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Part 3

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M. GIBBY, A. LEONARD & H. SCHNEIDER

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PHYLOGENETIC SYSTEMATICS AND EVOLUTION OF THE GENUS *HYMENOPHYLLUM* (HYMENOPHYLLACEAE: PTERIDOPHYTA)

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Key Words: *Hymenophyllum,* Hymenophyllaceae, phylogeny, morphological evolution, *rbcL*, *rps4-trnS*, *rbcL-accD*, morphology, cytology.

ABSTRACT

In this study we address the phylogenetic relationships within the genus *Hymenophyllum*. Our sampling includes the segregate monotypic genera *Cardiomanes*, *Serpyllopsis*, *Rosenstockia*, and *Hymenoglossum*, representatives of the five subgenera proposed for *Hymenophyllum* by Morton, and of the section *Microtrichomanes*. Using morphology, cytology, and nucleotide sequences (*rbcL*, *rps4-trnS*, *rbcL-accD*), we obtained a fully resolved topology with several clades well supported. We confirm the monophyly of two clades within the Hymenophyllaceae. *Serpyllopsis* and *Rosenstockia* are nested in *Hymenophyllum* within a derived clade, while *Cardiomanes* and *Hymenoglossum* are positioned within a basal grade. Although some of the phylogenetic associations that were previously proposed within *Hymenophyllum* are supported, many traditionally defined infrageneric taxa are not resolved as monophyletic: subg. *Hymenophyllum* and *Sphaerocionium* are paraphyletic, and the broad subg. *Mecodium*, whose homogeneity had never been questioned, appears polyphyletic.

INTRODUCTION

The debate surrounding the systematics of the Hymenophyllaceae originates mainly from the existence of intermediate shapes between the bivalved sorus typically described for *Hymenophyllum* Sm. and the tubular one characterising *Trichomanes* L. (Iwatsuki, 1977), as well as from the existence of very peculiar taxa. Consequently, many authors refuted the bigeneric system and proposed a number of genera ranging from 6 to 42 (Copeland, 1938, 1947; Morton, 1968; Pichi Sermolli, 1977; Iwatsuki, 1984, 1990). Recent phylogenetic studies (Pryer *et al.*, 2001; Ebihara *et al.*, 2002; Hennequin *et al.*, 2003) have suggested the existence of two clades, one corresponding to *Trichomanes* s.l., the other to *Hymenophyllum* s.l. This latter notably includes the "intermediate" taxa treated as separate genera even by advocates of an oligogeneric system (Morton, 1968; Iwatsuki, 1984, 1990) : *Cardiomanes* C.Presl, *Serpyllopsis* Bosch, *Rosenstockia* Copel. and *Hymenoglossum* C.Presl. It also includes many species of the genus *Microtrichomanes sensu* Copeland (1938, 1947), a taxon previously

considered closer to *Trichomanes* than to *Hymenophyllum* by most authors (Ebihara *et al.*, 2004).

In addition to the issue of the circumscription of *Hymenophyllum*, specialists of the family encountered another problem when studying the systematics of the taxon, even in a strict sense. The genus consists of about 350 species, which grow predominantly as epiphytes. In comparison with the *Trichomanes* lineage, the *Hymenophyllum* lineage appears quite homogeneous morphologically and ecologically with all species having long creeping rhizomes with thin roots and mostly pendant leaves. A thorough study reveals, however, that many features are quite variable. These are, for example, the sorus morphology (Iwatsuki, 1977), but also the indumentum, the stele anatomy, and the chromosome numbers (Tryon and Tryon, 1982). Copeland (1937) thus noted that "*Hymenophyllum* is even less homogeneous than *Trichomanes*". Nevertheless, this variability does not provide reliable characters for the systematics of the genus, and Copeland (1937) deplored "the absence of single conspicuous criteria for the recognition of the natural groups within *Hymenophyllum*". Several groups have however been proposed for *Hymenophyllum* since the 19th century. The four major 20th century classifications of the genus are reported in Table 1. The delimitation of the taxa proposed does not vary much among these classifications. The main conflicts lie in the taxonomic rank attributed to the taxa and in the affinities suggested among them.

Using a combination of two chloroplastic regions, Ebihara *et al.* (2002), Ebihara *et al.* (2003) (*rbcL* + *rbcL-accD*) and Hennequin *et al.* (2003) (*rbcL* + *rps4-trnS*) provided insights into the relationships within *Hymenophyllum*. By sampling 25 species of the *Hymenophyllum* lineage, Hennequin *et al.* (2003) obtained three main clades: 1) *Hymenophyllum* s.s., corresponding globally to the subg. *Hymenophyllum* Sm. proposed by Morton (1968) and including the genera *Rosenstockia* and *Serpyllopsis*, and the subg. *Craspedophyllum* C.Presl and *Hemicyatheon* Domin.; 2) a clade including several species of the subg. *Mecodium* Copel.; and 3) a clade composed of species of subg. *Sphaerocionium* (C.Presl.) C.Chr. including species of *Microtrichomanes* (Mett. ex Pantl) Copel. *Cardiomanes reniforme* (G.Forst.) C.Presl and *Hymenoglossum cruentum* (Cav.) C.Presl were retrieved at the base of the tree along with one species of *Mecodium*. Nevertheless, several clades lacked bootstrap support. In this study, we combine and extend the taxonomic sampling used by Ebihara *et al.* (2002), Ebihara *et al.* (2003) and Hennequin *et al.* (2003) and add *rbcL-accD* or *rps4-trnS* data for all species. In addition, we combine molecular with morphological and cytological data.

MATERIALS AND METHODS

Taxonomic sampling

We adopt here Morton's (1968) classification for the purpose of presenting the results. The name of the section *sensu* Morton (1968) will thus be placed in parenthesis when needed. In addition to the taxonomic samplings used by Ebihara *et al.* (2002), Ebihara *et al.* (2003) and Hennequin *et al.* (2003), our sampling (Table 2) includes *H. marginatum* Hook. & Grev., the type species of Morton's (1968) subgenus *Craspedophyllum*, three species of *Mecodium* (*H. australe* Willd., *H. demissum* (G.Forst.) Sw. and *H. polyanthos* (Sw.) Sw. and *H. (Sphaerocionium) hirsutum* (L.) Sw. This sampling represents all but four out of the 11 genera proposed by Copeland (1938) for *Hymenophyllum* s.l., and all but three out of the 10 sections proposed by Morton (1968) for the genus. We first performed an analysis based on *rbcL* using a broad sampling including five *Trichomanes* taxa and five non-Hymenophyllaceae as

Table 1. Comparison of the classifications of Copeland (1938, 1947), Morton (1968), Pichi Sermolli (1977) and Iwatsuki (1984, 1990), with species number in the studied sections, and their distribution.

Copeland (1938, 1947)		Morton (1968)		Pichi Sermolli (1977)		Iwatsuki (1984,1990)		Number of species	Distribution (7)
Genera	Genera	Sub-genera	Sections	Genera	Genera	Sub-genera	Sections		
Hymenoglossum	*Hymenoglossum*			*Hymenoglossum*	*Hymenoglossum*			1	Ch
Serpyllopsis	*Serpyllopsis*			*Serpyllopsis*	*Serpyllopsis*			1	Ch-Arg
Rosenstockia	*Rosenstockia*			*Rosenstockia*		*Rosenstockia*		1	NC
Mecodium		*Mecodium*	*Mecodium* (2, 3)	*Mecodium*		*Mecodium*	*Plumosa*	> 100	P
							Pseudomecodium		
							Mecodium		
							Diplophyllum	2	Aus, NZ
Craspedophyllum		*Craspedophyllum*		*Craspedophyllum*			*Corrugatae*		
							Pachyloma	2	Aus, NZ
Hymenophyllum	*Hymenophyllum*		*Hymenophyllum*	*Hymenophyllum*	*Hymenophyllum*	*Hymenophyllum*	*Hymenophyllum*	33	C
			Eupectinum				*Eupectinum*	4	Ch-Arg
Amphipterum		*Hymenophyllum*	(2)	*Amphipterum*			*Amphipterum*	4	A
Buesia			*Buesia*	*Buesia*			*Buesia*	5	NT
Meringium			*Ptychophyllum*	*Meringium*		*Chilodium*	*Chilodium*	70	P
Myriodon			*Myriodon*	*Myriodon*			*Myriodon*	1	A
Hemicyatheon		*Hemicyatheon*		*Hemicyatheon*			*Hemicyatheon*	2	Aus, NZ
Leptocionium			(1)	*Leptocionium*			*Leptocionium*	1	Ch-Arg
Sphaerocionium		*Sphaerocionium*	*Sphaerocionium*	*Sphaerocionium*	*Sphaerocionium*	*Sphaerocionium*		70	P
Apteropteris			*Apteropteris*	*Apteropteris*		*Apteropteris*		2	Aus, NZ
Cardiomanes	*Cardiomanes*			*Cardiomanes*	*Cardiomanes* (4)			1	NZ
Microtrichomanes	(6)			*Microtrichomanes*	(5)			9	PT

(1) included in *Sphaerocionium* as a sub-section; (2) included in *Mecodium* as a sub-section; (3) including the sub-section *Diplophyllum* ; (4) unique representative of the sub-family *Cardiomanoidae*; (5) included in *Trichomanes* section *Crepidomanes*; (6) included in *Trichomanes* under the unplaced sectional name *Flabellata*; (7) distribution: Arg, Argentina; A, Asia; Aus, Australia; Ch, Chile; C, Cosmopolitan, NT, Neotropical; NC, New Caledonia; NZ, New Zealand; P, Pantropical, PT, Paleotropical

Table 2. Names and sources of material sequenced, with GenBank numbers. LPP = Laboratoire de Paléobotanique et Paléoécologie, H = *Hymenophyllum*; T = *Trichomanes*.

Cardiomanes reniforme (G. Forst.) C. Presl - *rbcL* U30833, *rbcL-accD* AB083290 (Ebihara 011222-07 New Zealand (TI, CHR)), *rps4-trnS* AY095132 (Rumsey s.n., cult. RBG Kew); *H. acanthoides* (Bosch) Rosenst. - Ebihara Kinabalu 030, Malaysia (TI), *rbcL* AB064291, *rbcL-accD* AB064303, *rps4-trnS* DQ364196; *H. apiculatum* Mett. ex Kuhn - Dubuisson HV 1997-23 Venezuela (LPP, Duke), *rbcL* AF275642, *rbcL-accD* AY775438, *rps4-trnS* AY095131; *H. armstrongii* (Baker) Kirk - Smith 2610 New Zealand (UC), *rbcL* AY095109; *rbcL-accD* AB162691 (Ebihara 011219-09 New Zealand (TI)), *rps4-trnS* AY095128; *H. australe* Willd. – T. A. Ohsawa 001125-03, Australia (TI), *rbcL* AB191439; *rbcL-accD* AB191439; *rps4-trnS* AY775412; *H. baileyanum* Domin.- Streimann s.n. Australia (UC), *rbcL* AF275643, *rbcL-accD* AB191441 (Ebihara 010909-02, Australia (TI)), *rps4-trnS* AY095129; *H. barbatum* Baker - Ebihara 000319-01 Japan (TI) *rbcL* AB064287, *rbcL-accD* AB064299, *rps4-trnS* AY095124 (Munzinger & Engelmann 297 Laos (Duke)); *H. deplanchei* (Mett.) Copel.- Ebihara 001224-03 New Caledonia (TI), *rbcL* AB064288, *rbcL-accD* AB064300, *rps4-trnS* AY095136 (Munzinger 367 New Caledonia (P)); *H. demissum* (G.Forst.) Sw. - Glasgow B. G. 830, cult. RBG Edinburgh, *rbcL* AY775402, *rbcL-accD* AY775441, *rps4-trnS* AY775416; *H. dilatatum* (G.Forst.) Sw. - Brownsey & Birchard New Zealand (Duke), *rbcL* AY095111, *rbcL-accD* AB191444 (Ebihara 011219-06, New Zealand (TI)), *rps4-trnS* AY095138; *H. dimidiatum* Mett. - Ebihara 001225-08 New Caledonia (TI), *rbcL* AB064289, *rbcL-accD* AB064301, *rps4-trnS* DQ364197; *H. ferrugineum* Colla - Taylor 6074 Chile (UC), *rbcL* AF275644, *rbcL-accD* AB191445 (Ebihara 021224-02, Chile (TI)), *rps4-trnS* AF537124; *H. flabellatum* (G.Forst) Sw. - Unknown collector 42 Tahiti (UC), *rbcL* AY775403, *rbcL-accD* AY775442, *rps4-trnS* AY775417; *H. fucoides* (Sw.) Sw. - Dubuisson HV-1997-9 Venezuela (Duke), *rbcL* U20933; *rbcL-accD* AY775449, *rps4-trnS* AY095142; *H. fuscum* (Blume) Bosch - Ito 2000 0210-16 Java (TI), *rbcL* AB064292, *rbcL-accD* AB064304, *rps4-trnS* AY775408; *H. hirsutum* (L.) Sw.- Dubuisson HR-1999-6 La Réunion (LPP, Duke), *rbcL* AY775407, *rbcL-accD* AY775450, *rps4-trnS* AY775432; *H. hygrometricum* (Poir.) Desv. - Dubuisson HR-1999-13 La Réunion (LPP, Duke), *rbcL* AY095113; *rbcL-accD* AY775451, *rps4-trnS* AY095118; *H. lanceolatum* (Hook.& Arn.) Copel. - O'Brien s.n. Hawaii (UC), *rbcL* AF275646, *rbcL-accD* AY775452, *rps4-trnS* AY095119; *H. oligosorum* Makino - Ebihara 001105-01 Japan (TI), *rbcL* AB064293, *rbcL-accD* AB064305, *rps4-trnS* AY775422; *H. paniense* Ebihara et al. - Ebihara 001225-02 New Caledonia (P, TI, KYO, NOU), *rbcL* & *rbcL-accD* 001225-02, *rps4-trnS* AY775410; *H. pectinatum* Cav. - Wedin H41, Chile (UC), *rbcL* AY095115, *rbcL-accD* AB191450 (Asakawa 2017, Chile), *rps4-trnS* AY095134; *H. polyanthos* (Sw.) Sw. - Ebihara 991122-01 Japan (TI), *rbcL* AB064295, *rbcL-accD* AB064307, *rps4-trnS* AY775423; *H. polyanthos* (Sw.) Sw. – Dubuisson s.n. La Réunion (LPP), *rbcL* AY775405, *rbcL-accD* AY775445, *rps4-trnS* AY775424; *H. scabrum* A.Rich. - Ebihara 011223-05 New Zealand (TI), *rbcL* AB083278, *rbcL-accD* AB083278, *rps4-trnS* AY775428; *H. secundum* Hook. & Grev. - Taylor 6075 Chile (UC), *rbcL* AF275648, *rbcL-accD* AY775437, *rps4-trnS* AY095125; *H. sibthorpioides* Mett.- Dubuisson HR-1999-1 La Réunion (LPB, Duke), *rbcL* AY095117, *rbcL-accD*

AB192688, *rps4-trnS* AY095127; *H. subdimidiatum* Rosenst. – Ebihara 001226-01 New Caledonia (TI), *rbcL* AB064290, *rbcL-accD* AB064302, *rps4-trnS* AY095140; *H. tenellum* Kuhn. - Dubuisson HR-1999-27 La Réunion (LPP, Duke), *rbcL* AY095116, *rbcL-accD* AB191453, *rps4-trnS* AY095126; *H. tunbrigense* (L.) Sm. - Dubuisson NV. 2.1 France (LPP, Duke), *rbcL* Y09203[2], *rbcL-accD* AY775436, *rps4-trnS* AY095123; *H. wrightii* Bosch - Ebihara 000901-01 Japan (TI), *rbcL* AB064294, *rbcL-accD* AB064306, *rps4-trnS* AY775430; *Hymenoglossum cruentum* (Cav.) C. Presl - Wedin H38 Chile (LPP), *rbcL* AY095107, *rbcL-accD* AB191455 (Ohsawa 2015, Chile), *rps4-trnS* AY095133; *Rosenstockia rolandi-principis* (Rosenst.) Presl - van der Werff 16045 New Caledonia (UC), *rbcL* AY095110, *rbcL-accD* AB064286/AB04298 (Ebihara 001225-11 New Caledonia (TI)), *rps4-trnS* AY095143; *Serpyllopsis caespitosa* (Gaudich.) C.Chr. - Taylor 6076, Chile (UC), *rbcL* AF275649, *rbcL-accD* AB191456 (T. A. Ohsawa 2014 Chile (TI)), *rps4-trnS* AY095130; *T. digitatum* Sw. - Dubuisson HR-1999-11' La Réunion (LPP, Duke), *rbcL* AY095114, *rbcL-accD* AB162676, *rps4-trnS* AY095120; *T. javanicum* Blume - Hennequin 2001-7 cult. Indonesia (LPP), *rbcL* Y09195, *rbcL-accD* AY775453, *rps4-trnS* AY095141; *T. rigidum* Sw.- Dubuisson HV-1997-3 Venezuela (Duke), *rbcL* AY095108, *rbcL-accD* AY775447, *rps4-trnS* AY095137; *T. taeniatum* Copel. - Matsumoto 01-948 Vanuatu (TNS, TI)), *rbcL* AB162681, *rbcL-accD* AB162681, *rps4-trnS* AY095121 (Game 86/08, Cook Islands (UC)); *T. tamarisciforme* Jacq. - Dubuisson HR-1999-32 La Réunion (LPP, Duke), *rbcL* Y09202[3], *rbcL-accD* AY775448, *rps4-trnS* AY095135;

out-groups, as in Hennequin *et al.* (2003), to confirm the monophyly of the two clades within the family (results not shown). We then reduced the out-group to three species of *Trichomanes* as in Hennequin *et al.* (2003). All three markers were sequenced for the whole sampling, so that 44 sequences are newly produced.

Morphological and cytological characters

The coding of morphological and cytological character state changes is based on Hennequin (2003) and we used in this study the same matrix as in Hennequin (2004). Morphological and cytological characters states changes were reconstructed using MacClade 3.0. (Maddison and Maddison, 1992). All characters were treated as unordered and plotted onto the topology recovered in the maximum parsimony combined analysis (results not shown but used in the discussion). We performed and compared both ACCTRAN and DELTRAN optimization options.

DNA sequencing

All procedures for DNA extraction, amplification and sequencing follow Hennequin *et al.* (2003). We used primers rbcL1195F (5'-TTCTACAGTTCGGTGGTGG-3'; newly designed) and accD816R (5'-CCATGATCGAATAAAGATTCA-3'; Ebihara *et al.*, 2003), and newly designed internal primers HIF3 (5'-TGTCAGGTTCTAAC-ATGTGATTG-3') and HIR3 (5'-CCTATACCTGTTTGAACAGCATC-3') to amplify and sequence *rbcL-accD*, respectively.

Phylogenetic analyses

We treated indels as binary characters following Barriel's (1994) method. MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) allows the integration of data other than nucleotide

or protein sequence, so that morphological and cytological characters as well as characters resulting from the treatment of indels were integrated both in parsimony analyses ("MP"; run with PAUP*4.0b10; Swofford, 2001) and in likelihood analyses ("ML"; run with MrBayes 3.0). For MP, we conducted unequally weighted analyses as described in Pryer *et al.* (2001) and Hennequin *et al.* (2003). All searches used a heuristic approach (TBR branch-swapping, 10 replicates of random sequence addition, MulTrees option on). The robustness of each branch was assessed by bootstrap analysis (100 replicates; Felsenstein, 1985). For likelihood analyses, we used ModelTest 3.06 (Posada and Crandall, 2000) to determine the nucleotide substitution model that best fits our data. We performed ML analyses using a random tree and a GTR + I + G model. Clade credibility values were estimated by calculating the posterior probability for each node using a Bayesian procedure as implemented in MrBayes 3.0. 10,000 trees were sampled and the consensus tree was computed (PAUP*4.0b10; Swofford, 2001) on the last 9,450 trees, excluding the 550 trees found in the "burn-in period".

RESULTS AND DISCUSSION

Delimitation of *Hymenophyllum* s.l.

In both the MP strict consensus tree and the majority–rule consensus tree inferred from the Bayesian estimation of posterior probabilities (Figures 1 and 2), the four segregate genera *Serpyllopsis*, *Rosenstockia*, *Cardiomanes* and *Hymenoglossum* are embedded within *Hymenophyllum* s.l. These results are in agreement with previous phylogenetic studies and question all the classifications that were proposed for the family. They nevertheless confirm some hypotheses of affinity proposed between taxa and the group successively treated as genus "*Hymenophyllum* s.l." (Pryer *et al.*, 2001), "*Hymenophyllum* group", or tribes Hymenophylleae (Schneider, 1996) or Hymenophylloideae (Presl, 1843; Iwatsuki, 1984, 1990). The following taxa had been considered close to, or treated as included in, *Hymenophyllum* s.l.: *Hymenoglossum* (Presl, 1843; Pichi Sermolli, 1977; Schneider, 1996 who included it in his tribe Hymenophylleae), *Serpyllopsis* (Presl, 1843; van den Bosch, 1861; Schneider, 1996), *Rosenstockia* (Tryon et Tryon, 1982; Iwatsuki, 1984, 1990), and *Microtrichomanes* (Copeland, 1938; Pichi Sermolli, 1977). On the other hand, no author had suggested such a broad group, and even less the inclusion of *Cardiomanes reniforme* and *T. (Pleuromanes) pallidum* Blume in *Hymenophyllum*.

Hymenophyllum s.l. systematics

Morton (1968) proposed five subgenera for *Hymenophyllum*: *Mecodium*, *Craspedophyllum*, *Hymenophyllum*, *Hemicyatheon* and *Sphaerocionium* (Table 1). The subg. *Hymenophyllum* is further divided in five sections: *Hymenophyllum*, *Ptychophyllum*, *Eupectinum*, *Myriodon* and *Buesia*. Representatives of the first three only were available for this study. This subgenus globally corresponds to the clade *Hymenophyllum* s.s. retrieved in this study. *Hymenophyllum* s.s. (bootstrap support (BS) = 96%, posterior probability (PP) = 0.56) also includes taxa placed in the subgenera *Hemicyatheon*, *Craspedophyllum*, *Mecodium* (*H. oligosorum* and *H. fuscum*), and the species of the segregate genera *Serpyllopsis* and *Rosenstockia*. This clade is supported by several morphological apomorphic character states: the presence of a common hair type on fronds, a margin denticulation and five soral characters, the most conspicuous being the presence of a small thick base (Hennequin, 2004). This clade has a cosmopolitan distribution, with a few species observed in Europe and probably in North

America as well. Within the *Hymenophyllum* s.s. clade, relationships are poorly resolved and weakly supported, except for a very robust clade (*H. barbatum* - *R. rolandi-principis*, BS = 100%, PP = 1) already retrieved by Ebihara *et al.* (2002) and named "*H. acanthoides* clade". This exclusively Australasian clade includes many taxa of the section *Ptychophyllum* and is supported by the basic chromosome number $x = 21$. It may be broadened to include the two species of *Hemicyatheon* (BS = 95%, PP = 1). The inclusion, in this clade, of *H. fuscum* (Blume) Bosch, type species of the genus *Amphipterum sensu* Copeland (1938), was already obtained by Ebihara *et al.* (2003). *Amphipterum* thus appears close to species of the section *Ptychophyllum sensu* Morton (1968), in agreement with Copeland (1938), Pichi Sermolli (1977) and Iwatsuki (1984, 1990). Our results, on the other hand, refute Morton (1968)'s treatment of *Amphipterum*

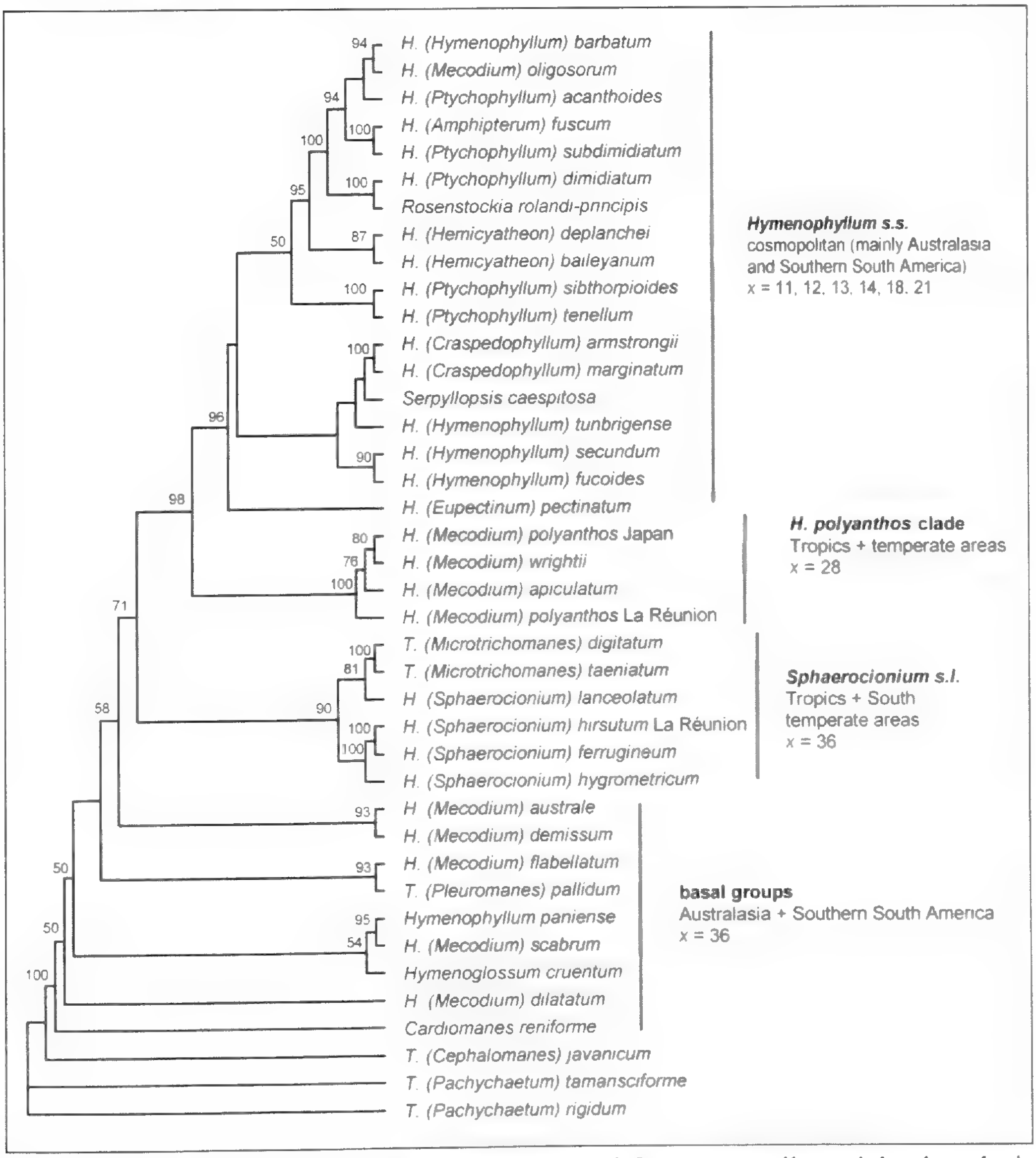

Figure 1. Single most parsimonious tree recovered from unequally weighted analysis of combined *rbcL* + *rbcL-accD* + *rps4-trnS* and morphological and cytological characters; tree length = 6056.93 steps, CI = 0.6178, RI = 0.6105 (Farris, 1989). Numbers at nodes are bootstrap values > 50%.

as a subsection in the subg. *Mecodium.* The remaining species of *Hymenophyllum* s.s. display a very variable soral morphology and various chromosome numbers. The acquisition of new cytological data, currently under study (Ebihara and Hennequin) may provide further insights into the relationships of these taxa.

With more than 100 species, *Mecodium* is a diverse putative subgenus whose monophyly had never been questioned (apart from the position of *Amphipterum*), mainly because it was considered to exhibit a high morphological homogeneity. Nevertheless, the subgenus appears characterised rather by the absence, than by the presence, of peculiar characters, such as margin denticulation or hairs on fronds. Our results confirm that *Mecodium* is polyphyletic as suggested by Hennequin *et al.* (2003). Several *Mecodium* species form a derived well supported (BS = 100%, PP = 1) clade, sister to *Hymenophyllum* s.s. (BS = 98%, PP = 1). This clade, named *H. polyanthos*

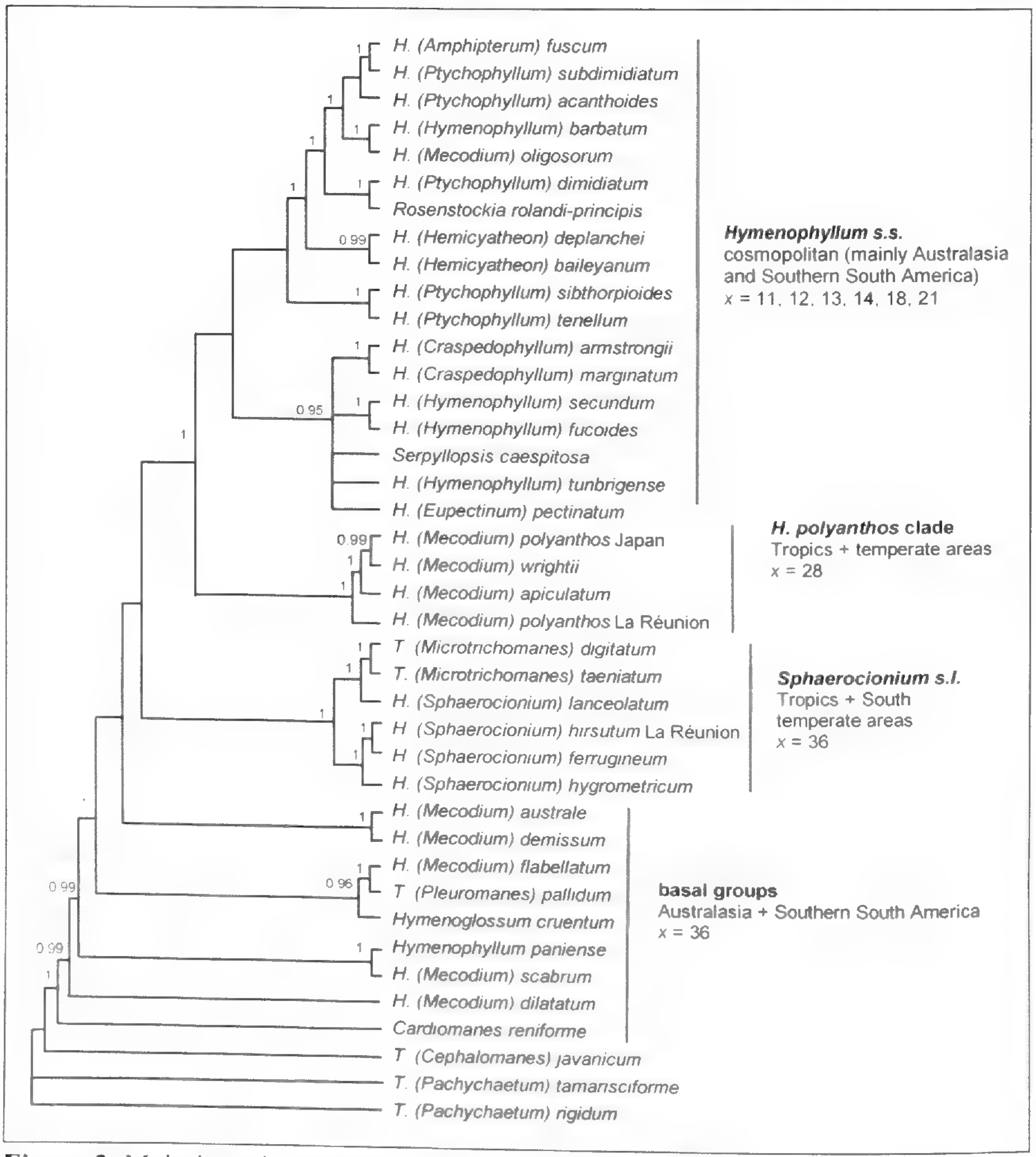

Figure 2. Majority-rule consensus tree of the 9,450 trees sampled during the Bayesian analysis of combined *rbcL* + *rbcL-accD* + *rps4-trnS* and morphological and cytological characters. Numbers at nodes are Bayesian posterior probabilities > 0.90.

clade based on one of the type species proposed for *Mecodium*, *H. polyanthos* (Sw.) Sw. (Morton, 1968; Pichi Sermolli, 1977; Iwatsuki, 1984, 1990), is supported by one exclusive apomorphic character state, the basic chromosome number $x = 28$. The *H. polyanthos* clade is distributed throughout the Neotropics, the Paleotropics and into temperate regions (Japan). Another species of subg. *Mecodium*, *H. oligosorum*, is nested within *Hymenophyllum* s.s. This position is in agreement with Iwatsuki (1984) and is notably supported by cytological data ($x = 21$; Tatuno and Takei, 1969). The remaining species of *Mecodium* included in our sampling are retrieved at the base of the tree in a grade comprising also the genera *Cardiomanes* and *Hymenoglossum*. These basal taxa can be distinguished from the *H. polyanthos* clade by characters that appear plesiomorphic for the genus, *i.e.* the chromosome number $x = 36$, also present in *Sphaerocionium* s.l., a stele where internal xylem is reduced in comparison to the massive protostele (reduced or dorsi-ventral stele) but not subcollateral as in the other clades of the genus, and a tendency to display a rougher habit than the other species of *Hymenophyllum* (thicker rhizomes, large fronds, and for some species a thicker (2-4 cells thick) lamina). In addition, these basal species have a typical austral distribution (Chile, New Zealand, Australia, New Caledonia).

Species of the subg. *Sphaerocionium* are all retrieved in a clade named *Sphaerocionium* s.l. (BS = 90%, PP = 1), which includes in addition some species of the problematic section *Microtrichomanes*. This clade appears sister to *Hymenophyllum* s.s. + *H. polyanthos* clade (BS = 71%, PP = 0.65). The monophyly of *Sphaerocionium* was not questioned, as it was characterised by distinctive stellate hairs at the lamina. Morton (1968) divided his subgenus in two sections: *Sphaerocionium* and *Apteropteris*, and according to analyses not shown here (Hennequin, 2004) the latter is embedded in *Sphaerocionium* s.l. *Microtrichomanes* was placed by several authors in *Trichomanes* but Copeland (1938) suggested an affinity between this taxon and *Sphaerocionium*. Our results corroborate his hypothesis and they are discussed in more detail in a related study (Ebihara *et al.*, 2004). *Sphaerocionium* s.l. is a pantropical clade, with some taxa expanding in southern temperate areas. It is supported by several apomorphic character states, the most conspicuous being the presence of marginal setae, with stellate marginal hairs in *Sphaerocionium* and unicellular marginal hairs in *Microtrichomanes*. It has been proposed that the latter hair type is the result of a reduction from stellate hairs (Copeland, 1938; Holttum, 1963).

Craspedophyllum and *Hemicyatheon* are small subgenera, made up of only two species. The monophyly of both subgenera is confirmed here, but there is no support for their treatment at the subgeneric level. A treatment as sections, as realised by Iwatsuki (1984, 1990), appears more appropriate.

CONCLUSIONS AND PROSPECTS

The results obtained in this study corroborate those previously obtained on a reduced sampling and using less data (Ebihara *et al.*, 2002; Hennequin, 2003; Hennequin *et al.*, 2003; Ebihara *et al.*, 2003). New results include the monophyly of the two species of subg. *Craspedophyllum* and the confirmed polyphyly of subg. *Mecodium*. The analyses based on three molecular markers combined with morphological and cytological data provide much better support for the clades previously obtained, although robust support is still lacking for the clades retrieved within *Hymenophyllum* s.s. and for the basal relationships within the *Hymenophyllum* lineage. By exploring the branch-length distribution, we recognized the short distances between clades in the poorly supported

parts of the phylogeny. This could be either explained as the result of rapid radiations or through low mutation rates. Hopefully, by broadening the taxonomic sampling and adding more molecular, morphological and cytological data, we will be able to improve our understanding of the complex history of the genus.

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MICRO-FUNGAL PTERIDOPHYTE PATHOGENS

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Key words: Uredinales, rust fungi, pteridophytes.

ABSTRACT

Of the 225 genera of pteridophytes listed in Kubitzki (1990) there are 131 (58%) genera with no known fungal association, according to the most comprehensive fungal database. The remaining 94 genera are represented by 524 taxa at the species and subspecies level which form about 1848 mainly parasitic interactions with 822 fungal taxa. Around 450 of these interactions are parasitic associations with rust fungi (Uredinales, Basidiomycetes), which are represented by four genera (and two form genera) and around 130 species and subspecies. Fungal synonymies have been resolved as far as possible, however, for this presentation pteridophyte synonymies have only partly been resolved, due to my lack of experience with ferns.

This paper examines the taxonomic distribution of fern - fungus interactions in general and the importance of the fern rusts in particular. Examples of interactions are illustrated with the aim of raising awareness among pteridologists and mycologists.

INTRODUCTION

Pteridophytes are not normally associated with plant disease problems, and, whilst the current information is far from complete, it appears that many genera are relatively free from fungal pathogens. The rust fungi (Uredinales) are the most important pathogens affecting ferns (only one record of rust for Lycopodiatae, none for Equisetatae), with around 100 species in two rust families (Cummins & Hiratsuka, 2003). The largest genus is *Milesina* with 34 recognised species and 22 species of the form genus *Uredo* (asexual form only) believed to belonging to this genus. Together with *Uredinopsis* (26 species and 2 form species) and *Hyalopsora* (7 species and 5 possible form species) it belongs to the family Pucciniastraceae and uses species of *Abies* as alternate hosts (Hiratsuka, 1958). The fourth genus, *Desmella*, is a member of the Uropyxidaceae, infecting ferns in South and Central America. It is only known from its uredinial and telial stages (no known alternate host).

MATERIALS AND METHODS

For this study the herbarium records and database of the US National Fungus Collection at Beltsville (BPI) (Farr et al. 2004) and other online herbaria were searched, and selected specimens were examined using light and scanning electron microscopy. Over 2500 records were processed, their synonymies cleared as far as possible, and results plotted against relevant host data.

Table 1. The twenty most common genera of microfungi on ferns and fern allies.

Fungal genus	**Fern hosts**
Milesina	118
Uredinopsis	69
Uredo	60
Hyalopsora	55
Cercospora	47
Mycosphaerella	42
Desmella	29
Taphrina	28
Phyllosticta	27
Rhizoctonia	25
Pleospora	24
Pythium	24
Phaeosphaeria	22
Leptosphaeria	21
Trichopeltheca	18
Pseudocercospora	16
Clathrospora	14
Phytophthora	14
Dasyscyphus	13
Fusarium	13

Table 2. The 20 pteridophyte genera, most commonly attacked by microfungi.

Pteridophyte genus	**Fungi**
Pteridium	116
Athyrium	100
Dryopteris	96
Equisetum	73
Cibotium	58
Cyathea	58
Pteris	54
Selaginella	47
Polypodium	44
Adiantum	43
Lycopodium	38
Asplenium	35
Osmunda	35
Polystichum	33
Blechnum	29
Nephrolepis	28
Dicksonia	27
Rumohra	27
Platycerium	20
Matteuccia	19

RESULTS

Tables 1 and 2 show the 20 most common genera of microfungi found on ferns and fern allies, and the 20 pteridophyte genera most commonly attacked by microfungi respectively. The most commonly recorded fern pathogens are the rust genera *Milesina*, *Uredinopsis* and *Hyalopsora*, together with the anamorphic form genus *Uredo*. Several fern families are host to all the fern rust genera, whereas others are restricted in their susceptibility, such as the Vittariaceae, Lomariopsodaceae (only one rust recorded respectively) and Schizaeaceae (probably all restricted to *Desmella*), Aspleniaceae and Davalliaceae (restricted to *Milesina*).

Of particular interest is the record of *Uredo vetus* on *Selaginella* sp. in China, the only record of a rust on Lycopodiatae (Hennen, 1997). Conversely, there appears to be no obvious pattern in the distribution of fern families as host plants of the four rust genera.

Key to the fern rusts:

1 only urediniospores present *Uredo*
1' teliospores present 2
2 teliospores external, 2-celled on pedicells *Desmella*
2' teliospores formed inside host epidermal cells 3
3 urediniospores more or less lanceolate, smooth or with a few lines of coglike warts *Uredinopsis*
3' urediniospores usually echinulate or verrucose, orange pigment present in cytoplasm *Hyalopsora*
3'' as above, no pigment present *Milesina*

CONCLUSIONS

At present, there is only a limited database on the distribution and frequency of parasitic microfungi on ferns. Further collecting should be encouraged and pteridologists will play a crucial role in this endeavour.

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PHENOLOGICAL ASPECTS OF FROND PRODUCTION IN *ALSOPHILA SETOSA* (CYATHEACEAE: PTERIDOPHYTA) IN SOUTHERN BRAZIL

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Key words: phenology, frond production, growth rates, ecology, spore production.

ABSTRACT

Two populations of *Alsophila setosa* Kaulf. in secondary semi-deciduous subtropical forest remnants in the State of Rio Grande do Sul, Brazil were studied with attention to frond formation, expansion and senescence rates, as well as to phenology of sporangia formation and spore release, during a 15 month period. Plants of various sizes were marked at a site at Morro Reuter (45 plants) and another at Sapiranga (48 plants) municipalities. The average frond production rates were 5.51 fronds/year at Morro Reuter, and 4.14 fronds/year at Sapiranga. After frost occurrence in early winter, all the exposed young croziers were irreversibly damaged with necrosis of the tissues. A new set of croziers was formed in October (spring), with all the croziers uncoiling almost simultaneously, 84.4% of the specimens in Morro Reuter and 66.7% in Sapiranga presenting one or more croziers in the initial expansion stages. The senescence rates were 6.97 fronds/year at Morro Reuter, and 4.33 fronds/year at Sapiranga. Low temperatures (including the occurrence of frost) and low rainfall during winter coincide with the highest frond senescence, with some plants losing all the fronds. The species presents the capacity to compensate for the occasional loss of all the young fronds in a short period of time, keeping the number of fronds relatively stable at a given development stage. The data indicate ecological limits to the occurrence of this species in Southern Brazil. Spore production occurred only in a few plants, which were at least 2.5m tall. Spore formation is seasonal and maturation gradual to irregular even in a single frond.

INTRODUCTION

Information on reproductive aspects and growth patterns of ferns are relevant for a better understanding of their role in forest formations. Ferns may set patterns in early succession stages as well as influence the establishment of other species in regeneration processes. Interesting data discussed by Coomes *et al.* (2005) suggest that the competition of the ground layer herbs may reduce regeneration opportunities for some seed plants. However, tree ferns and other caudex forming ferns may act as substrate for seed germination and initial establishment of certain woody angiosperms and gymnosperms, thus having an important role in their regeneration.

Biological data are also important for the conservation effort for endangered species, such as the tree ferns which are subject to commercial (even if illegal)

exploitation. Some species are the source of fibrous material formed by the adventitious root mass covering the caudex which is used as substrate for growing orchids and aroids, while others have plants removed from the field and used in landscaping, decoration or as shelter building material. Even with the adherence to the CITES agreements restricting the trade, local use still represents a major pressure on some fern populations.

Ecological data for Neotropical ferns are mostly found dispersed in the taxonomic and floristic literature and generally only of descriptive nature. There is a lack of studies as to their reproduction, growth and development. Among the groups that need urgent attention are the tree ferns (Dixit, 1986). *Alsophila setosa* Kaulf. (Cyatheaceae), one of the species currently extracted from primary and secondary semi-deciduous subtropical forests in the State of Rio Grande do Sul, Brazil (Windisch, 2002; Schmitt & Windisch, 2005), is the object of the present study.

In southern Brazil *Alsophila setosa* is found in the mixed humid forests with *Araucaria* as well as in semi-deciduous forests. The removal of plants is not only a problem for the conservation of this species but also leads to the alteration of the forest formations. In contrast, *Dickonia sellowiana* Hook. is not under protection by any local or national legislation. Even if not used for tree-fern fibre extraction, its aesthetic properties make it an object of commercial exploitation for ornamentation, including church decoration for weddings in certain localities (Windisch, 2002). The caudexes support several epiphytic species, which themselves may also face conservation problems due to the disappearance of the sporophyte. In the present study the frond formation of *A. setosa* has been followed *in situ* in order to establish frond production, expansion and senescence rates, as well as the phenology of sporangial production and spore release.

MATERIAL AND METHODS

Two populations were studied, one in the municipality of Morro Reuter at 29° 32' S and 51° 04' W, 700m alt., another in Sapiranga at 29° 38' S and 51° 00' W, 570m alt., from May 2000 to August 2001. These localities present secondary semi-deciduous seasonal forest formations.

The regional climate is subtropical with the absence of drought. Average compensated monthly temperatures are normally under 15°C during four months of the year, this cold period being a determinant for physiological seasonality (Teixeira *et al.*, 1986). The closest Meteorological Station (Ivoti, 127m alt, data for the years 2000-2001) indicated 13.3° C as the average of the coolest month (July) and 25.1° C for the warmest (February), absolute minimum of –1.0° C (July) and maximum of 35.7° C (September and January). At the Morro Reuter, local residents registered –5°.0 C in the winter of 2000. During the period of the present study, precipitation data for Ivoti indicated 2138mm yearly rainfall, with less during May (average 59.4 mm), and maximum in September (232.2mm), October (303.9 mm) and January (308.6mm). Data for an extended period of time could not be obtained. Frost may occur several times during some winters.

Monthly visits were made in order to follow the frond production phenology of 45 plants in Morro Reuter and 48 plants in Sapiranga. The plants were selected and marked by closely placed numbered stakes in the ground. During the 15-month period crozier formation, frond maturation and senescence were observed on every visit. At the beginning of the survey the youngest crozier was marked by a loose loop of plastic line

(not interfering with the crozier development), and the marked crozier serving as a reference in relation to the existing fronds and subsequently produced ones. In mature plants sporangia formation and spore release were recorded. The selection of two populations was decided upon in order to guarantee results in case of predatory extraction in one of the sites. The 15 months observation period ensured data for a complete seasonal growth-year.

Soil samples were collected for NPK macronutrients analysis, with phosphorous and potassium measured by atomic emission spectrophotometry and nitrogen by standard nitrometric methods (Greenberh, 1992; Page *et al.*, 1982). Statistical analyses were performed using methods described by Vieira (1980) and Watt (1998) and through the SPSS 9.0 software program (SPSS Inc., Chicago IL, USA) at the Universidade do Vale do Rio dos Sinos data processing facility.

RESULTS AND DISCUSSION

Alsophila setosa is an understory arborescent plant, forming an erect caudex up to 10m tall and up to ca. 10cm wide, covered by the spiny remnants of frond bases (and dry frond remnants at the distal part), with a crown of up to 3m long, and fronds which are tripinnate-pinnatisect at the base. The stipe is ascending, with blackish spines, presenting 2-4 pairs of aphlebia (with laminar tissue) at the basal portion.

The Morro Reuter soil samples showed an average of 0.35 (±0.20)% nitrogen, 4.5 (±1.36) ppm phosphorous, and 175.16 (±51.09) ppm potassium. The average values for the Sapiranga samples were 0.36 (±0.15)% nitrogen, 3.8 (±0.69) ppm phosphorous and 229.33 (±92.81) ppm potassium. Soil analyses demonstrated heterogeneity in macronutrients composition (NPK) among sites of each locality, but the application of t-test for independent homogenized samples indicated that contents of nitrogen ($P = 0.981$), phosphorous ($P = 0.440$) and potassium ($P = 0.282$) in the soil are statistically equal in both localities. Although the macro-nutrients (NPK) did not show significant differences, considering the probability of different histories of soil usage in the two sites, differences in micronutrients may be expected between the study sites.

The average yearly frond production in the samples was 5.51 (±3.55) fronds/plant for Morro Reuter and 4.14 (±1.93) fronds/plant for Sapiranga, the difference being statistically significant ($P = 0.024$). After the occurrence of frost between May and the beginning of July 2000, all young croziers at the apex of the caudex were damaged, with necrosis of the tissues, keeping the frond production at zero until August (Fig. 1) in both populations.

With the arrival of the spring season, new croziers were formed and fronds started to expand, almost simultaneously, with 84.4% (Morro Reuter) and 66.7% (Sapiranga) of the specimens presenting one or more croziers in the initial phases of expansion. The largest frond production occurred during the period of higher rainfall (Fig. 1), with the peak in October (4.4±3.80 in Morro Reuter and 1.89±1.90 fronds/plant in Sapiranga). These observations agree with Luederwaldt (1923), who commented that in Southeastern Brazil new fronds of *Alsophila setosa* (cited as *Hemitelia setosa*) started to bud in the spring, approximately by the end of October. Shreve (1914) observed that *Cyathea pubescens* Mett. ex Kuhn, in humid mountain forests of Jamaica, formed new fronds during the winter and spring. Tanner (1983) working in Jamaica observed that the increase in the frond production of *C. pubescens* occurred in October, November and December after an increase in rainfall. Durand & Goldstein (2001) observed the native species of genus *Cibotium* Kaulf. in Hawaii produced most of their fronds

between February and April, characterizing a marked seasonality, which does not seem to be related with the rainy season. Seiler (1981) registered that the frond production in *A. salvinii* Hook. was not synchronous, but occurred at the end of the dry season and the beginning of the humid season. Data on the frond production of *Dicksonia blumei* Moore from Java was presented by Jaag (1942), but unfortunately not covering a complete growth year. The same author marked plants of *A. glauca* J. Sm, evaluating the production and life-span of fronds, with the complete renewal of the crown occurring between 182 and 254 days.

The 55 croziers marked at Morro Reuter expanded on average 5.41 cm/day between October and November, 0.93 cm/day between November and December. From December to January 2001, 31 fronds continued to increase in length, but at a much slower rate (0.083 cm/day). In the fourth month only 11 fronds still presented some expansion (0.07 cm/day average). The maximum expansion rate was 7.48 cm/day between October and November 2000. The 31 croziers marked at Sapiranga expanded on average 5.38 cm/day between October and November, 1.24 cm/day between November to December. From December to January 22 fronds increased in length, with an average of 0.092cm/day. From January to February 2001 only eight fronds continued expansion with an average of 0.037cm/day. These data agree with the observations by Shreve (1914), who described different growth/expansion rates in different stages. The highest expansion rate presented in his study, 4.94 cm/day for *Cyathea pubescens* growing in the humid mountain forests of Jamaica, comes close to the values recorded in the present study.

The average total number of fronds varied throughout the year. In Morro Reuter the smallest average was of 3.04 mature fronds/plant (October 2000), and the maximum 7.46 mature fronds/plant (May 2000). Comparing the average number of fronds in May

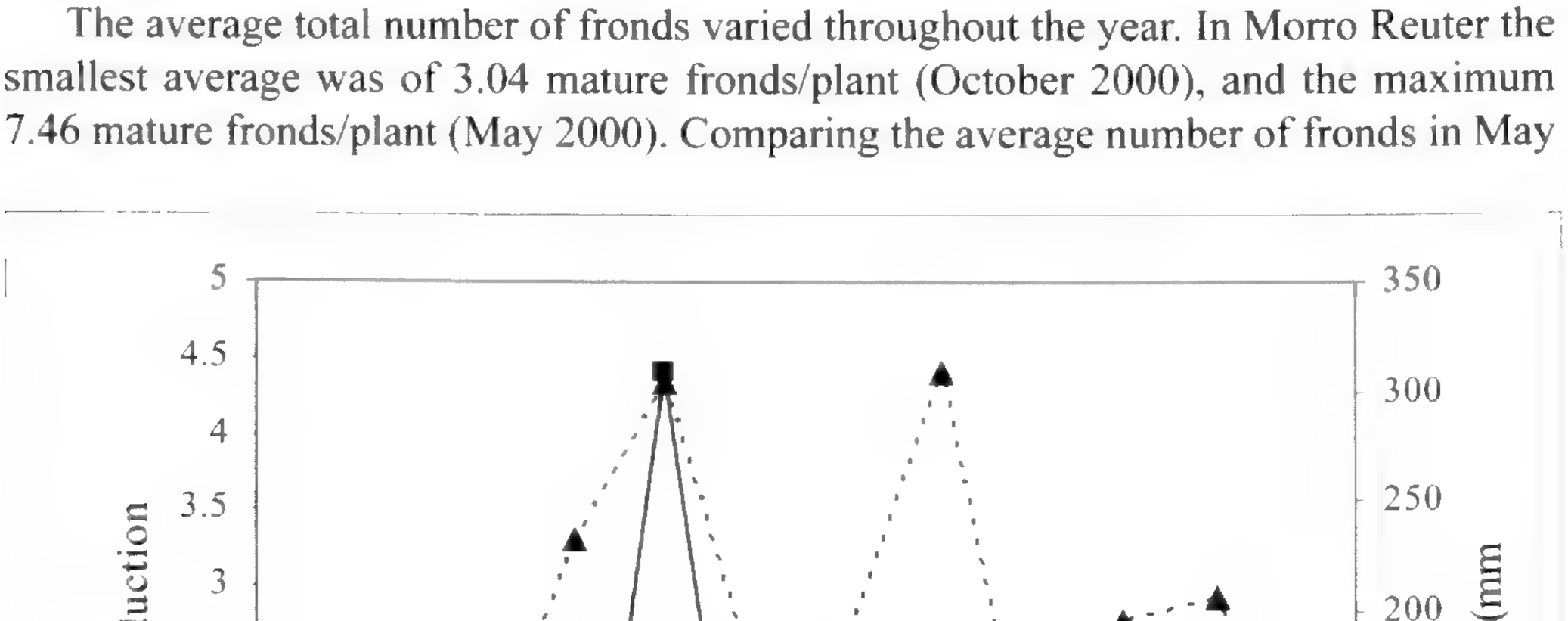

Figure 1. Average monthly frond production of *Alsophila setosa* populations in Morro Reuter and Sapiranga compared with precipitation (June 2000 to May 2001).

2000 with that in May 2001 (5.77 mature fronds/plant) a statistically significant difference can be observed ($P < 0.0001$, $n = 45$). In Sapiranga, the smallest average was of 3.68 mature fronds/plant (October 2000), while the highest value was 5.95 mature fronds/plant (December 2000). Comparing the average number in June 2000 (5.62 mature fronds/plant) with that of June 2001 (5.33 mature fronds/plant), the values are statistically equivalent ($P = 0.644$, $n = 48$).

The average yearly senescence rate of fronds in Morro Reuter was 6.97 fronds/plant, being superior to the new frond production rate. In Sapiranga, the plants presented an average yearly senescence rate of 4.33 fronds/plant, about the same as the new frond production rate (Fig. 2). Our field observations indicate that with the intense cold and frost occurrences, the fronds initially presented loss of photosynthetic surface, by partial damage to parts of the laminar tissue, followed by the total drying out of the fronds (Schmitt & Windisch, 2001). Reduction of the mature fronds was gradual, with eight individuals in the total sample (both localities) losing all of their fronds.

In the Sapiranga population, the similar yearly average production and senescence of fronds indicates the maintenance of a constant number of fronds from one year to the next. The significant reduction of the total number of mature fronds in Morro Reuter, comparing data from May 2000 and May 2001, may be due to different local conditions including exposure to frost. The canopy of the forest in Morro Reuter is more open than that of Sapiranga, increasing the degree of exposure. These data may also indicate ecological limits for the occurrence of this species in Southern Brazil. *Alsophila setosa* presents the capacity to compensate for the occasional loss of all the young fronds in a short period of time, and so the number of fronds is kept relatively stable at a given development stage, although in a few cases no new fronds were produced.

The production of new fronds and total number of fronds are similar to those found in the literature for other tree-ferns (Table 1) in other parts of the world. Our data for

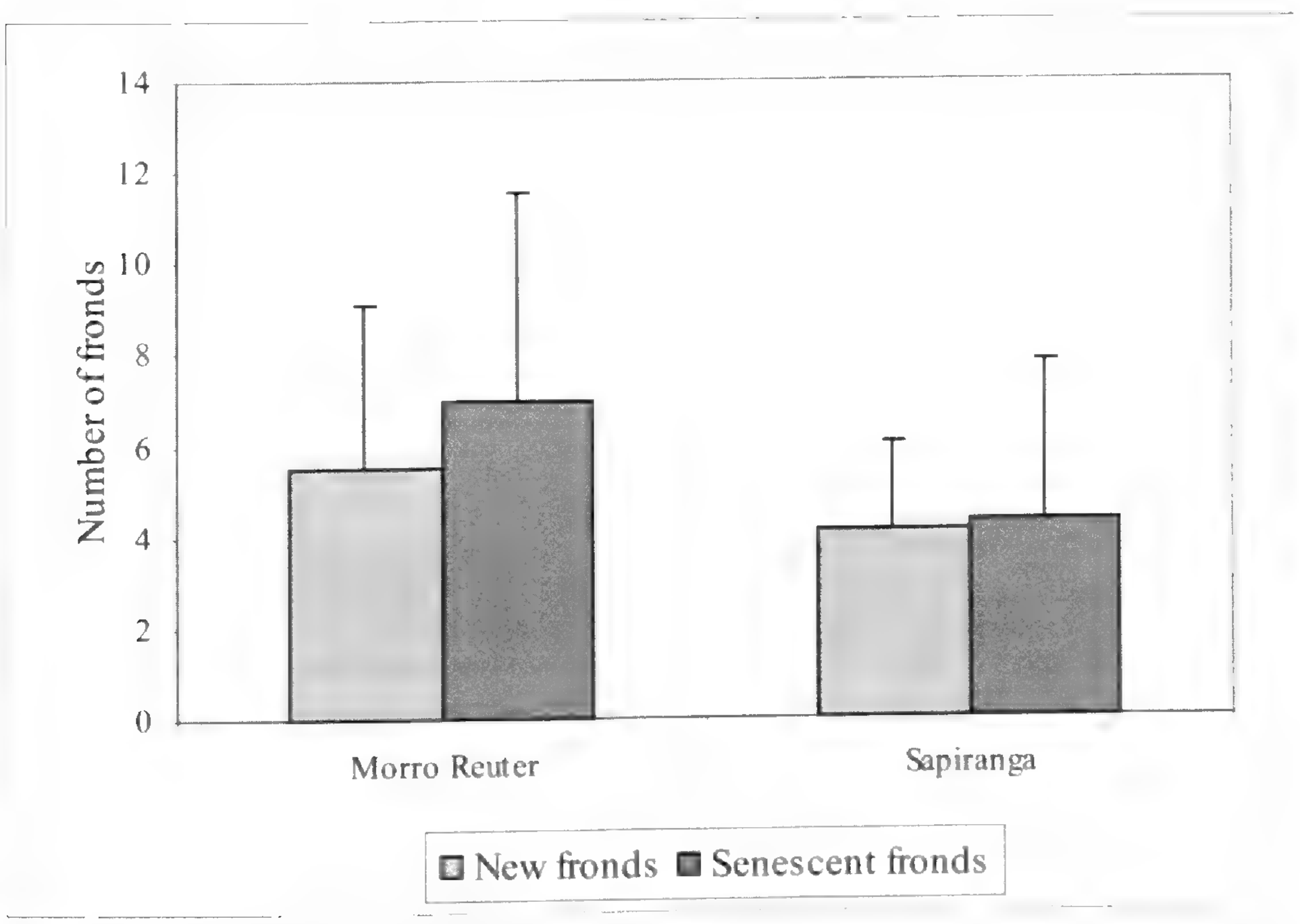

Figure 2. Average yearly frond production and senescence in the Morro Reuter and Sapiranga populations of *Alsophila setosa* (standard deviation indicated).

Alsophila setosa seem to be the first record of total loss of fronds. Low temperatures (including frost occurrence) and low rainfall during the cold season coincide with the loss of fronds. Seasonality in the loss of fronds was also observed in other tree ferns such as *A. salvinii* (Seiler, 1981), *Cibotium glaucum* (Sm.) Hook. (Walker & Aplet, 1994), while it was less pronounced in *Cyathea pubescens* (Tanner, 1983) and *C. hornei* (Baker) Copel. (Ash, 1987).

Herbivory was observed in some fronds, but always partial and with preference to young fronds. No total loss of fronds due to herbivory was observed. It was not possible to identify the herbivore. Ants of the genus *Iridomyrmex* Mayr found on the plants probably only use cavities at the stipe bases for nesting.

In Morro Reuter 8.88% of the plants produced fertile fronds, but only 2.08% in Sapiranga. All the fertile plants had caudexes at least 2.5m in length. Most of the fertile fronds presented developing sporangia between February and March, while in April the liberation of spores had already started. Spore maturation and liberation was gradual and irregular even with respect to the position on a single frond. Rosenstock (1907) observed that *Alsophila setosa* plants less than 8m tall are normally sterile. The low fertility values observed are related to the age structure of the populations, as there is a predominance of younger plants. Similar observation was made by Young & León (1989) in the Peruvian Amazon region, where only two specimens of *Trichipteris nigra* (Mart.) R. M. Tryon, from a total of 25 plants, were fertile and those were at least 5.5m tall.

In *Alsophila setosa* vegetative reproduction by underground structures (Schmitt & Windisch, 2005) seems to be quite effective to allow for a low production of fertile

Table 1. Production of new fronds and total number of fronds in tree-ferns.

Locality	Species	Fronds/plant	Fronds/year
Java (Tjibodas)[a]	*Alsophila glauca*	6-12	6-13*
El Salvador[b]	*Alsophila salvinii*	6	2.5
Jamaica[c]	*Cyathea pubescens*	7	8
Fiji[d]	*Cyathea hornei*	3-11	3-9
Hawaii[e]	*Cibotium glaucum*	5-16	3-5
Brazil (Morro Reuter, RS)[f]	*Alsophila setosa*	0-21	0-14
Brazil (Sapiranga, RS)[f]	*Alsophila setosa*	0-17	0-11

[a] Jaag 1942, [b]Seiler 1981, 1995, [c]Tanner 1983, [d]Ash 1987, [e]Walker & Aplet 1994, [f]present study, *data from 10 month observation period.

fronds. Page (1979) pointed out that spore production might be low when a plant is in vegetative growth or high when the plant is under more severe ecological conditions, competing with other plants. Furthermore, Sato (1982) suggested that the cold climate has a restrictive effect on frond expansion as well as in the spore production period. The present data for *A. setosa* seem to agree with that suggestion. The gradual spore liberation over a period of time may be a positive aspect for species survival, allowing for higher chances of dispersal to new recently exposed microhabitats (Ranal, 1995).

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CONSERVATION OF TWO ENDANGERED FERNS, *ARCHANGIOPTERIS SOMAI* AND *A. ITOI* (MARATTIACEAE: PTERIDOPHYTA), BY PROPAGATION FROM STIPULES

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Key words: *Archangiopteris itoi*, *Archangiopteris somai*, conservation, Marattiaceae, endangered plants, stipule propagation, Pteridophyte, reproduction.

ABSTRACT

Archangiopteris somai Hayata and *A. itoi* Shieh are ferns endemic to Taiwan and are categorized as endangered and critically endangered species respectively. Five fresh stipules were removed from each of 10 sporophytes of *A. somai* and *A. itoi* growing in Wu-lai, northern Taiwan. After rinsing in clean water and placing on medium (4:1, soil: peat moss) 50 stipules of each species were cultured at room temperature with 12 hr fluorescent light each day. After one year plantlets were produced by 40% of *A. somai* stipules and 90 % of *A. itoi* stipules. Within each species, the mean sprouting rate and sprouting time of stipules from stems of different sizes did not differ significantly. Sprouting and non-sprouting stipules were not significantly different in size. The relationship between average sprouting time and stipule size was very weak (*A. somai*) or non-existent (*A. itoi*). The growth of the mother plants from which stipules were stripped was not significantly different from their growth in the previous year, nor did it differ from the growth of control plants. This simple method of propagation from stipules provides an effective means of propagating these two species for horticulture, *ex situ* conservation and *in situ* restoration.

INTRODUCTION

Archangiopteris Christ & Gies. is recognized as one of the ancient lineages of pteridophytes. Eleven species of *Archangiopteris* have been found in southeast China, northern Vietnam and Taiwan (Ching, 1958). Most taxa are endemic to these areas. The origin of *Archangiopteris* can be traced back to the Middle Jurassic period in the fossil record (Hill & Camus, 1986). This genus is phylogenetically closely related to *Angiopteris* Hoffm., another marattialean genus endemic to Southeast Asia, and *Protomarattia* Hayata, which is restricted to northern Vietnam (Hayata, 1919; Chang, 1975). Extant species of these genera represent relics of an ancient lineage that evolved through several glaciation and vicariance events. As a relic taxon, *Archangiopteris* provides information invaluable for tracing the evolutionary history of eusporangiate ferns, although until recently species of *Archangiopteris* have received little attention (Hsu et al., 2000; Chiang et al., 2002). Furthermore, many species of *Archangiopteris* are rare or endangered.

Two species of *Archangiopteris*, *A. somai* Hayata and *A. itoi* Shieh, have been documented in Taiwan (DeVol & Shieh, 1994). Both species are endemic. *Archangiopteris somai* is an endangered species and *A. itoi* is critically endangered (Kuo, 1997; Moore, 2001). Only two populations of each species have been reported,

one of each in Wulai, in northern Taiwan, and one of each in Lienhwachi, in central Taiwan. However, the population of *A. itoi* in Lienhwachi is known only from the original collection on which the species description is based, with no recent records from this site. Population sizes of both species are very limited, with c. 1000 individuals of *A. somai* and less than 100 of *A. itoi*, and in the field young sporophytes are very rare. *Archangiopteris somai* gametophyte growth is very slow and after 2.5 years in culture only about 1% of gametophytes produced sporophytes (Chiou and Huang, unpublished). For *A. itoi*, spore germination and gametophyte culture have never been documented. In addition, there are no reports of tissue culture of the sporophytes of either species. Thus, conservation of these two endangered ferns is critical.

Species within the Marattiaceae produce stipule buds and although expansion of these is rarely seen in the field (for example in *Danaea wendlandii*, Sharpe & Jernstedt 1991) some horticulturists have attempted to propagate marattialean ferns from stipules (Hoshizaki & Moran, 2001; Jones, 1987). To aid their conservation, an attempt was made to propagate the two species of *Archangiopteris* from stipules. Sprouting rate and sprout time were documented and the effect of stem and stipule size on sprouting analyzed. The morphology of young fronds was studied to assist further investigation in the field. Propagation from stipules proved to be an easy and effective method of propagating both species for conservation and horticulture.

MATERIALS AND METHODS

Because *Archangiopteris somai* and *A. itoi* are so rare, great care has to be exercised in using any of these plants or their parts for experimentation and propagation. First we tried to remove above ground stipules, but found they were firmly attached to the stems. To avoid damaging the "mother plants", we removed underground stipules, which were not firmly attached to the stems, from sporophytes of the population in Wulai. For each species, five stipules were removed from each of 10 sporophytes. As soon as they were harvested, stipules were sealed in plastic bags to prevent dehydration. In the laboratory the stipules were rinsed for three minutes with clean water and then placed on, and half-covered by, medium (4:1, soil: peat moss) in plastic boxes. All cultures were maintained under white fluorescent illumination (24 μmole $m^{-2}s^{-1}$, 12 h/d) at 20 to 28°C.

The effects of mother plant size (stem diameter) and stipule size (width x length) on sprouting were analyzed (Table 1). Stipules were examined for sprouts every month for one year. In addition, the plants from which stipules had been removed were monitored throughout the experiment to determine whether stipule removal affected plant growth.

RESULTS

The stems and stipules of *Archangiopteris itoi* were significantly larger than those of *A. somai*. Plantlets began to sprout from *A. itoi* stipules after three months in culture and sprouting peaked at 90 % after seven months. For *A. somai*, plantlets first sprouted after four months in culture. Cumulative sprouting increased slowly throughout the year to 40 % (Fig. 1). The stipules of *A. itoi* had a higher sprouting rate and shorter sprouting time than stipules of *A. somai* (Table 1).

Within each species, stem size did not affect sprouting rate significantly. However, the sprouting rate of stipules taken from the small stems of *A. somai* was somewhat higher than the sprouting rate of stipules removed from large stems. Similarly, stem size did not affect sprouting time significantly, but there was a tendency for stipules from small stems of *A. somai* to produce plantlets more quickly than stipules from large

Table 1. *Archangiopteris somai* and *A. itoi* stem diameter, stipule size, and sprouting rate and time.

	A. somai	*A. itoi*	t-test[1)]
Diameter of stem (cm)	4.1±0.9	8.6±1.9	**
Stipule size			
Width (cm)	1.7±0.3	2.7±0.6	**
Length (cm)	2.3±0.4	3.4±0.5	**
Width x Length	3.9±1.3	9.2±3.0	**
Sprouting rate (%)	40.2±21.1	90.0±4.1	**
Sprouting time (mo)	7.2±2.4	4.6±1.1	**

[1]All comparisons between these two species were significantly different ($P < 0.01$).

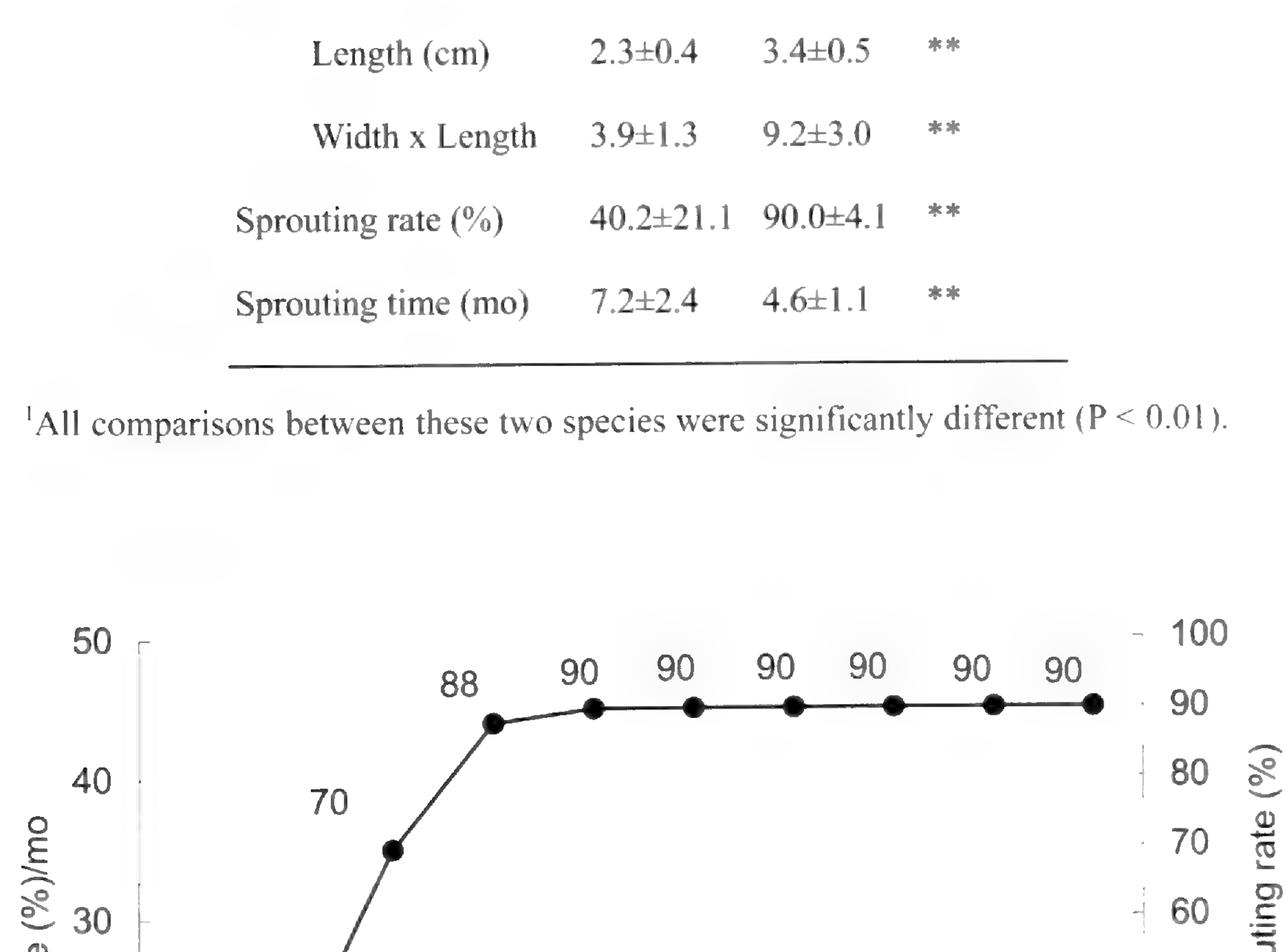


Figure 1. Sprouting rates (left axis, vertical bars) and cumulative sprouting rates (right axis, curves) of *Archangiopteris somai* (grey) and *A. itoi* (black) stipules cultured for one year.

Table 2. Mean sprouting rates and times for stipules taken from *Archangiopteris somai* and *A. itoi* stems of different sizes.

Archangiopteris somai

Diameter of stem (cm)	3 (n=3)	4 (n=2)	5 (n=5)
Sprouting rate (%)[1]	60	30	32
	(40-80)[2]	(0-60)	(0-60)
Mean sprouting time (mo)[1]	6.1	7.0	8.4
	(4-12)[2]	(6-8)	(6-11)

Archangiopteris itoi

Diameter of stem (cm)	6 (n=1)	7 (n=1)	8 (n=4)	9 (n=2)	10 (n=1)	13 (n=1)
Sprouting rate (%)[1]	100	60	95	90	80	100
Mean sprouting time (mo)[1]	3.8	5.3	4.6	4.6	4.8	5.2

[1]None of the data in the same row were significantly different (t-test).
[2]Numbers in parentheses are the range of values.

stems (Table 2). For both species, stipules that gave rise to plantlets did not differ significantly in size from stipules that did not produce plantlets (Table 3). For *A. somai* there was a significant, weak, negative correlation between stipule size and sprouting time (Fig. 2). For *A. itoi* the correlation was not significant. The majority of first fronds emerging from *A. somai* stipules were simple fronds (75%), but most first fronds emerging from stipules of *A. itoi* had one to three pairs of pinnae (93 %) (Table 4).

None of the plants from which stipules were removed for the study exhibited obvious signs of injury or damage during the next year. For each plant, growth, the number of new fronds, and the timing of spore production were similar to the year before and the year after stipules were removed. In addition, the growth and other characteristics of plants from which stipules were removed did not differ significantly from the growth of control plants from which no stipules had been removed (data not shown).

DISCUSSION

During the study we observed no expansion of stipule buds for either species, *A. somai* or *A. itoi*, in the field. However, stipules have been used to propagate offspring of

Table 3. The relationship between sprouting status and stipule size in *Archangiopteris somai* and *A. itoi*.

Species	*Archangiopteris itoi*			*A. somai*			
Sprouting status[1]	a	b	t-test	a	b	c	t-test
Stipule size (cm^2)	9.3±3.0	7.6±1.7	ns	3.5±1.1	4.4±1.4	3.7±1.5	ns
Stipule number	45	5		20	24	6	

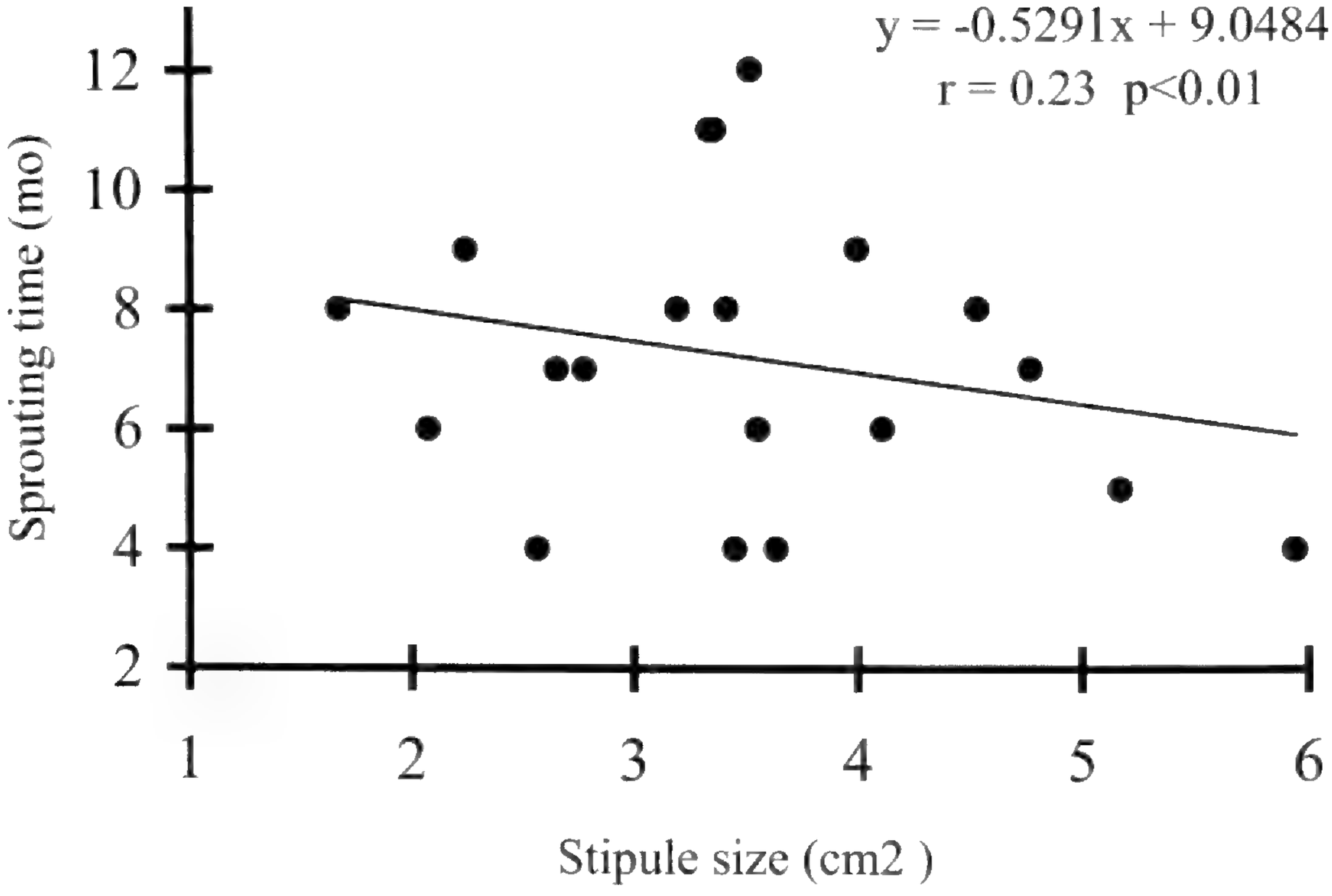
[1]a = sprouting stipules; b = non-sprouting stipules that died in culture; c = non-sprouting stipules alive after one year in culture.

several species in this family. One method for propagation of some marattialean species is to use the stipule buds (Jones, 1987). However, each sporophyte of *A. somai* and *A. itoi* produces only 2 to 5 fronds per year so removing entire fronds could injure or kill these rare ferns. In this study, we removed underground stipules that did not subtend a living frond. Many of these stipules produced plantlets, and their removal did not damage the living fronds or affect the phenological characteristics of the mother plant in the following year.

The sprouting rate and sprouting time for stipules removed from stems of different sizes were not significantly different within the range of stem sizes used in this study. However, given the small population size of both species and our concerns about their conservation, only 10 plants of each species were used in this study. A larger sample size may yield somewhat different results. This is most likely for *A. somai*, where the mean sprouting rate of stipules from 3 cm stems was about twice the rate for stems of other sizes, and sprouting time was about one month shorter. Thus, to propagate *A. somai*, we recommend using stipules from 3 cm stems, especially where parent plants are limited. In contrast, the sprouting rate of *A. itoi* reached 90%, sprouts formed in three to seven months, and the effect of stem size was negligible. Underground stipules from stems of any size appeared to be suitable for propagating this species. Stipule size had no significant effect on the sprouting rate or time of either species. Therefore, it appears not to be a factor in the vegetative propagation of these ferns.

Table 4. Frequency of different types of first fronds emerging from stipules of *Archangiopteris somai* and *A. itoi*.

	A. somai (%)	*A. itoi* (%)
Simple frond	75	7
Frond with one pair of pinnae	20	51
Frond with two pairs of pinnae	5	31
Frond with three pairs of pinnae	0	11



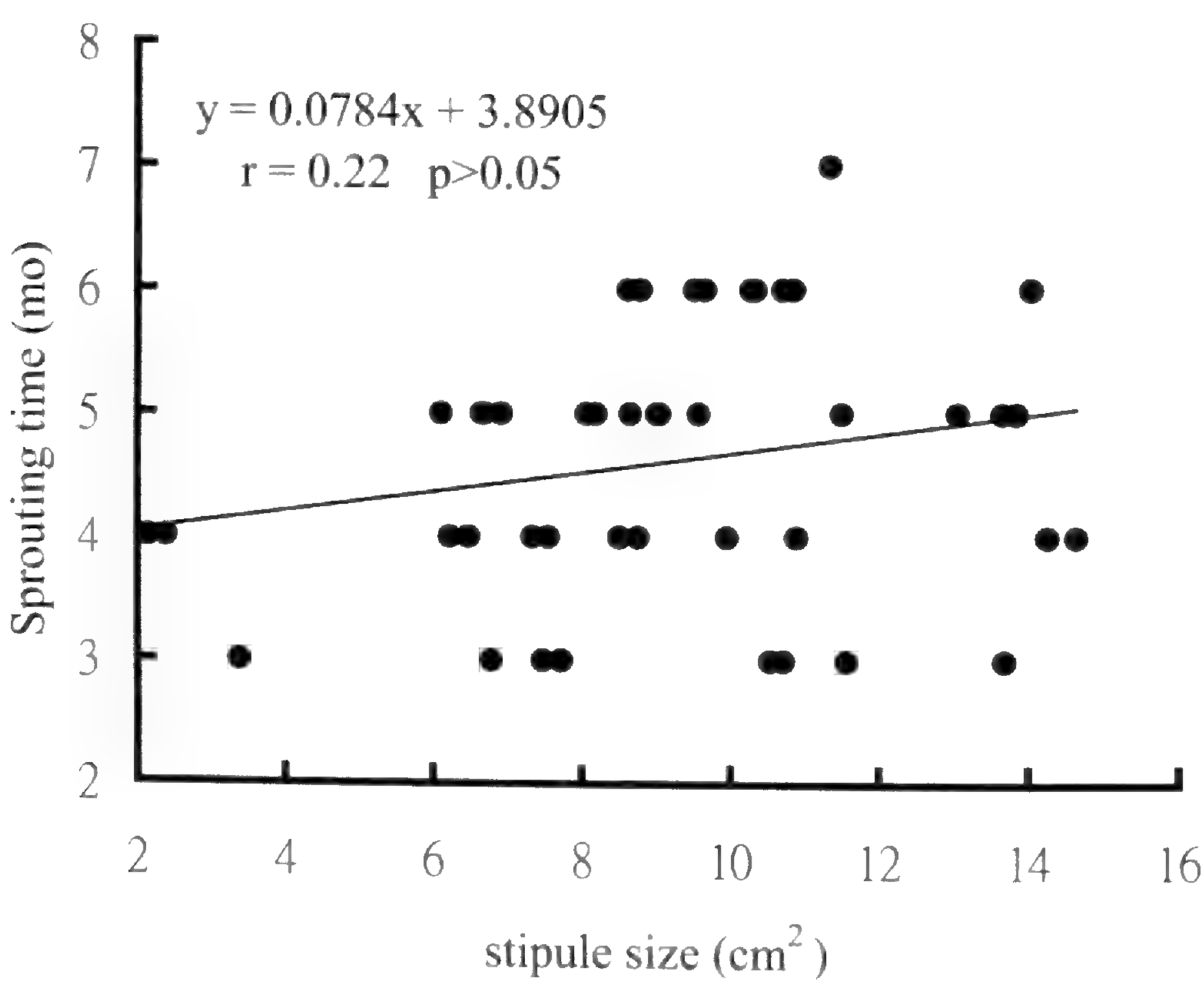


Figure 2. Correlation between stipule size (width x length) and the time for plantlets to sprout from stipules of *Archangiopteris somai* (top) and *A. itoi* (bottom).

Ferns can be propagated from spores under natural conditions and in the laboratory or greenhouse. When spores are limited, ferns can be propagated vegetatively, most commonly from frond buds, but also by tissue culture. *Archangiopteris somai* spores will produce gametophytes. However, the gametophytes grow very slowly and after 2.5 years only 1% had produced sporophytes in multi-spore cultures (unpublished). There are no published reports of spore germination for *A. itoi* and we failed to get the spores of this species to germinate. Propagation of the two species by tissue culture was also unsuccessful (Gen Chang, personal communication). Stipule culture appears to be a feasible and efficient method of propagating fern plantlets for conservation, both *in situ* and *ex situ*, e.g., in a botanical garden (Ranker, 1994). This method also could facilitate the propagation and conservation of other rare species of Marattiaceae.

Another advantage of stipule culture over spore culture is that plants mature earlier. Usually, the first frond of sporophytes produced from gametophytes is simple. About 25% of *A. somai* plantlets produced from stipule cultures had one or two pairs of pinnae and 93 % of the *A. itoi* plantlets had one to three pairs of pinnae. Plantlets with more than one pinna grew faster and reached maturity earlier than plantlets with only a single frond, which is very important for horticulture, *ex situ* conservation and *in situ* restoration.

The primary disadvantage of vegetative propagation is that populations of ferns derived from these plants will have limited genetic diversity. However, based on a study of the *atp*B-*rbc*L intergenic spacer of chloroplast DNA, the genetic diversity of populations of *A. somai* and *A. itoi* is surprisingly high (Chiang et al., 2002). Thus, propagating plantlets from the stipules of a number of different plants will help maintain a large proportion of the genetic diversity of each population.

A further limitation of stipule culture is that the sporophytes of *A. somai* and *A. itoi* produce only two to five new fronds each year (unpublished) and, consequently, only two to five new stipules each year. However, we do not know how long stipules survive. Clearly, the number of stipules is limited and care must be exercised when determining when and how many stipules should removed from a plant.

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FILICALEAN FERNS FROM THE TERTIARY OF WESTERN NORTH AMERICA: *OSMUNDA* L. (OSMUNDACEAE : PTERIDOPHYTA), *WOODWARDIA* SM. (BLECHNACEAE : PTERIDOPHYTA) AND ONOCLEOID FORMS (FILICALES : PTERIDOPHYTA)

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ABSTRACT

Recently discovered frond remains assignable to *Osmunda wehrii* Miller (Osmundaceae), as well as several new records of *Woodwardia* (Blechnaceae), and a new onocleoid fern are reported from the Tertiary of western North America. Pinnule morphology of *O. wehrii* supports the inclusion of this species in *Osmunda* subgenus *Osmunda*, as originally proposed by Miller and suggests a close affinity to *O. regalis* L. and *O. japonica* Thunb. New occurrences of the *Woodwardia aerolata* clade are noted for the Late Paleocene of western North Dakota and of a highly reticulate-veined form from the Miocene of western Washington. Re-evaluation of specimens of *W. deflexipinna* H. Smith (Succor Creek, Miocene) confirms its close affinity to *W. virginica* J. Smith. A fern with onocleoid anatomy is recognized from the middle Eocene Clarno Nut Beds of Oregon. Together, these examples demonstrate that the presence of critical taxonomic features, even in fragmentary remains, can increase our knowledge of filicalean fern evolution, biogeography and ecology in the Tertiary.

INTRODUCTION

Despite a widely held belief that the fossil record provides little information about them, filicalean ferns, including derived forms, are a relatively common component of many Tertiary floras. However, the impact of the fossil record in deciphering Tertiary fern evolution has been limited for several reasons. The focus for many Tertiary researchers has been on the collection and comparison of angiosperm remains, although ferns are typically included and occasionally highlighted in published treatments of floras (e.g., Pabst, 1968). Often paleobotanists focus on investigating major groups of plants during what they perceive as key moments of evolutionary times. In the case of ferns, which have been thought by many to undergo their major radiations primarily during the Paleozoic and Mesozoic, the pulses and subsequent evolution of many filicalean ferns in the Tertiary previously have been ignored. Fragmentary remains, particularly of nonfertile fronds, have not been studied in detail, because of the perceived difficulty of relating fossil material to modern taxa. Occasionally, florules

have been recovered that contain fern remains in essentially monotypic stands (e.g., Smith, 1938; Crabtree, 1988); however, these localities have been rarely studied. In recent years, several whole plant reconstructions of fossil ferns have been produced (e.g., Rothwell and Stockey, 1991; Pigg and Rothwell, 2001) and new emphasis has been focused on fern biogeography and ecology (Collinson, 2001, 2002; Page, 2002). With this new attention accorded to the fossil ferns of the Tertiary, the potential now exists for a better understanding of their evolutionary, biogeographic, and ecological significance.

Our recent work in the middle Miocene, permineralized Yakima Canyon flora of central Washington, USA has provided information on three fern genera that occur there: *Osmunda wehrii* Miller (Osmundaceae); *Woodwardia virginica* J. Smith (Blechnaceae), and a small fern with onocleoid vegetative anatomy, *Wessiea yakimaensis* Pigg & Rothwell (Miller, 1982; Pigg and Rothwell, 2001). In this contribution we report new occurrences of related taxa in several Tertiary localities of western North America. Together, these examples show that even fragmentary fern remains, when demonstrating critical taxonomic features, can provide considerable new information about filicalean fern evolution in the Tertiary.

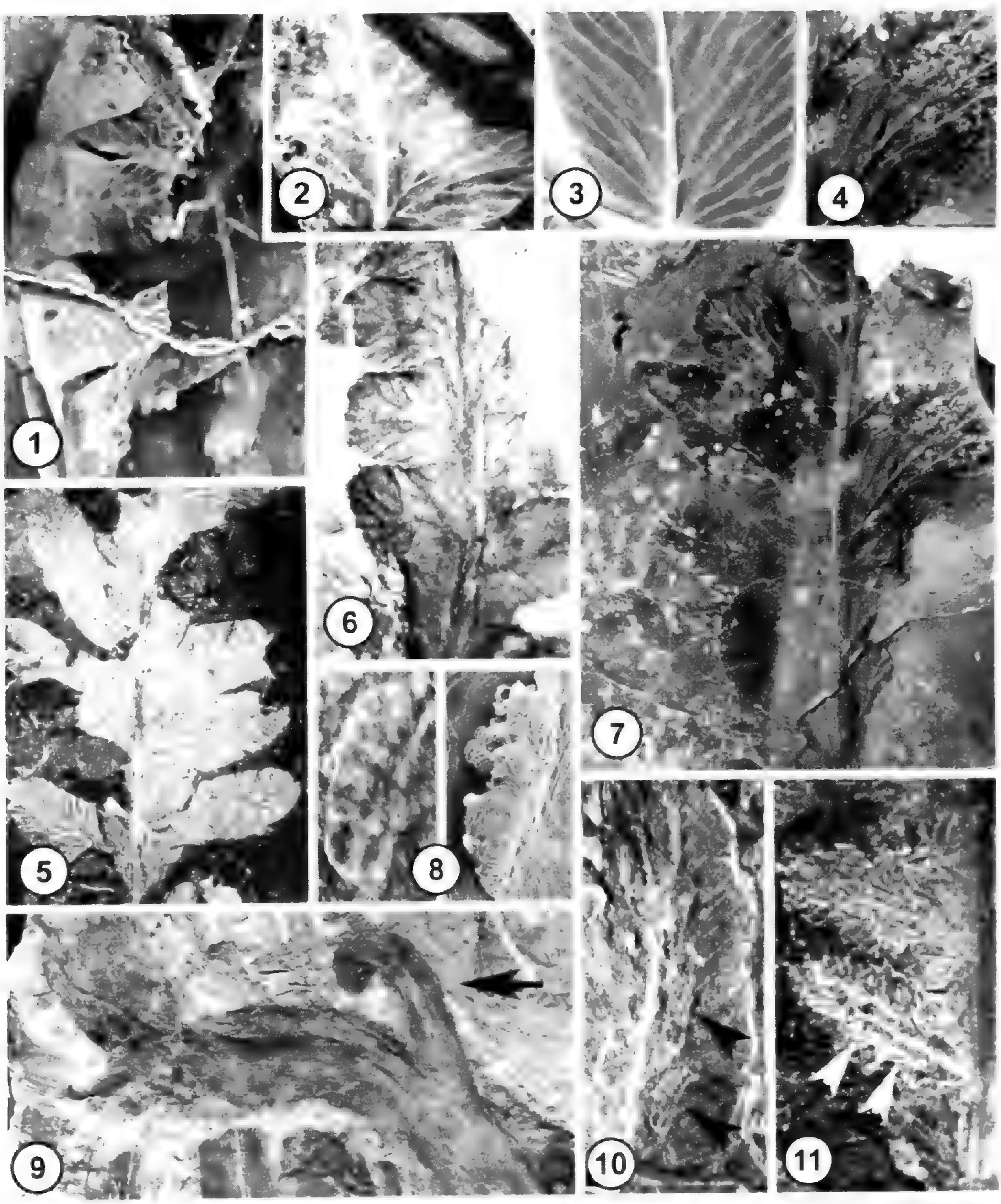
RESULTS AND COMMENTS

Osmunda wehrii (Osmundaceae)

Osmunda wehrii was described from permineralized rhizomes and leaf bases by Miller (1982) from Yakima Canyon, central Washington, USA, where it occurs intermingled with taxodiaceous foliage, stems and cones, the abundant foliage and smaller rhizomes of *Woodwardia virginica* and rhizomes and stipes of *Wessiea yakimaensis* (Pigg and Rothwell, 2001). Several pinnules of *Osmunda wehrii* have recently been recognized in attachment or closely associated to *O. wehrii* stipes (Figs. 1, 2). Fronds are bipinnate and the most extensive segments are up to around 2.8 cm long and represent portions of pinnae and individual pinnules. The pinnules are up to 8 mm long and 3.5 mm wide and each has an oblique base (Fig. 1, 2). Venation is dichotomous with the initial forking occurring close to the point of divergence of lateral veins from the pinnule midvein with a second and third order of dichotomies occurring in the basal part of pinnules (Fig. 2). Based on these morphological features, *Osmunda wehrii* pinnules are assignable to the subgenus *Osmunda*, which includes the extant royal fern, *O. regalis,* and the two Asian species *O. japonica* and *O. lancea.* Pinnules are most similar to those of *O. regalis* (Fig. 3) and *O. japonica* both of which share with *O. wehrii* the features of an oblique pinnule base and multiple vein dichotomies, features that *O. lancea* lacks (Hewitson, 1962). Anatomical details still under investigation will provide additional information about this middle Miocene member of *Osmunda* subgenus *Osmunda.*

Woodwardia aerolata (Blechnaceae)

A fern with possible affinities to *W. aerolata* is recognized from Late Paleocene Beicegel Creek flora of western North Dakota, USA (Figs. 4, 7). This flora is very similar to the Almont flora of central North Dakota (Crane, et al., 1990), both in composition and preservational type. However, in comparison to the Almont flora, material from the Beicegel Creek flora tends to have a greater percentage of permineralized specimens and provides an unparalleled opportunity to critically examine both the anatomy and morphology of Late Paleocene taxa (DeVore et al., 2003). Previous to our studies no ferns have been described from Almont or Beicegel Creek, however several fragmentary fern pinnules that represent at least two different



Figures 1,2. *Osmunda wehrii* pinnules from Yakima Canyon, Middle Miocene, Washington, USA. **Figure 1.** Overview of pinnae attached to rachis, x 4. **Figure 2.** Detail of pinnule to show dichotomising venation, x 4.3. **Figure 3.** *Osmunda regalis* pinnule for comparison, x 5. **Figures 4,7.** *Woodwardia* pinnule from Beicegel Creek, Late Paleocene, North Dakota, USA. Note elongate, pinnatifid pinna and diamond-shaped aeroles characteristic of extant *W. aerolata*. **Figure 4.** Detail of pinnule, x 3.8. **Figures 5,6,8.** (left), 9-11. *Woodwardia deflexipinna* vegetative and fertile pinnae, Middle Miocene Succor Creek, Oregon, USA. **Figures 5,6.** Overviews of disarticulated pinnae, x 1.5, and x 2.7, respectively. **Figure 7.** Overview of Beicegel Creek specimen, x 2.5. **Figure 8.** Sori with flap-like indusia, at left: fossil *W. deflexipinna*, x 15; at right: SEM of extant *W. virginica*, x 16. **Figure 9**. Rhizome with crozier (at arrow) previously illustrated by Smith (1938) and Graham (1965), x 1. **Figures 10,11.** Fertile pinnae of *W. deflexipinna* showing indusiate sori (arrows), x 3, and x 4.3, respectively.

types of ferns recently have been recovered. The *Woodwardia* specimen is 3.5 cm long x 2.7 cm wide, and is comprised of a pinnatifid, possibly apical, portion of the frond showing several pinnule lobes (Fig. 4, 7). The venation includes relatively coarse elongate, diamond-shaped aeroles 4-6 mm long x 6-8 mm wide and is marginally freely dichotomising, confirming its identification as *Woodwardia* rather than *Onoclea* (Collinson, 2001). A second taxonomically important character, the presence or absence of marginal teeth could not be determined from available material.

A second group of *Woodwardia* taxa with an excellent fossil record are those of the *W. virginica* clade (=*Anchistea* sensu Cranfill and Kato, 2001). *Woodwardia deflexipinna* was originally described from the Miocene Succor Creek of Oregon based on material collected from a small florule where these ferns occurred in a nearly monotypic stand. Fronds, rhizomes, crosiers and fertile remains were described and briefly illustrated by H. Smith (1938) and later by Alan Graham (1965). Reinvestigation of this material confirms that the vegetative and fertile remains are remarkably like those of both fossil and extant *W. virginica* (Figs. 5, 6, 8-11; Pigg and Rothwell, 2001). Frond remains are pinnatifid pinnae preserved for up to 6.5 cm long wide with up to 10 pinnule segments (Figs. 5, 6). Pinnules are 11mm long x 4 mm wide. Like *W. virginica*, this species has a single series of areoles that parallel the midvein, and relatively few anastomoses in the lateral part of the pinnule. Meshes are 3-4 mm long x 0.5-0.7 mm across (Fig. 6). Fertile frond fragments consist of elongate sori around 2 mm long x 0.5

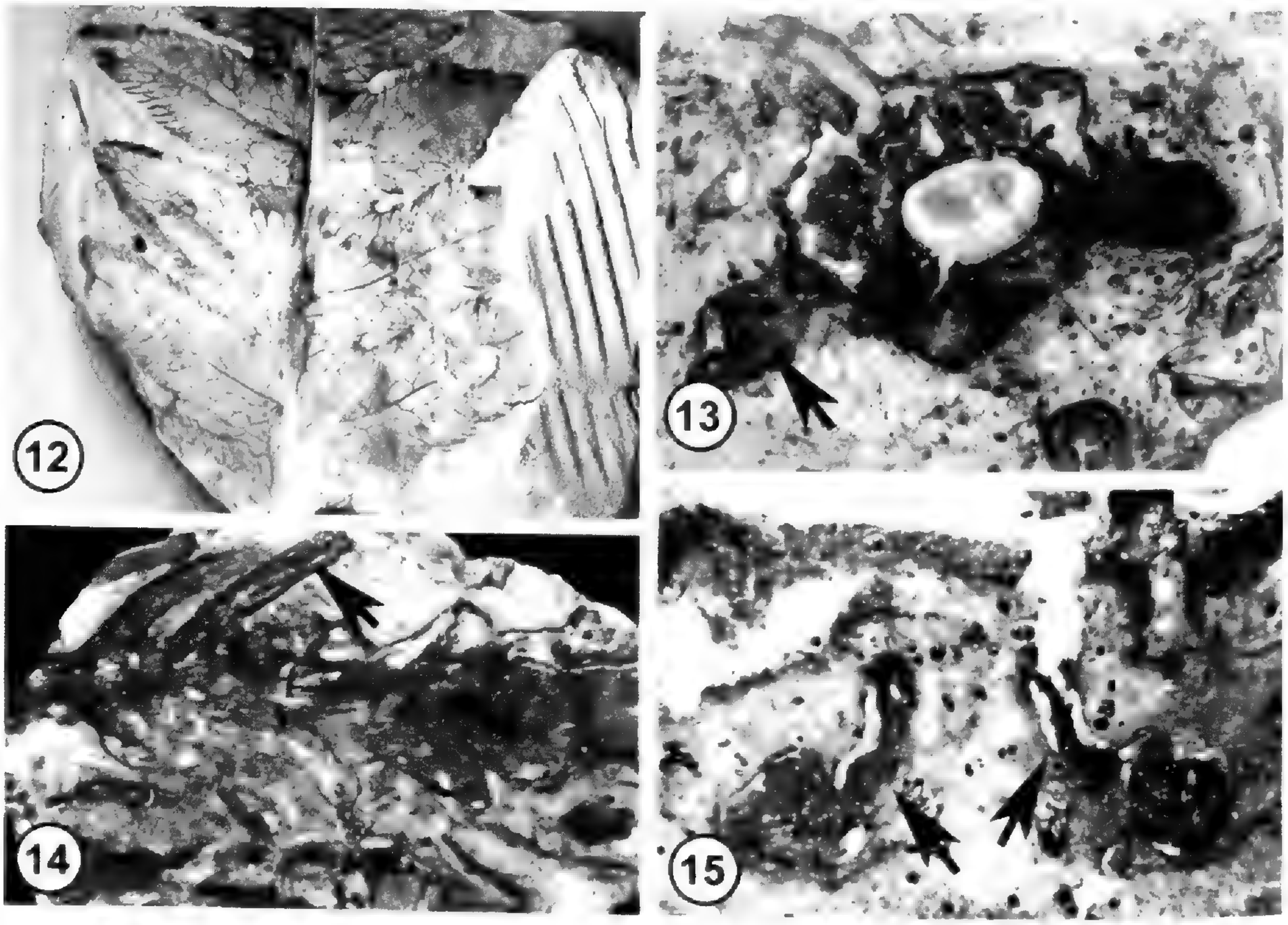


Figure 12. *Woodwardia* Miocene, Vasa Park, Washington, USA. Vegetative frond segment showing highly reticulate pattern of venation, x 1.3. **Figures 13-15.** Onocleoid fern, Middle Eocene Clarno Nut Beds, Oregon, USA. **Figure 13.** Transverse section of rhizome showing helically arranged, attached stipe bases, x 1.5. **Figure 14.** External view of rhizome showing numerous attached stipes and adventitous roots, x 0.9. **Figure 15.** Detail of stipe trace (arrows), x 5.

mm wide, each partly covered by an elongate, flap-like indusium that hinges along its outer side like those of *W. virginica* (Figs. 8, 10, 11). A crozier-bearing rhizome, previously illustrated by Smith (1938) and Graham (1965) is around 9 cm long and 1.2 cm thick (Fig. 9). Spores have not been recovered.

A third new occurrence of fossil *Woodwardia* is from the Miocene Vasa Park locality near Seattle. Only a few fragments have been discovered to date. The largest is up to 3.8 cm long x 3.4 cm wide and includes several pinnules. Pinnules are 1.8 cm long x 0.9 cm across, and veins are highly reticulate, resulting in short, polygonal meshes 2-3 mm long x 0.8-1.0 mm (Fig. 12). In comparison with extant species, these remains mostly closely resemble those species with numerous, short, polygonal reticulations in their leaves, such as *W. fimbriata,* a species from western North America today.

ONOCLEOID fern

A permineralized fern with onocleoid anatomy has been discovered in the middle Eocene Clarno Nut Beds of central Oregon. This locality is well known for its beautifully preserved fruits and seeds (Manchester, 1994). Rhizomes are preserved for up to 7.2 cm in length and 1.5 - 2 cm in diameter and produce numerous stipes (Fig. 14). The stipes extend up to 1.9 cm in length from their attachment to the rhizome and are around 0.3 cm across. Anatomical structure is similar to that of *Wessiea yakimaensis* Pigg & Rothwell, from Yakima Canyon and *Makotopteris* from the middle Eocene Princeton chert of southern British Columbia (Stockey, et al. 1999; Pigg and Rothwell, 2001). In this pattern, paired hippocampiform frond traces are helically arranged and are produced without leaf gaps (Fig. 13, 15). In contrast, adventitious root traces are produced from the stipe bases, and leave behind a root gap. This pattern is exhibited by onocleoid ferns and is common to a large number of higher filicalean genera (Stockey et al., 1999; Pigg and Rothwell, 2001). As additional vegetative and hopefully fertile remains become available for these ferns, their affinities will be more closely resolved.

DISCUSSION & CONCLUSIONS

Although such factors as angiosperm bias and fragmentary preservation have often limited a detailed description of Tertiary filicalean ferns, the careful study of their remains may help flesh out the record of their evolution and diversification into habitats they occupy today. Whole plant reconstructions (e.g., *Onoclea sensibilis*, Rothwell and Stockey, 1991; *Woodwardia virginica*, Pigg and Rothwell, 2001) have previously demonstrated that essentially modern filicalean fern species were established in present day habitats and persist in similar sites today. The examples provided in this study are mostly fragmentary in nature but still possess critical features that can be used for identification and provide additional new data to the record.

The Osmundaceae have the most extensive fossil record of the filicalean ferns, extending back to the Permian based on characteristic vegetative anatomy of rhizomes and leaf bases (Serbet and Rothwell 1999; Collinson, 2001). Of the three genera in the family, *Osmunda* is the best represented and most diverse. The genus is traditionally divided into three subgenera, *Osmunda*, *Osmundastrum* and *Plenasium*. Molecular studies generally support this subgeneric classification, however the genus *Osmunda* appears to be paraphyletic to *Todea* and *Leptopteris* (Yatabe et al., 1999). The subgenus *Osmunda* is well represented in Cretaceous and Tertiary localities by both anatomically preserved rhizomes and compressed foliage. Permineralized stems include *O. pluma* (North Dakota, Paleocene), *O. oregonensis* (Oregon, Eocene), *O. iliaensis* Romania (Miocene-Pliocene), and *O. nathorstii* (Spitzbergen, Eocene; Miller, 1967, 1982). Most

recently, *O. shimokawaensis*, a fern very similar to *O. wehrii* has been described from the Miocene of Japan (Matsumoto & Nishida, 2003). Foliage of subgenus *Osmunda* is known from several localities in the Late Cretaceous and Tertiary (Miller 1967; Collinson, 2001).

The discovery of pinnules of *Osmunda wehrii* supports Miller's earlier (1982) placement of the species into subgenus *Osmunda* and the fossil material is similar to *O. regalis* and *O. japonica* (Hewitson, 1962). The *Osmunda* subgenus is well established by the Miocene, so its presence at Yakima Canyon is no surprise. Evolution and paleoecology of the Osmundaceae are, however, interesting in light of the recent resurgence of interest in the relationship of angiosperms to the Tertiary radiation of the higher filicalean ferns (Lovis, 1977; Rothwell, 1987; Schneider, et al. 2004). Unlike the higher filicalean ferns, the Osmundaceae has been around since the Permian, and thus predate angiosperm influences by over 140 million years. Osmundaceous ferns are often associated in the fossil record in conifer-dominant environments. *Osmunda regalis* often occurs as the dominant fern in *Taxodium* swamps of southeastern North America today, as did *O. wehrii* in the middle Miocene Yakima Canyon flora of Washington state. Presumably, osmundaceous ferns evolved in relation not to angiosperms, but to conifers.

In contrast, the first evidence of *Woodwardia* is in the Late Cretaceous (Collinson, 2001). This genus was an important component of the circumboreal flora of the Northern Hemisphere Paleocene (Kvaček, 1994; Collinson, 2001). Although the fossil record of *Woodwardia* is in need of revision, it is now evident that the occurrence of several clades sensu Cranfill and Kato (2001) among woodwardioid ferns can be recognized in the Tertiary. The basalmost clade, represented by *W. aerolata* (=*Lorinseria aerolata* sensu Cranfill and Kato, 2001) appears in the Late Cretaceous and Paleocene. The next clade is represented by *W. virginica* (=*Anchistea virginica* sensu Cranfill and Kato, 2001) and related taxa. It is interesting to note that although this clade is estimated to be ancient, it does not appear in the fossil record until the Miocene. The Miocene, however, is the heyday for the *W. virginica* clade with not only permineralized *W. virginica* remains in Washington at Yakima Canyon (Pigg and Rothwell, 2001), but the very similar *W. deflexipinna* at contemporaneous localities in the Succor Creek flora of Oregon (Smith, 1938; Graham,1965). Asian Miocene occurrences of the *Woodwardia virginica* clade are also known, as are those of central and western Europe (some fossils of *W. munsteriana*, *W. maxonii*; Hurnick, 1976; Pigg and Rothwell, 2001). Of the other, apparently more derived clades that may also have a fossil record, the Vasa Park site may document an early occurrence for one of the more highly reticulate-veined forms such as W. *fimbriata*, a species native to western North America today.

Lastly, three onocleoid ferns with characteristic stipe and root trace anatomy are now known: the Princeton chert *Makotopteris* and the Clarno Nut Beds fern, both from the middle Eocene, and *Wessiea* from the middle Miocene. These ferns cannot be placed into modern genera. *Makotopteris* cannot be named to a genus because although fertile remains are known, some critical taxonomic characters could not be gleaned from the fossil and comparative anatomical information of remains known in the fossil is not available for all pertinent living relatives. *Wessiea* and the Clarno fern are presently known only from their rhizome and stipe anatomy. Nevertheless they demonstrate that middle Miocene and Eocene floras, respectively, hosted a variety of highly derived filicalean ferns.

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GROWTH IMPAIRMENT OF HUMAN CELLS BY FERN SPORE EXTRACTS

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INTRODUCTION

Our review of the literature (Simán *et al.*1999) posed a question – are there human health risks from fern spores?. Our conclusion was that there may be. Carcinogenesis in humans caused by tissues of ferns is well established (e.g. Alonso-Amelot & Avendaño, 2002) and spores of some ferns cause allergic reactions and contact dermatitis in some people. We first obtained evidence for DNA-damage and carcinogenicity from experiments with spores of a single species (*Pteridium aquilinum* - bracken) fed to mice. Similar experiments have since established that spores of five fern taxa (including northern and southern hemisphere bracken) induce DNA adducts in upper gastrointestinal tissue of mice (Simán *et al.* 2000a).

Experimental administration of spores to whole humans is not practical, but experiments with human cells strengthen the conclusion that fern spores can cause DNA damage. Administration of extracts of spores of *Dicksonia antarctica, Pteris vittata, Sadleria pallida, Anemia phyllitidis* and *Pteridium aquilinum* to human premyeloid leukaemia cells induces breaks in their genomic DNA (Simán *et al.* 2000b). DNA damage is strongly correlated with carcinogenic events (e.g. Fairbairn *et al.* 1995) but even if such damage was caused in whole humans after inhalation or ingestion of spores, it is of course possible that repair mechanisms would mend the breaks before tumourigenesis was triggered.

The aim of the research reported here was to establish whether fern spore extracts prevent the growth and proliferation of human cells.

MATERIALS AND METHODS

Fifty mg of spores of six ferns: *Anemia phyllitidis* (L.) Swartz, *Cyathea arborea* Sm., *Dicksonia antarctica* Labill., *Dryopteris filix-mas* (L.) Schott, *Pteridium aquilinum* (L.) Kuhn and *Pteris vittata* (L.) were ultrasonicated and extracted with 0.5 ml sterilised distilled water overnight. *P. aquilinum* and *D. filix-mas* were examined because of the established toxicity of their vegetative tissues, the remaining species were included to provide breadth of taxonomic differences and/or horticultural significance.

The HaCaT keratinocyte human cell line used adopts a fibroblast morphology in culture. Cells were seeded at 2-4 x 104 cells ml^{-1} in 1.5 cm diameter wells in 24-well dishes and fed 1 ml complete Dubecco's modified Eagle medium (DMEM) containing 5% fetal calf serum. After 24 h (start of "day 0") the medium was replaced by complete DMEM containing only 0.5% serum. (This reduction in serum sensitises the cells.) At the same time the cells were fed 50 ml spore extract. "Vehicle controls" were fed 50 ml sterilised, distilled water, "controls" were given no treatment.

Cell number in a fixed amount of liquid extracted was counted in a haemocytometer

every 24h for 5d. The average of 3 counts per well for 3 wells per day formed the basis for a calculation of the mean of means. The mean final number of cells ml^{-1} on day 5 of each treatment, and the growth rate between day 3 and 5, were compared with the controls using *t* tests for unmatched samples.

RESULTS

Over the five days of the experiment cell number increased in control treatments at a rate typical of this cell line, by a factor of x10-15. Control cell counts increased by means of x12.4 and 12.6, respectively. Cell number after treatment with extracts of *A. phyllitidis, C. arborea, P aquilinum* and *P. vittata* increased less than those of controls (see Figure 1). Cell cultures exposed to *P. aquilinum* extracts showed an increase in cell number of x2.0 - highly significantly less than that of both the controls and vehicle controls (t = 5.29 for control, 6.28 for vehicle control, df=4, $p \leq 0.01$). The final concentration of cells in wells treated with *P. aquilinum* extracts was about six times lower than those of the controls.

Extracts of 3 other species: *P. vittata, C. arborea* and *A. phyllitidis*, impaired the growth of the cultured cells - increase factors of x6.4, 7.4 and 10, respectively. (Not significantly different from either of the controls at the 99% confidence level, but *C. arborea* treatment was significantly impaired at 95% confidence level.)

D. filix-mas extract had no detectable effect on the growth of the cultured cells (increase factor x12.4). Cells treated with extract of the tree fern *D. antarctica* increased more than any others (x15) over the five days of the experiment, but this was not significantly different from the controls.

Between days 2 & 3 cultures treated with *P. vittata* extracts experienced a significant inhibition of cell proliferation (t>9.6, df=4, $p \leq 0.01$,see Figure 2). From day 3 these cultures started to recover. After day 3 all growth rates stabilised. Between days 3 & 5

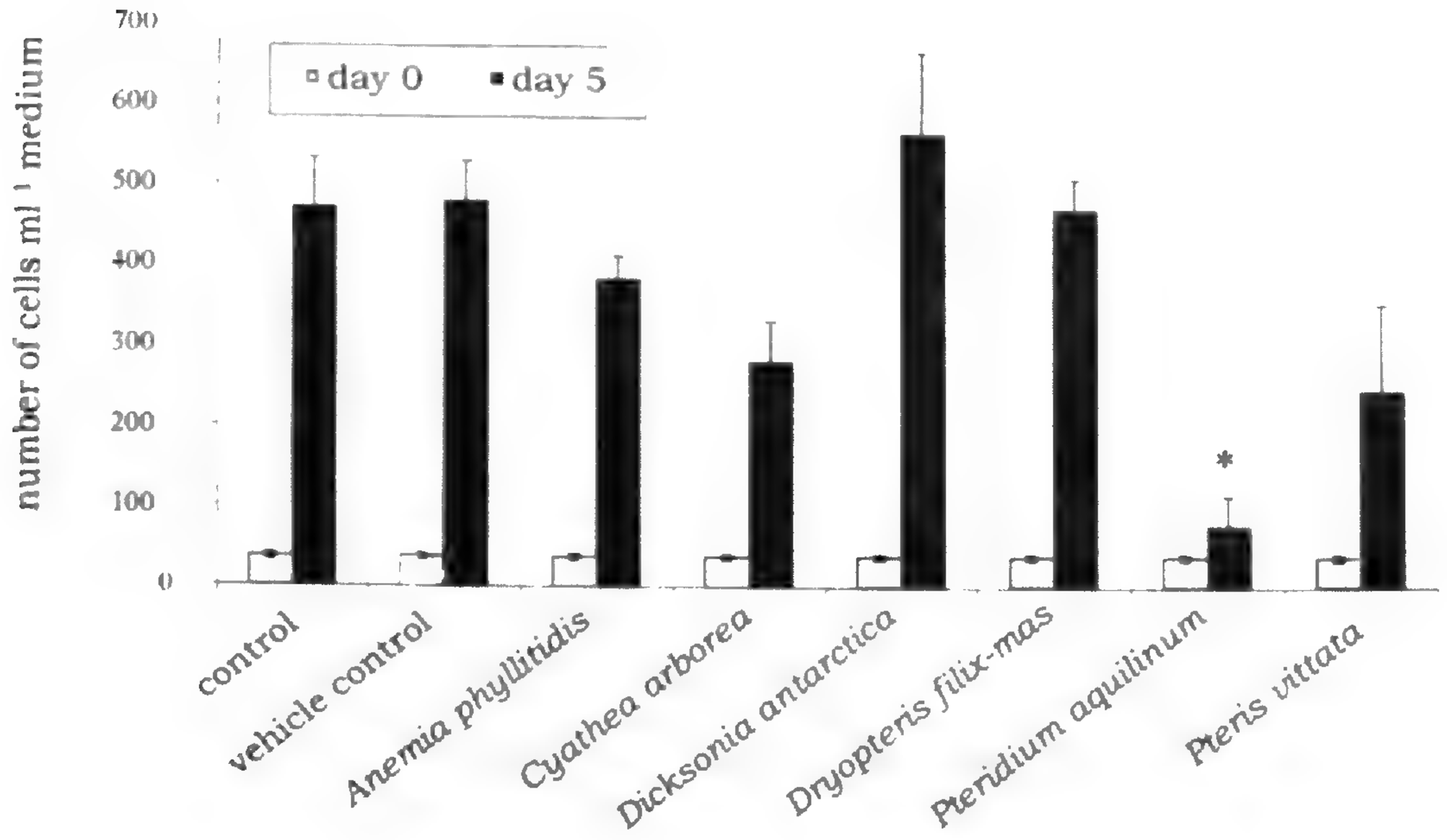

Figure 1. HaCaT cells treated with fern spore extracts then cultured for 5 days. Column marked (*) differs from controls at $p \leq 0.01$.

the vehicle control cells and the cells treated with *D. antarctica* and *D. filix-mas* extracts had a higher proliferation rate than cells of any of the other treatments. Growth rate was highest in *D. antarctica*-treated cells (t=2.3, df=4, p≤0.1). The growth rate of *P. aquilinum*-treated cells was lower than that of the vehicle controls (t=2.3, df=4, p≤0.1).

DISCUSSION

Cytotoxicity.

Spore extracts of 4 of the 6 ferns tested impaired the growth of the human cells used in the conditions of this study. Cytotoxicity was strongest in *P. aquilinum* treatments (the results indicate that the cells in these cultures would eventually have died).

P. vittata is a common glasshouse weed, a popular garden ornamental, and a potential phytoremediation treatment (Ma *et al.* 2001). *P. vittata* extract treatment generated the second lowest cell number and can be said to have had a cytostatic effect. *C. arborea*-treament generated the third lowest final cell density. This growth impairment was not mirrored by the other tree fern species used, however, (the horticultural favourite, *D. antarctica)* which, if anything, appeared to promote cell growth. Vegetative tissues of the garden ornamental *D. filix-mas* have been used to kill tapeworms and insects. Perhaps spores are not as toxic as vegetative tissues, or non-mammalian cells respond differently to the toxins, since cells treated with spore extracts of this species were the ones least different from the controls.

Explanation for cell growth impairment.

Extracts of *P. aquilinum, D. antarctica, P. vittata*, and *A. phyllitidis* induce DNA strand-breaks in human cells (Simán *et al.* 2000b). In the present study, only *P. aquilinum* and *P. vittata* extracts showed a pronounced adverse effect on the cells – those of *D. antarctica* did not. Growth impairment may therefore be due to something other than damage resulting from DNA strand-breaks in some or all ferns, or experimental differences in the study reported here perhaps reduced the strand-breaks

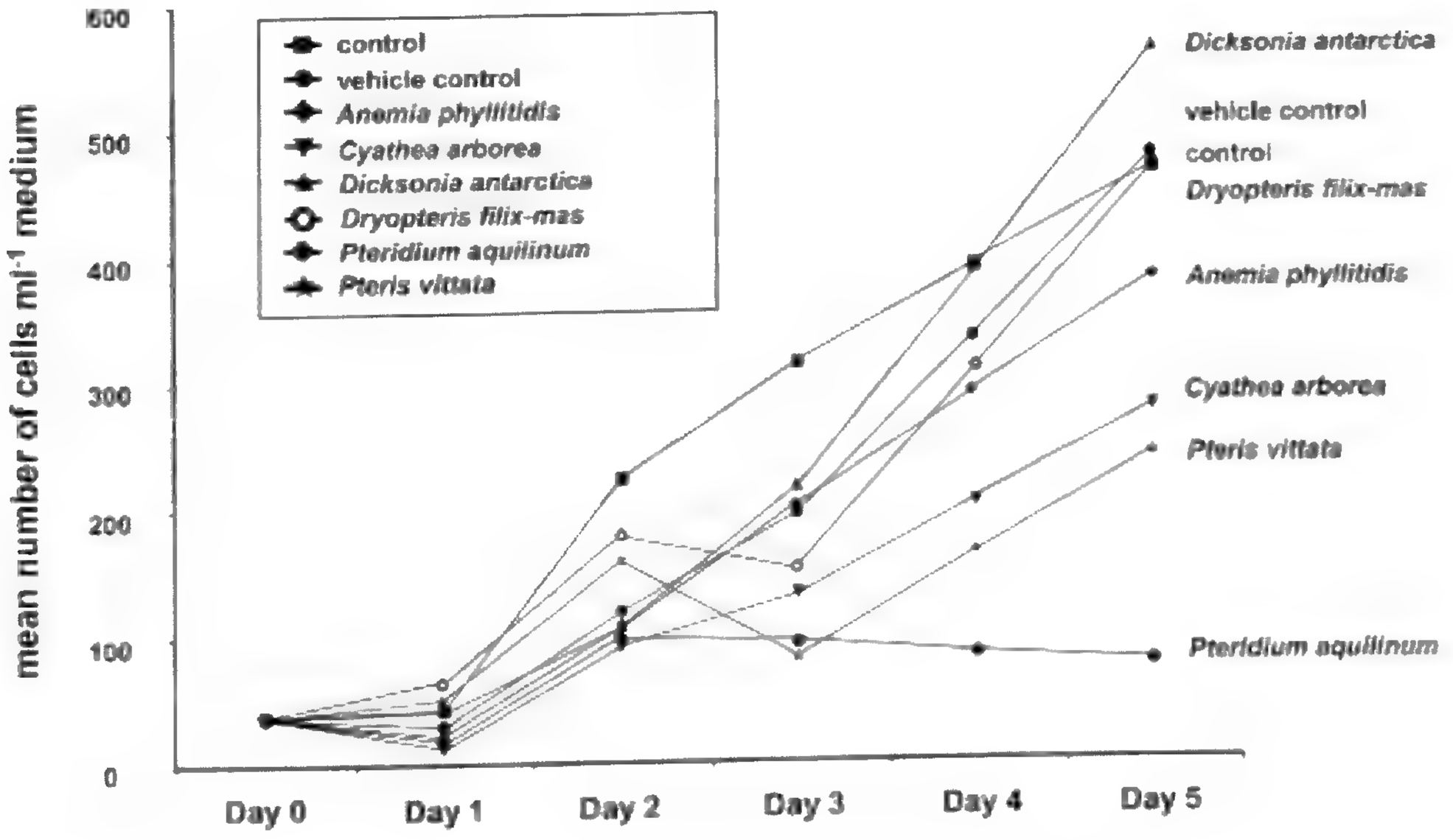

Figure 2. HaCaT cell number after treatment with fern spore extracts.

caused by some species and not others.

One difference between the experiments is that spore extracts used in the study of DNA strand-breaks were made with the solvent dimethyl sulphoxide (DMSO), rather than water. Damaging compounds differ in solubility between media, and as ferns differ markedly in biochemical constituents, this could explain the difference in effects. Of course neither DMSO or water constitute realistic solvents for the human cell surfaces that encounter spores. It could be argued, however, that water constitutes the milder solvent, so deleterious effects that are seen with both water and DMSO provide strong evidence for toxicity of the spores in question.

Another explanation for growth impairment is the formation of adducts (which form when compounds bind to DNA). Adducts may be repaired by the cell, or can initiate events leading to the creation of a cancer cell. Povey *et al.* (1996) established that spores of *Pteridium* can induce DNA adducts in tissues of mice, and we have shown that spores of *P vittata* and *A. phyllitidis* share this property (Simán *et al.*, 2000a).The main *Pteridium* carcinogen, ptaquiloside, can cause adduct formation (Smith & Seawright 1995), is water soluble (Ojika *et al.* 1987), and is found in bracken tissues including spores (Schmidt *et al.* 2005; Rasmussen, Schmidt & Sheffield unpubl.) so it is possible that this compound is responsible for the cytotoxicity of *P. aquilinum* spore extracts.

Human health implications?

This *in vitro* study strengthens the extensive in vivo evidence of the toxicity of *Pteridium*. Health warnings about walking through bracken stands during the sporing season may therefore be reasonable, although far from all *Pteridium* stands are fertile (Wynn *et al.* 2000), and there is no way to relate likely numbers of spores inhaled to potential risk. Bracken remains the most damaging fern examined to date, in all the studies reviewed, but the evidence presented herein suggests we should not be complacent about other ferns. Toxins may of course differ between species, and there is no defensible way to extrapolate from effects on starved cells *in vitro* to risk for entire healthy humans (either in terms of effects seen, or numbers of spores used).

However, humans are not good at dealing with health risks. For example, there is probably no safe dose of ionising radiation, yet people choose to live in areas dominated by granite, and therefore experience high levels of natural background radiation. Smokers do not start "complaining" about their health until long after taking the first puff (cf. Hainsworth 2000). **If** health risk **may** be incurred from inhaling spores of ferns other than bracken, there is no reason to "never touch a fern again" (cf. Hainsworth 2000). Anyone regularly exposed to large numbers of spores need spend only a few pence on a mask (available from most DIY stores) which excludes particles. Our advice to those involved with caring for sporing ferns, or harvesting spores is simple – better safe than sorry!

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RESPONSES OF PTERIDOPHYTE SPORES TO ULTRAFREEZING TEMPERATURES FOR LONG-TERM CONSERVATION IN GERMPLASM BANKS

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Key Words: fern spores, long term conservation, ultra freezing, viability.

ABSTRACT

There are many unresolved questions around the loss of viability of pteridophyte spores and the most suitable conditions for long term conservation. The effects of humid and dry conditions, different temperatures, and the short exposure of spores to liquid nitrogen have been occasionally studied by various authors.
The work presented here is the first result of a project focussed on long-term conservation of spores of pteridophytes. Using species from different ecological habitats, we show the effects of ultra-freezing, at -80ºC and -196ºC (LN) for six months of storage, on the germination process as well as on the development of the gametophyte until it reaches sexual maturity.

We analyze and comment on the results obtained for the final germination percentage and the germination rate, the final percentage of gametophytes that reach the laminate developmental phase, and of gametophytes that attain the sexual phase under the two conditions. All these data are referenced to the initial viability of the samples used as well as to a control of spores stored at room temperature (approx. 25ºC).

INTRODUCTION

Conserving biodiversity involves maintaining the genetic variability of the different groups of living species in all aspects. The pteridophytes are the oldest group of vascular plants on Earth. As far as shapes, sizes and species are concerned, it was a very varied and diverse group in days gone by. Nowadays, even though they are just a shadow of that former abundance, they greatly contribute to the richness of plant biodiversity, not only because of their floral significance but also for their phylogenetic value.

A large percentage of pteridophytes tends to be associated with ecosystems that are particularly sensitive to degradation (mature forest formations, humid areas and riparian habitats, etc.), some of which are considered by present-day legislation as natural habitats of community importance. Some of these taxa are included in the lists of species of community interest which require strict protection. Other than contributing to the conservation of species of a greater scientific interest and also to the maintenance of biological biodiversity, in all the aspects this entails, their protection and conservation also contribute to ensuring that these ecosystems are indeed maintained.

In view of the clear need of conserving and regenerating ecosystems under threat, and given that pteridophytes are very sensitive to environmental changes, it is essential that their spores should be included in a germplasm bank in which material is to be conserved for the biological-experimental study of the species as well as for the conservation of biodiversity on a long-term basis.

Germplasm banks play an important role in the long-term *ex situ* conservation of plant species. Seed conservation in germplasm banks is a reality supported by numerous studies (Ellis *et al*., 1985; Dickie *et al*., 1990; Gómez-Campo, 2001). Nonetheless, no conclusive studies exist as far as the conservation of pteridophytes is concerned to guarantee a long-term conservation methodology.

Various studies verify the relatively rapid loss of spore viability under environmental conditions. This loss occurs in a few days (*Equisetum* sp.), a few months (*Osmunda regalis*) or even a year or so (*Onoclea* sp. and *Matteuccia* sp.) in the case of green spores; in a few months (*Gleicheniaceae* and *Thyrsopteris elegans*), between 1 year and a decade (the majority of species), and in exceptional cases, several decades (*Pellaea* sp., *Asplenium serra*, *Marsilea* sp.) where non-green spores are concerned (Lloyd & Klekowsky, 1970; Dyer, 1979; Page, 1979; Windham *et al*., 1986; Lindsay et al. 1992).

The different studies that have been conducted on this theme indicate causes of this rapid viability loss: biochemical and metabolic factors, such as the deficiency of respiratory substrates, the lack of membrane integrity, the inactivity of growth enzymes and substances in non-green spores (Beri & Bir, 1993), or genetic factors such as chromosomal mutations (Page *et al*., 1992). It has been indicated that the loss of viability in green spores may be due to their high respiratory rate (Lloyd & Klekowsky, 1970), or to the loss of the capacity to recover photosynthetic activity after drying (Lebkuecher, 1997). However, impacts of these causes are not completely clear because experimentation has been conducted on this theme.

On the other hand, most of the few studies that actually deal with the conservation of pteridophytes have focussed on analysing which conditions are optimum to maintain spore viability during storage. Different temperatures in germplasm banks have been tested (4ºC, 5ºC, -12ºC, -20ºC, -70ºC), with both humid and dry conservation methods. Even cryoconservation techniques using liquid nitrogen have been tested. Some studies indicate that the autecology of the species may be a significant factor when establishing conservation protocols. Other factors, such as the ploidy level, have been analysed in different studies (Windham *et al*., 1986; Lindsay *et al*. 1992; Agrawal *et al*., 1993; Simabukuro *et al*., 1998; Pence, 2000; Constantino et al., 2000; Quintanilla *et al*., 2002; Aragon & Pangua, 2004).

Extending spore viability up to 2 or 3 years in green spores has been achieved in the *Equisetum* genus (Jones & Hook, 1970). Furthermore, spores from species such as *Woodwardia radicans*, *Culcita macrocarpa*, *Athyrium filix-femina*, *Phyllitis scolopendrium* and many others, remained in storage and suffered no viability loss over more than 12 or 24 months (Lindsay *et al*. 1992; Quintanilla *et al*., 2002; Aragon & Pangua, 2004); also spores immersed in liquid nitrogen are still viable for at least 75 months (Pence, 2000). Generally speaking however, few quantitative and long-term data exist that determine the best and most lasting way to conserve spores.

The study by Page *et al*. (1992) indicated that when spores are being stored, a need exists to maintain not only their viability, but also both their growth capacity and genetic integrity. In this respect, and as a novelty with regard to the rest of existing works undertaken in this particular field, the effects of tested conditions regarding germination and early development, the effects of late gametophyte development, along with their capacity of completing the life cycle, will be analysed. This is the main objective since what is being dealt with here is the discovery of which conditions are optimum for the long-term storage of fern spores in germplasm banks. For this reason

the spores need to be viable and capable of producing sporophytes, which are to be subsequently used in both habitat restoration and for research purposes.

MATERIAL AND METHODS

This study has been carried out with 10 pteridophyte species present in the Mediterranean area. The species and populations collected are shown in Table 1. In order to obtain a significant sample of the variability in the natural population, work was conducted with spores of at least 20 individuals per species.

For spore obtaining, collection sheets are prepared in glossy paper for the fronds from the individuals collected in the field. These sheets are placed in a dry, aired place under light pressure, and are left at room temperature for a week. After this time, the spores which had fallen on the paper were collected and stored in glass vials once they had been sifted with a 0.074 mm sieve in order to eliminate any remains of sporangia and paleae.

The spores were placed into Eppendorf tubes, approximately 1 mg/spores per tube. The tubes were stored in the dark at 25ºC (laboratory temperature), -80ºC and –196ºC (liquid nitrogen) for 6 months. After a fast defrost at 40ºC for 5 minutes, 1 mg spores was suspended in 1 ml of liquid Dyer culture medium and dispensed 5 drops with a micropipette among seven 5.5 mm petri dishes on culture medium with 1% agar (Dyer, 1979). The dishes were sealed thoroughly with Parafilm and incubated at 12 h light photoperiod (daylight fluorescent tubes, photon irradiance 25-50 µmol m-2 s-1 in the 400-700 nm regions) at 20°C.

In order to observe the start of germination and how it developed, the percentage of germinated spores was noted daily after 10^{th} day, afterwards every three days. The final spore viability was checked by analysing the germination percentage after 30 days, by randomly counting 100 spores per dish and by considering those spores with either a primary rhizoid or a first chlorophyllous cell as having germinated.

The percentage of gametophytes that reached the laminar developmental phase was also analysed after 30 days. The laminar phase was taken as the initiation of the 2D growth (the transition stage), that is, the first division in a perpendicular plane of the prothallium cells. This date was chosen since it was the time when all the gametophytes in the controls taken at zero time reached this phase. Subsequently gametophytes were transferred to soil in order to check the correct appearance of the sexual phase in each replica of the three treatments after 120 days.

An arcsine transformation was applied to the percentage data, and a one-way ANOVA or a t-test was used to analyse them. The Tukey test was used ($p>0.05$) on the means to identify homogeneous groups. All statistical tests were carried out with the SPSS program, version 11.0.

RESULTS

The obtained results (Table 2) show a 24-72 hour delay in the start of the germination and in reaching T_{50} among the spores conserved at room temperature (25ºC) with respect to those that were ultrafrozen. This delay does not occur in the species whose germination began after 6 or more days, as is the case of *Polystichum lonchitis*, *Notholaena marantae* and *Ceterach officinarum*, species from high mountainous terrain and xerophilous environments, respectively.

If we look at Figure 1 we see that a significant viability loss has been revealed in all the species used in the study (ANOVA, p-value <0.05) when they were kept at room

Species	Locality	UTM	Altitude	Date
Asplenium onopteris	Embalse del Pasteral La Cellera de Ter, Girona.	31TDG64	600 m	20/04/2003
Notholaena marantae	Órganos de Benitandús Alcudia de Veo, Castelló.	30SYK22	650 m	23/05/2003
Cystopteris fragilis	Maset del Zurdo Vistabella del Maestrat, Castelló.	30TYK26	1380 m	12/06/2003
Pteris vittata	Barranco de la Safor Villalonga, València.	30SYJ30	260 m	03/07/2003
Dryopteris filix-mas	Maset del Zurdo Vistabella del Maestrat, Castelló.	30TYK26	1380 m	17/07/2003
Athyrium filix-femina	Barranco del Mançanar Vistabella del Maestrat, Castelló.	30TYK26	1400 m	17/07/2003
Polystichum aculeatum	Font del Tilde Vistabella del Maestrat, Castelló.	30TYK26	1200 m	18/07/2003
Thelypteris palustris	Ullals Riu Verd Massalavés, València.	30TYJ13	35 m	13/08/2003
Polystichum lonchitis	Coma d'Amitges Espot, Lleida.	31TCH31	2420 m	15/09/2003
Ceterach officinarum	Barranco de Tiero Benagéber, València.	30SXJ69	760 m	02/10/2003

TABLE 1. Collection details of the species used.

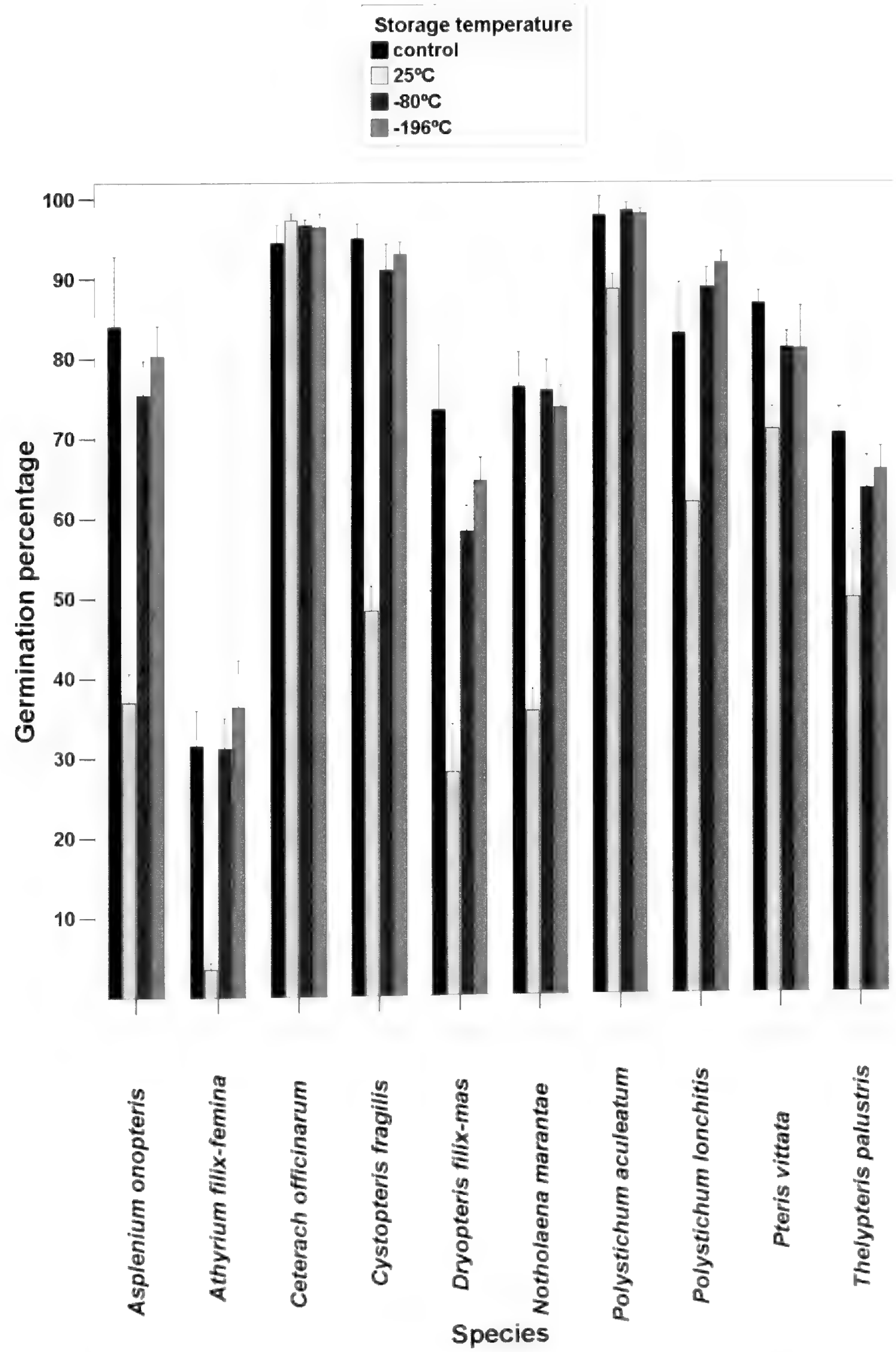

Figure 1. Germination percentage of the different species after 6-month of storage at 25°C, -80°C and –196°C in comparison with the initial value.

Taxon	Storage temperature	Germination's beginning day	T_{50}	Gametophytes in laminar phase (%)	Homogeneous groups	Plates with sexual gametophytes
Asplenium onopteris	25°C	5	6.5	98.4 ± 0.3	a	7/7
	-80°C	4	6	99.1 ± 0.7	a	7/7
	-196°C	4	6	99.6 ± 0.2	a	7/7
Athyrium filix-femina	25°C	6	8	91.6 ± 1.8	a	7/7
	-80°C	4	5	95.6 ± 0.6	a	7/7
	-196°C	4	5	95.0 ± 1.5	a	7/7
Ceterach officinarum	25°C	7	10	86.9 ± 8.4	a	5/7
	-80°C	7	9.5	52.3 ± 12.9	a	3/7
	-196°C	7	9.5	71.4 ± 12.3	a	5/7
Cystopteris fragilis	25°C	6	8	98.4 ± 0.4	a	7/7
	-80°C	5	7.5	98.3 ± 0.5	a	7/7
	-196°C	5	7.5	99.0 ± 0.4	a	7/7
Dryopteris filix-mas	25°C	5	7	99.3 ± 0.3	a	7/7
	-80°C	4	5.5	99.3 ± 0.4	a	7/7
	-196°C	4	6	99.3 ± 0.2	a	7/7

Notholaena marantae	25°C	6	9	97.0 ± 0.6	a		7/7
	-80°C	6	9	95.4 ± 0.8	a		7/7
	-196°C	6	9	96.6 ± 0.6	a		7/7
Polystichum aculeatum	25°C	5	6.5	98.1 ± 0.3	a		7/7
	-80°C	4	5.5	98.1 ± 0.6	a		6/7
	-196°C	4	5.5	98.3 ± 0.6	a		7/7
Polystichum lonchitis	25°C	6	9.5	93.7 ± 1.8	a		5/7
	-80°C	6	8	95.1 ± 0.9	a		5/7
	-196°C	6	8	98.1 ± 0.4		b	7/7
Pteris vittata	25°C	4	5.5	99.3 ± 0.3	a		7/7
	-80°C	3	5.5	98.6 ± 0.6	a		7/7
	-196°C	3	5.5	93.4 ± 0.9		b	7/7
Thelypteris palustris	25°C	6	8	91.0 ± 1.4	a		6/7
	-80°C	5	6	94.0 ± 1.1	a		7/7
	-196°C	5	6	90.3 ± 2.0	a		7/7

TABLE 2. Germination's beginning day, T_{50}, homogeneous groups (test tuckey $\alpha=0.05$), the percentage of germinated spores that had become two-dimensional gametophytes after 60 days, as well as the number of dishes with sexual gametophytes, are shown for each species and temperature.

temperature (25°C), except for *Ceterach officinarum*, which maintains the same germination percentage after a 6-month storage as those spores either stored at –80°C or at –196°C in liquid nitrogen do. These germination percentages of those spores stored in an ultra frozen state were no different to the controls taken after collecting plant material, so therefore no viability loss was observed. Furthermore, those spores that do not germinate were also seen to not swell in the same way as those that do germinate, that is, there is no proper imbibition.

As for late gametophyte development, no negative effects to it were observed at any of the temperatures tested in the study, as Table 2 shows, since more than 90% of germinated spores in all the species (except *Ceterach officinarum*) and treatments reach the two-dimensional gametophyte stage after a 30-day culture. Furthermore, sexual structures develop in the gametophytes in virtually all the dishes after 120 days of culturing, grow normally after transference to soil and completed their development. This point is of great relevance when the aim is to obtain mature plant for populational reinforcements.

DISCUSSION AND CONCLUSIONS

Agrawall *et al.* (1993) and Pence (2000) were pioneers in observing the effects that both cryoconservation and long-term storage at a temperature of –196°C (reached with liquid nitrogen) have on spores. Their results are positive since they not only established that cryofreezing does not kill spores, but also that spores remain viable after being stored at this temperature for up to 75 months. However, the results from these studies are qualitative and/or only focus on germination. Therefore, the real effect that cryoconservation has on spore viability and on the subsequent gametophyte development remained unknown.

Whittier (1996) also studied the effects that ultrafreezing at –70°C had on *Equisetum hyemale* green spores by observing that the viability of these spores could be prolonged. After 16-month storage, more than a quarter of the spores stored remained viable, when these do not survive more than 3 or 4 weeks at room temperature.

After a six-month work period, our results provide the first quantitative data as well as data regarding gametophyte development after ultrafreezing (-80°C and –196°C) in non-green spores. We notice that not only does ultrafreezing not affect spore viability after 6 months, but also that the gametophyte develops in perfect conditions, with no sign of it being negatively affected.

We could conclude a priori from this that ultrafreezing is seen to be a good conservation method for both spore viability and genetic integrity of the conserved material since no effects are provisionally observed on its development, that is, on its phenotype. This is coherent with what is expressed in the bibliography on the conservation of biological material at cryogenic temperatures (cryopreservation), a process able to suppress biological reactions completely, therefore permitting this material to be stored indefinitely. Thanks to cryobiology, cryopreservation processes may be controlled in such a way that the damage cells suffer is the minimum, therefore meaning that recovery is the maximum, providing the process is conducted properly.

By contrast, a considerable loss of viability exists in those spores stored at room temperature (25°C) in all the species tested, except for *Ceterach officinarum*. Furthermore, a delay was seen in the start of those species whose germination initiation is under 6 days. We have also seen that the non-germinated spores do not swell in the same way as those that do germinate, that is, there is no proper imbibition, just as Beri

& Bir (1993) demonstrated with *Pteris vittata*.

The viability percentage of some species has dropped by more than 30%, as is the case with *Asplenium onopteris*, *Dryopteris filix-mas*, *Cystopteris fragilis*, *Polystichum lonchitis*, *Athyrium filix-femina* and *Notholaena marantae*. There are also some species whose germination percentage has dropped less than 15%, as is the case of *Polystichum aculeatum*, *Pteris vittata* and *Thelypteris palustris*. As far as these differences are concerned, no relation of any kind has been observed as to them being able to influence the autecology of the species, the lesure-type spores, the ploidy level, nor the taxonomic group to which the different species belong.

In all the species where a viability loss occur after storage at 25ºC however, the late gametophyte development of germinated spores occurs correctly without any differences with regard to those conserved at ultra low temperatures. This provisionally indicates that the viability loss after 6 months storage is produced by the alteration to germination. However, there appears to be no damage in the genetic integrity of the material. It will be necessary to await further data with longer storage times in order to confirm this, and to also check the anomalous gametophyte proportion (digitate or callus-like forms) that is produced in the two-dimensional phase, just as Smith & Robinson (1975) point out.

We have selected these parameters as a development measure because those that measure early gametophyte development only indicate whether a delay in gametophyte development exists (see for example Beri & Bir (1993) and Camloh (1999)). However, we needed to record whether gametophyte development is completed correctly because it is the data relating to germplasm bank spore conservation that interest us.

The fact that no delay to the germination initiation is noted in spores that germinate from the sixth day, even though they lose viability at 25ºC, could be related to the ecology of the species, since these species correspond to environments with water deficiencies in the three cases observed: high mountainous terrain and xerophilous environments. Without going into physiological questions, even though it would be an interesting field to analyse, this might occur because the spore displays some germination-delaying mechanism, even when imbibed in such a way that it increases the chance of water availability during the subsequent gametophyte development. Therefore, the delay may assume the slower imbibition which was pointed out by Beri & Bir (1993). This behaviour could be a dormancy status as a survival strategy in extreme xeric environments (Kornas, 1985).

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HERBIVORY ON EPIPHYTIC FERNS OF A MEXICAN CLOUD FOREST

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Key words: cloud forest, epiphytes, leaf age, fertility, herbivory, Mexico

ABSTRACT

The often-stated hypothesis that ferns are attacked less by herbivores than are angiosperms has not been confirmed for terrestrial ferns. Several authors reported for terrestrial ferns and angiosperms the same number of insect pest species, and similar leaf damage of 5-38 percent, depending on species, leaf age, and type of vegetation. We studied five epiphytic species: *Pleopeltis crassinervata* (Fée) T. Moore, *Polypodium furfuraceum* Schltdl. & Cham., *P. plebeium* Schltdl. & Cham., *P. polypodioides* (L.) Watt, and *P. rhodopleuron* Kunze , in a Mexican cloud forest to test the hypothesis that epiphytic ferns have less leaf damage than terrestrial ferns. For each species we tagged 14-30 sections of tree branches and marked each fern leaf individually. For each leaf, herbivory was estimated as leaf area loss for each pinna, using a scale of seven damage classes (0%, less than or equal to 10%, ≤ 25%, ≤ 50%, ≤ 75%, ≤ 100%, 100%), in February 2003 and February 2004. In 2004, we counted the number of marked and unmarked new leaves to calculate leaf life-span. Leaf damage depended strongly on species and leaf life-span, but generally did not differ from the values reported for three terrestrial fern species in the same forest site (5.8-11.1%). *P. furfuraceum* and *P. rhodopleuron* were the least damaged species in both years with 8.4 - 10.7 % mean leaf area loss, while *P. plebeium* had the highest leaf area losses of 21.2 - 22.0 %. The highest leaf damage in *P. plebeium* might be a consequence of its longer leaf life-spans of 29.5 ± 3.4 months, while *P. rhodopleuron*, the least damaged species, had the shortest leaf life-span of less than 12 months.

INTRODUCTION

Although the hypothesis that ferns are attacked less and damaged less by insects than are angiosperms is long established (Schneider 1892), it did not receive much attention until the publication of the classical article of Ehrlich and Raven (1964), who stated that "In fact, very few insects feed on ferns at all, a most surprising and as yet unexplained fact with no evident chemical or mechanical basis". As possible reasons, the lack of food sources such as flowers and fruits and the consequently lower co-evolution between insects and ferns, and the probable better biochemical defences were discussed. Biochemical analyses revealed tannins, cyanogenic glycosides, phytoecdysones and other toxic or deterring substances in ferns. However, their effect as defending mechanisms against insects was not stronger than that of biochemical compounds of angiosperms (SooHoo & Fraenkel, 1964; Southwood, 1973; Cooper-Driver, 1976; Lawton, 1976).

Table 1. Insect families with highest numbers of fern feeding species (summarized from Balick *et al.* 1978).

Insect family	Insect order	No. of fern feeding species
Aphididae	Homoptera	104
Curculionidae	Coleoptera	85
Tenthredinidae	Hymenoptera	48
Noctuidae	Lepidoptera	44
Miridae	Heteroptera	35
Diaspididae	Homoptera	27
Anthomyiidae	Diptera	20
Aleyrodidae	Homoptera	18
Lygaeidae	Heteroptera	18
Cixiidae	Auchenorrhyncha	14
Lecaniidae	Homoptera	12
Pyralidae	Lepidoptera	12
Pseudococcidae	Homoptera	11
Tortricidae	Lepidoptera	11

Balick *et al.* (1978) conducted the first preliminary field studies on this topic and found no evidence that ferns are damaged less than angiosperms. Hendrix (1980) concluded that some insect groups might be under-represented on ferns. Both authors ascertained that research data are too incomplete for a final conclusion. Balick's review lists data for 75 insect families feeding on ferns, with dominance of insects with sucking (vs. chewing) mouth parts. Homoptera, Coleoptera, Hymenoptera, Lepidoptera, and Heteroptera comprise the highest number of fern feeding species (Table 1). Most herbivorous species were reported for the cosmopolitan weed *Pteridium aquilinum* due to the search for biological control organisms (Kaplanis *et al.,* 1967; Carlisle & Ellis, 1968; Weiczorek, 1973; Lawton, 1976; Hendrix, 1977), while for other fern genera data obviously reflect our lack of knowledge, but not the real numeric interactions between insect herbivores and ferns (Table 2). Only 128 interactions refer to fern species, and not to genera or entire groups (Balick *et al.* 1978). Hendrix's (1980) review lists 465 fern-feeding insect species, but few ferns were identified to species level.

Other field studies by Hendrix and Marquis (1983) and Mehltreter and Tolome (2003) estimated damages to three terrestrial fern species, the first ones at La Selva (Costa Rica), the later ones at the same forest site as the present study in Mexico. Both studies confirmed that leaf damage on ferns is similar to that on angiosperms (5-15 %), although these may be caused by a smaller number of insect species.

Until now, leaf damage was studied only on terrestrial ferns. Consequently, our objective was to investigate whether epiphytic ferns present lower levels of leaf damage than terrestrial ferns, because we supposed that the limited availability of water and nutrients would result in lower growth rates and consequently better defence mechanisms to avoid leaf damage. Additionally, we investigated whether leaf age, leaf fertility and leaf life-span are positively correlated with leaf damage.

Table 2. Fern genera with highest numbers of attacking insect species (summarized from Balick *et al.* 1978).

Fern genus	No. of insect species
Pteridium	119
Asplenium	50
Cibotium	31
Dryopteris	26
Adiantum	26
Polypodium	23
Pteris	23
Nephrolepis	20
Cyathea	17
Athyrium	16
Polystichum	15
Blechnum	14

METHODS

The study site is a tropical lower montane forest 2.5 km south of Xalapa, Veracruz (19°30'N, 96°57'W), at 1300 m elevation. Climatic conditions are seasonal with a dry period from November to April, a mean annual temperature of 18°C and a mean annual precipitation of 1500 mm. We studied herbivory on five epiphytic fern species: *Pleopeltis crassinervata* (CR, 22 individuals), *Polypodium furfuraceum* (FU, 19), *Polypodium plebeium* (PL, 30), *Polypodium polypodioides* (PO, 14), *Polypodium rhodopleuron* (RH, 25). Each leaf was tagged, and classified as young (not fully expanded) or mature (fully developed), and as sterile or fertile. Finally we checked each leaf for the presence of miners, and estimated herbivory as leaf area loss for each pinna, using a scale of seven damage classes (0%, less than or equal to 10%, <=25%, <=50%, <=75%, <100%, 100%). Herbivory data were estimated during the dry season in February 2003 and one year later. Estimations for each pinna were averaged for each leaf and individual using the median of each damage class. Data were analysed with ANOVAS on ranks. Paired t-tests comparing individuals/branch sections were applied for each species to test for differences between years. T-tests were applied for differences between leaf ages and differences between sterile and fertile leaves. Leaf life-span was calculated as the mean leaf number per plant (of both years) divided by leaf production and multiplied by the number of months of the observation period. Statistical analyses were performed with SIGMASTAT (1995).

RESULTS AND DISCUSSION

In 2003, *Polypodium plebeium* had the highest leaf damage of 21.2 ± 1.9 %, while the other four species had similar and significantly lower levels of damage, 8.4 – 9.3 % (ANOVA on ranks, $H = 37.15$, $df = 4$, $P < 0.001$, Dunn's Method, $P < 0.05$)(Figure 1). In 2004 damage for *Pleopeltis crassinervata* and *Polypodium polypodioides* increased significantly (paired t-test, $t = -4.00$, $df = 20$, $P < 0.001$, and $t = -2.28$, $df = 11$, $P = 0.031$, respectively) to 17.9 ± 2.2 % and 16.0 ± 2.3 %, respectively, and was similar to that recorded for *P. plebeium* (22.0 ± 2.2 %). *Polypodium rhodopleuron* had the lowest

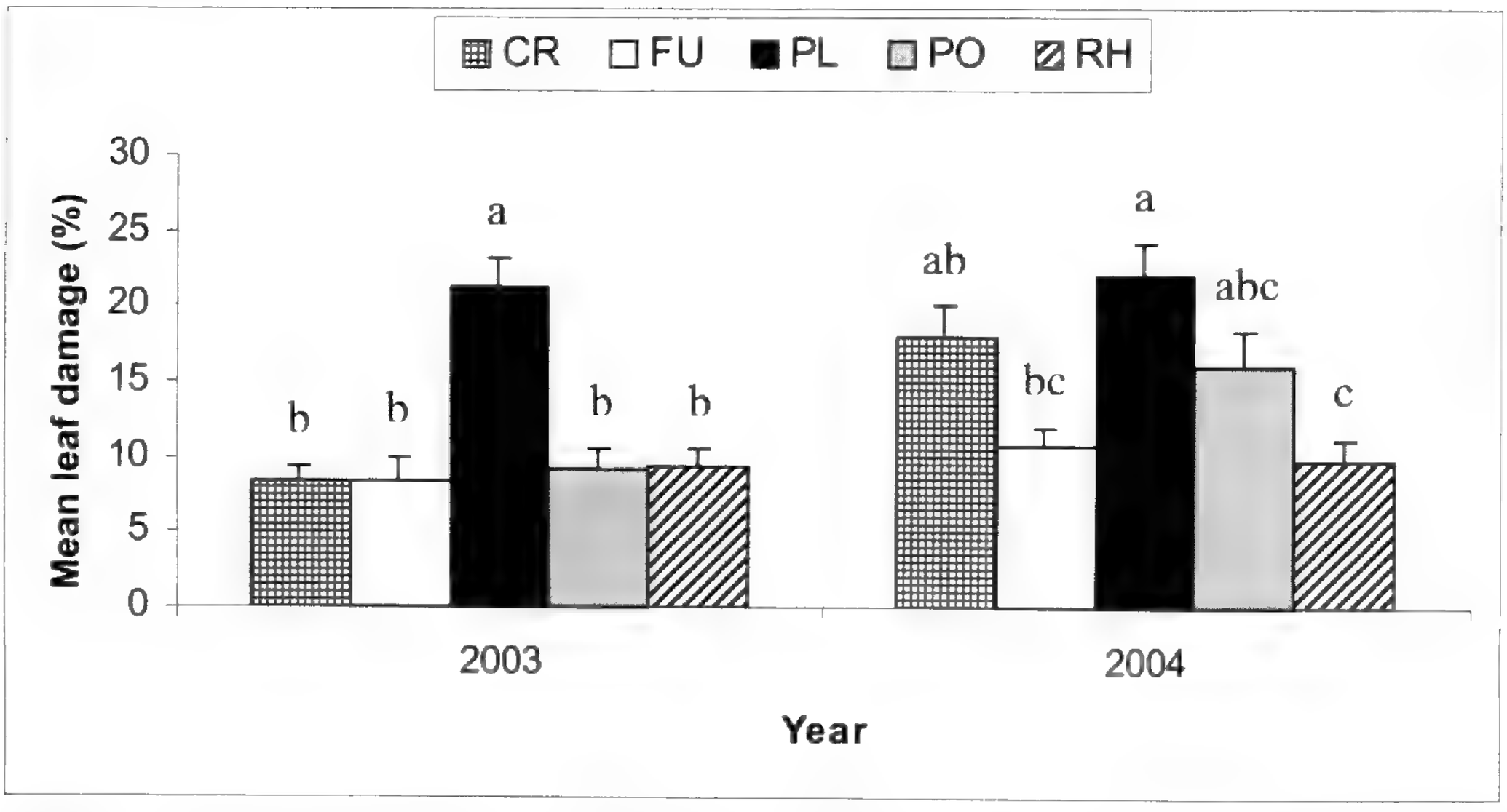

Figure 1. Mean leaf damage (+ 1 SE) of five epiphytic fern species in 2003 and 2004. Different letters indicate significant differences (ANOVA on ranks, $P < 0.05$) among fern species within the same year. CR = *Pleopeltis crassinervata* (n = 22 individuals in 2003, 21 in 2004), FU = *Polypodium furfuraceum* (19, 17), PL = *Polypodium plebeium* (30, 30), PO = *Polypodium polypodioides* (14, 12), RH = *Polypodium rhodopleuron* (25, 24)

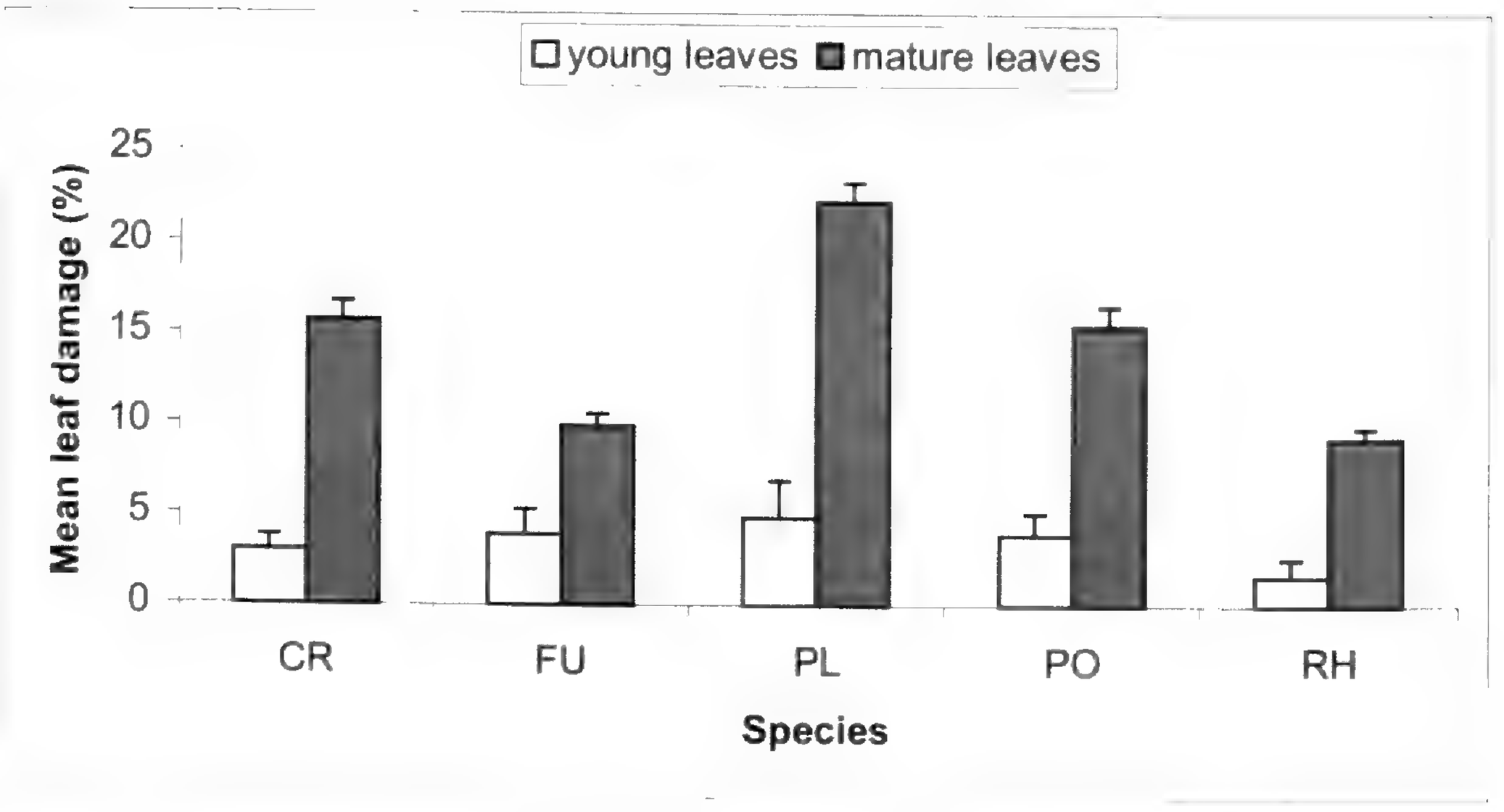

Figure 2. Mean leaf damage (+ 1 SE) of young and mature leaves of pooled data of 2003 and 2004. Differences between leaf ages were significant for all species (t-test, $P < 0.001$). CR = *Pleopeltis crassinervata* (n = 93 young leaves, 331 mature leaves), FU = *Polypodium furfuraceum* (31, 190), PL = *Polypodium plebeium* (27, 347), PO = *Polypodium polypodioides* (41, 244), RH = *Polypodium rhodopleuron* (6, 374).

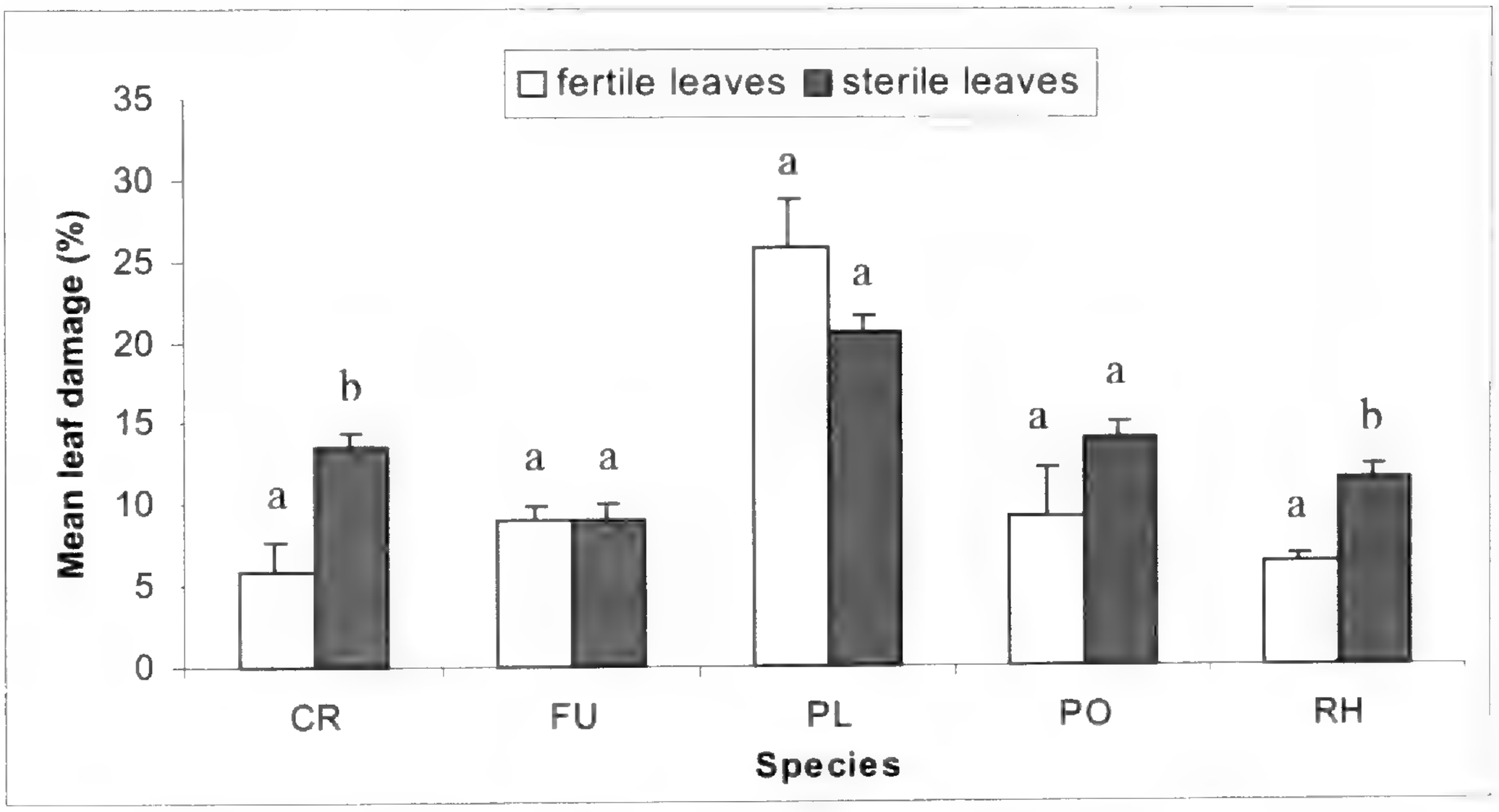

FIG. 3. Mean leaf damage (+ 1 SE) of fertile and sterile leaves of pooled data of 2003 and 2004. Different letters indicate significant differences (t-test, $P < 0.05$) between leaf types of the same species. CR = *Pleopeltis crassinervata* (n = 36 fertile leaves, 388 sterile leaves), FU = *Polypodium furfuraceum* (133, 88), PL = *Polypodium plebeium* (25, 349), PO = *Polypodium polypodioides* (21, 264), RH = *Polypodium rhodopleuron* (176, 204).

levels of damage of 9.7 ± 1.5 %. High leaf damage on *P. plebeium* may be explained by a higher number of feeding insect species or a higher number of individuals of the same species. The leaf damage to three terrestrial fern species at the same site (5.8-11.1 %, Mehltreter & Tolome, 2003) was similar to or slightly lower than that on the epiphytic species, but this may be a consequence of their shorter life-span (K. Mehltreter, pers. obs.). By contrast, leaf damage of epiphytic orchids and bromeliads in the same forest was about an order of magnitude lower than in ferns (Winkler et al., 2005).

We found no significant correlation between leaf damage and leaf life-span, possibly because of the low number of studied species. However, *Polypodium plebeium* had the greatest damage and the longest leaf life-span of 29.5 ± 3.4 months while the leaves of *Polypodium rhodopleuron* had the shortest life-span of 12.0 ± 0.0 months and the less damage. In fact, the leaf life-span of this species is probably less than 12 months, because all leaves appear to be shed in the dry season, but an annual data survey cannot resolve shorter time-spans. The other three species had intermediate leaf life-spans of 20.5 ± 2.9 months (*Polypodium polypodioides*), 20.6 ± 2.0 months (*Polypodium furfuraceum*) and 20.8 ± 3.3 months (*Pleopeltis crassinervata*) and intermediate levels of leaf damage (Figure 1).

On all species mature leaves were significantly more damaged than young leaves (t-test, $P < 0.001$, Figure 2), which indicates that herbivores do not feed exclusively on young leaves of understory plants as reported by Coley & Aide (1991), but continue feeding on mature leaves, as we also directly observed.

Sterile leaves showed greater damage than fertile leaves in *Pleopeltis crassinervata* ($t = -2.489$, $df = 422$, $P = 0.013$) and *Polypodium rhodopleuron* ($t = -5.021$, $df = 378$, $P < 0.001$), but in the other species there was no difference (Figure 3). These results

confirm the observations of Mehltreter & Tolome (2003) on terrestrial ferns that fertile leaves are not exposed to a higher selective pressure of herbivores, especially if we suppose that both leaf types of species with monomorphic leaves possess similar life-spans (Mehltreter & Palacios-Rios, 2003).

Miners affected four of the five fern species, and were found on 0.9 % (*Pleopeltis crassinervata*), 2.4 % (*Polypodium plebeium*), 3.6 % (*Polypodium furfuraceum*) and 17.4 % (*Polypodium rhodopleuron*) of the leaves. *Polypodium polypodiodes* was not damaged by miners. *Polypodium rhodopleuron* was the species with the shortest leaf life-span, but the heaviest attack by miners. Short-living leaves normally possess fewer biochemical defences, and consequently might be more likely to be infested with miners.

CONCLUSIONS

We cannot confirm our hypothesis that epiphytic ferns are better protected against herbivores and consequently less damaged than terrestrial ferns. However, we are able to conclude that:

1. Leaves are attacked by herbivores during their entire life-span and not only during their early development.

2. Leaf life-span may be one of the most significant factors to interpret results on herbivory, and should be registered within herbivory studies.

3. Damage of fertile leaves is similar to sterile leaves.

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BIODIVERSITY AND CHOROLOGY OF PTERIDOPHYTES FROM BUENOS AIRES PROVINCE, ARGENTINA

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Key words: Biodiversity, Chorology, Pteridophytes, Buenos Aires, Argentina.

Buenos Aires Province is situated between 33° 16′- 41° 02′ S and 56° 39′- 63° 23′ W. It has an area of 307.571 km^2 and is the biggest and the most populated province in Argentina. Studies of pteridophytic diversity and chorology for this region were carried out as a first step for the conservation of species. In the studied area 20 families, 41 genera and 87 specific and infraspecific taxa are recorded. Pteridaceae is the most diversified family. The biodiversity of Buenos Aires is less rich than that of northwestern and northeastern Argentina but richer than the boundary provinces. For the chorological study, 0° 10′ x 0° 15′ latitude x longitude squared maps were elaborated and the presence of taxa was represented by dots. The analysis of the maps shows that the species are concentrated in three areas of biodiversity: 1) Tandilia hills 2) Ventania hills 3) La Plata estuary environs. Between these three areas there is a notable decrease in the number of squares occupied by Pteridophytes, with the only records being for Azollaceae and Marsileaceae. The squares situated at 38° S have the richest biodiversity of the province and in these areas conservation should be prioritised.

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The BRITISH PTERIDOLOGICAL SOCIETY was founded in 1891 and today continues as a focus for fern enthusiasts. It provides a wide range of information about ferns through the medium of its publications and other literature. It also organises formal talks, informal discussions, field meetings, garden visits, plant exchanges, a spore exchange scheme and fern book sales. The Society has a wide membership that includes gardeners, nurserymen and botanists, both amateur and professional. The Society's journals, *The Fern Gazette*, *Pteridologist* and *Bulletin*, are published annually. *The Fern Gazette* publishes matter chiefly of specialist interest on international pteridology, the *Pteridologist*, topics of more general appeal, and the *Bulletin*, Society business and meetings reports. **WWW site**: http://www.eBPS.org.uk

Membership is open to all interested in ferns and fern-allies. **SUBSCRIPTION RATES** (due on 1st January each year) are Full Personal Members £20, Personal Members not receiving *The Fern Gazette* £16, Student Members £10, Subscribing Institutions £33. Family membership in any category is an additional £2. Applications for membership should be sent to the Membership Secretary (address above) from whom further details can be obtained. (Remittances made in currencies other than Sterling are £5 extra to cover bank conversion charges.) **Airmail** postage for all journals is an extra £4, or for those not receiving *The Fern Gazette* £2.50. **Standing Order** forms are available from the Membership Secretary and the BPS website.

Back numbers of *The Fern Gazette*, *Pteridologist* and *Bulletin* are available for purchase from P.J. Acock, 13 Star Lane, St Mary Cray, Kent BR5 3LJ; E-mail: BackNumbers@eBPS.org.uk

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