35.798$, 1 df, $P < 0.001$). However, the direction of the effect was different: in *C. macrocarpa*, archegoniate neighbors reduced the relative frequency of presexual-to-male transitions, while in *W. radicans* archegoniate neighbors increased the relative frequency of presexual-to-male transitions (Table 3).

Discussion

Gametangium sequence

Under the culture conditions used in the present study, gametophytes of *C. macrocarpa* were initially male and subsequently hermaphrodite, while gametophytes of *W. radicans* passed from female to hermaphrodite. These sequences were the same independently of whether the gametophytes were maintained in isolation (condition 1) or as one of a pair (conditions 2 and 3). In *C. macrocarpa*, however, the time-courses of this sequence differed between the two populations, probably reflecting genetic differences (Cousens, 1979). Nevertheless, the two species have clearly characteristic sequences, which coincide with those described for *C. macrocarpa* by Stokey (1930), Rezende-Pinto (1943) and Mukherjee and Sen (1986), and with Klekowski's (1969b), experimental findings for *W. radicans*.

Table 3. Frequencies of each type of first sexual phenotype transition in paired gametophytes, in the presence of an archegoniate (i.e. female or hermaphrodite) neighbour, and in the presence of a non-archegoniate (i.e. presexual or male) neighbour

		Phenotype of neighbouring gametophyte	
Species	First transition	Archegoniate	Non-archegoniate
Culcita macrocarpa	Presexual-to-male	1	361
	Presexual-to-female	0	4
	Presexual-to-hermaphrodite	0	1
	Presexual-to-dead	5	11
	No-transition	0	1
Woodwardia radicans	Presexual-to-male	6	0
	Presexual-to-female	37	336
	Presexual-to-hermaphrodite	1	1
	Presexual-to-dead	3	0
	No-transition	0	0

The category *no-transition* refers to gametophytes remaining presexual at the end of the 51-week culture period.

Breeding systems

The balance between automixis (intragametophytic selfing) and intergametophytic reproduction can be expected to depend on the lengths of the different sexual phases, on whether or not they overlap, and on the length of the period of overlap (Cruden and Lloyd, 1995). Both species studied here are dichogamous, i.e. the male and female phases do not exactly coincide. The sequence seen in *C. macrocarpa* (initially male gametophytes that then become hermaphrodite) is present also in other Dicksoniaceae (Mukherjee and Sen, 1986). According to morphological studies, it may also be the most common one in homosporous ferns (reviewed in Atkinson and Stokey, 1964; Stokey, 1951; Nayar and Kaur, 1971). It has been suggested that this sequence is that which maximizes the probability of automixis (Klekowski and Lloyd, 1968; Klekowski, 1969a). The sequence seen in *W. radicans* (initially female gametophytes that then become hermaphrodite) is presumably more favourable to intergametophytic reproduction (i.e. autogamy or xenogamy), and this sequence has also been reported in other ferns including Blechnaceae (Holbrook-Walker and Lloyd, 1973; Klekowski, 1969b, 1970).

The balance between xenogamy (intergametophytic crossing) and autogamy (intergametophytic selfing) can be expected to depend on the degree of relatedness of gametophytes in the populations. Degree of relatedness will reflect the genetic structure not only of the sporophytes, but also of the spore bank (Dyer and Lindsay, 1992; Schneller, 1998). Several authors have pointed out that fern spore dispersal distances typically show a leptokurtic distribution (e.g. Schneller, 1975); in other words, most spores locate close to the parent sporophyte. If we also take into account that populations of *C. macrocarpa* and *W. radicans* predominantly comprise long-lived individuals and that population turnover is slow, it seems reasonable to suppose that gametophyte genotypes and spore-bank genotypes will be largely determined by the genotypes of the nearest sporophytes. The sporophyte allozyme variation in the populations of *C. macrocarpa* and *W. radicans* used in the present study has already been analysed (Quintanilla, 2002) and will be reported elsewhere. In short, random mating could not be tested in *C. macrocarpa* owing to the lack of genetic variation within their populations. The genotype frequencies of *W. radicans* showed Hardy–Weinberg equilibrium, in accordance with intergametophytic crossing.

Factors limiting fertilization

In northwest Spanish populations of both species, sporangium dehiscence occurs at around the spring equinox, when temperatures are suitable for germination (Quintanilla et al., 2000). Since the proportion of sexually mature gametophytes peaked after 4 months (17 weeks) of culture in *W. radicans*, and after 5 months (21 weeks) of culture in *C. macrocarpa*, fertilizations are most likely to occur in the wild state in the summer. On many days in the summer, light intensity in the populations in question is up to double that used in our trials (unpublished own data). This may be one of the factors that prevented fertilization in our trials (see Page, 1979).

Gametophyte reproduction is also affected by the substrate used for culture (e.g. Korpelainen, 1994; Rubin et al., 1985). Generally, natural substrates give better results than artificial substrates. For example, under conditions similar to those used in our cultures,

sexually mature gametophytes of *Asplenium septentrionale* did not produce sporophytes in mineral agar, but on transplantation to soil numerous fertilizations were observed (Aragón and Pangua, 2003). The underlying causes of this hindering of fertilization by non-natural substrates are unknown, as in the case of the effects of insufficient illumination.

In any case, the scant (*W. radicans*) or zero (*C. macrocarpa*) obtention of sporophytes in the present study means that we are unable to evaluate the efficacy of the different mating systems under conditions of isolation or gametophyte pairing. Klekowski (1969b) had similar problems in cultures of *W. radicans*, and was unable to compare the proportion of sporophytes produced by isolated and paired gametophytes. This author detected certain abnormalities in the development of sporophytes produced by automixis, which he interpreted as due to endogamic depression. This is another characteristic indicating cross-fertilization in this species. In the case of *C. macrocarpa*, none of the authors who have cultured this species refer to sporophyte production (Mukherjee and Sen, 1986; Rezende-Pinto, 1943; Stokey, 1930).

Antheridiogens and sexual systems in the study populations

In our cultures of *W. radicans*, archegoniate gametophytes favoured production of antheridia by nearby presexual gametophytes, indicating the action of an antheridiogen. This favours intergametophytic fertilizations. The influence of this pheromone was not detected when we compared the frequency of sexual phenotypes of isolated gametophytes (condition 1) or in pairs (conditions 2 and 3). The reason was synchrony in the state of development of each pair of gametophytes, since antheridiogens generally affect immature gametophytes (for reviews see Näf, 1979; Schneller et al., 1990; Yamane, 1998). In nature, we can expect that asynchrony in gametophyte germination and development will offer more opportunities for the antheridiogen to act (Hamilton and Lloyd, 1991), and it thus seems probable that the sexual system of *W. radicans* will be trioecious (Table 4), as noted by Klekowski (1969a, b). By contrast, in *C. macrocarpa* there is a relative deficit of gametophytes that become male in the company of archegoniate gametophytes, which makes the action of an antheridiogen improbable in this species; its populations must thus be androdioecious.

Table 4. Sexual systems in pteridophytes

	♀	♂	♀ + ♂
Monoecious	·	·	+
Dioecious	+	+	·
Gynodioecious	+	·	+
Androdioecious	·	+	+
Trioecious	+	+	+

♀, female gametophytes; ♂, male gametophytes; ♀ + ♂, hermaphrodite gametophytes. Adapted from Cruden and Lloyd (1995).

In conclusion, the present study shows that two sympatric ferns sharing numerous life-history traits nevertheless have markedly different breeding systems. In *C. macrocarpa*, automixis is favoured by protandry and the absence of antheridiogens. In *W. radicans*, by contrast, protogyny and antheridiogen activity favour autogamy and xenogamy.

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