ALLOZYME, SPORE AND FROND VARIATION IN SOME SCOTTISH POPULATIONS OF THE FERNS *CYSTOPTERIS DICKIEANA* AND *CYSTOPTERIS FRAGILIS*

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Cystopteris dickieana R. Sim is a fern species that currently receives legal protection in Britain on the basis that it is endemic to Scotland and extremely rare. However, ever since it was first discovered in the 1830s, there has been considerable debate about its taxonomic status within the *Cystopteris fragilis* complex. This debate centres on the relative importance of two characters, the architecture of the fronds and the surface sculpturing of the spores, in delimiting *C. dickieana* from other taxa in the *C. fragilis* complex and is complicated by the fact that most comparative studies reputed to have included '*C. dickieana*' have failed to include either the type material of *C. dickieana* or material from the 'type population' (i.e. the population of plants, of uniform and distinctive morphology, that still grows at the type locality for *C. dickieana*). The principal aim of this investigation was to generate some of the critical data and information currently missing from the debate concerning the taxonomic status of *C. dickieana* by including material from the 'type population' in an allozyme study of several Scottish *Cystopteris* populations. The frond architecture, spore sculpturing, and allozyme banding patterns were investigated in five natural and two cultivated populations of the *C. fragilis* complex. All plants from the 'type population' of *C. dickieana* had the distinctive frond architecture and rugose spores of the type material. Two populations comprised plants that had the frond architecture and echinate spores typical of *C. fragilis*. Also examined were two populations containing plants that had frond architecture typical of *C. fragilis* but rugose spores similar to those of the type material of *C. dickieana*. These plants occurred with typical *C. fragilis* in one of the populations and with typical *C. dickieana* in the other. In the latter population, situated close to the 'type population' for *C. dickieana*, a third, 'smooth' spore type occurred in nine out of 55 fertile plants. Consistent and analysable allozyme results were obtained for seven enzymes at ten loci: eight loci were polymorphic and all but the smallest population showed variation at one or more loci. In total, 22 allozyme phenotypes were recorded in 226 individuals. No consistent correlation was found between frond architecture, spore sculpturing and allozyme banding pattern. Our results provide no support for the recognition of *C. dickieana* as a distinct species related to *C. fragilis*.

Keywords. *Cystopteris baenitzii*, spore bank, spore sculpturing, spore type, conservation, frond architecture.

INTRODUCTION

Cystopteris dickieana R. Sim is a fern species that currently receives legal protection in Britain on the basis that it is endemic to Scotland and extremely rare. However,

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ever since it was first discovered in the 1830s, there has been considerable debate about its taxonomic status within the *Cystopteris fragilis* complex. The complexities of this debate have recently been published (Dyer *et al*., 2000), so only the most crucial points are presented here. The debate centres on the relative importance of two characters, the architecture of the fronds and the surface sculpturing of the spores, in delimiting *C. dickieana* from other taxa in the *C. fragilis* complex. The type specimens of *C. dickieana* have distinctive fronds. Plants with similar frond architecture have, to date, been recorded growing naturally only at the site in Scotland from which the type specimens were collected and at one other site nearby. The type specimens of *C. dickieana* also have mature spores with surface sculpturing often described as 'rugose'. These are distinctive and unusual in the genus *Cystopteris*, in which most taxa have 'echinate' spores. However, rugose-spored plants have been recorded not only at, and near, the type locality in Scotland but also at many other sites in the northern hemisphere in populations of plants defined largely on the basis of frond architecture as *C. fragilis* (L.) Bernh. or *C. baenitzii* Dörfl. This indicates that spore sculpturing should not be used alone to delimit *C. dickieana* from other taxa within the *C. fragilis* complex but, despite this, the literature on '*C. dickieana*' contains many reports of studies on material identified as *C. dickieana* solely on the basis of spore sculpturing. This, combined with the fact that most comparative studies have also failed to include material known to have come from the type locality, has resulted in considerable and continuing uncertainty over the taxonomic status and distribution of *C. dickieana.*

The uncertainty over the taxonomic status and distribution of *C. dickieana* has important implications for the Scottish and British fern flora and its conservation (Lusby & Wright, 1996). *C. dickieana* currently qualifies for legal protection because it is one of five fern species now listed in Schedule 8 of Part 1 of the Wildlife and Countryside Act, 1981. The collection of plants or even small frond or spore samples, for any purpose, is allowed only after a collecting licence has been obtained from *Scottish Natural Heritage*. Inclusion within the taxon '*C. dickieana*' of plants with *dickieana*-like spores but *fragilis*-like fronds would extend this legal protection to all such plants elsewhere in Britain (e.g. Tennant, 1996), even though these plants can be distinguished from the common, unprotected, *C. fragilis* only after removal of fertile frond material and microscopic examination of spores in the laboratory. As such, it would, in practice, be impossible for the statutory conservation organizations (*Scottish Natural Heritage*, *English Nature* and *Countryside Council for Wales*) to enforce the legal protection. On the other hand, if *C. dickieana*, however defined, were to become accepted as a variety of *C. fragilis*, this legal protection would be lost even for the *C. dickieana* 'type population' (i.e. the population of plants, of uniform and distinctive morphology, that still grows at the type locality for *C. dickieana*) because currently no plants below species level are listed in Schedule 8 of the Wildlife and Countryside Act.

It was against this background that we investigated allozyme and spore traits of the *C. dickieana* 'type population' and compared it with six other Scottish popu-

lations. These included: a nearby population containing plants that resembled the type material of *C. dickieana*, as well as others with frond architecture more typical of *C. fragilis*; a population of plants, all of which had frond architecture typical of *C. fragilis* but which had been reported to produce echinate and rugose spores; two populations of plants that were in all ways typical of *C. fragilis*; and two populations in cultivation at the Royal Botanic Garden Edinburgh (RBGE). This was the first allozyme study of *Cystopteris* to include material from the 'type population' for *C. dickieana* and its principal aim was to generate some of the critical data and information currently missing from the debate concerning the taxonomic status of *C. dickieana.*

MATERIALS AND METHODS

Populations examined

The Cave population (or *C. dickieana* 'type population'). This population grew within an east-facing sea cave in schist-gneiss layers dipping c.45 degrees south which form headlands overlooking the North Sea near the village of Cove in Kincardineshire, Scotland. This is the type locality for *C. dickieana* (for details see Marren, 1984). Because of the restricted distribution of this fern and the nature of the site, this locality can be identified with greater precision and confidence than almost any other classic fern site in Scotland. There can be no doubt that this was the population visited by a succession of botanists over a period of more than 150 years. We visited the site twice in 1993. All the *Cystopteris* plants grew within the cave, some in deep shade, and many of them were out of reach across the roof. They all appeared to have the same characteristic frond architecture as the type material of *C. dickieana.*

The Rocky Overhang population. This population grew in a smaller, more sheltered site about 0.8km north of the *Cave* population. The site was a shallow cave (or recess) at the foot of a rocky overhang, half-way up a cliff, above a small cove separated from the type locality by a headland. This was probably the locality observed by Dickie in about 1845: 'About half a mile North from the Cove, there is a dripping cave where there is a variety near the last. I have known it there for the last fifteen years' (Dickie, 1860). *Cystopteris* growing within the recess exhibited frond architectures ranging from that which characterizes the type material (and 'type population') of *C. dickieana*, to that more typical of *C. fragilis*. This has probably been a feature of this population for a long time. Sutherland (1858) noted that the most typical specimens of *C. dickieana* came from one locality (presumably the cave) while 'in the other places there is a tendency to become identical with *dentata*.' [note: *dentata* is a member of the *C. fragilis* complex that has sometimes been given varietal or higher taxonomic status. It is only occasionally recognized in Britain today and then only as a variety of *C. fragilis* (Tennant, 1996; Dyer *et al*., 2000).] Marren (1984) was probably referring to this locality when he stated '. . .the nearest known station is separated from the type locality by a rocky headland and there the

fern is said to be morphologically dissimilar from the type and closer to *C. fragilis*.'. Although there is some uncertainty regarding the existence and position of other local populations, none has been reported between the *Cave* and the *Rocky Overhang* populations (Marren, 1984).

The Ben Lawers population. This *Cystopteris* population grew in a narrow, sheltered, rocky stream gorge, on the lower slopes of Ben Lawers, Perthshire (altitude 230m, about 100km inland and 140km south-west of the *Cave* and *Rocky Overhang* populations). It was first examined by Mr David Tennant (pers. comm.), who determined that 14 or more plants with rugose spores were present (unpublished data, 1992; see also Tennant, 1996). Although the fronds resembled *C. fragilis*, these plants were thus identified as *C. dickieana* and came under legal protection.

The Schiehallion population. This population grew in a small area of exposed limestone pavement (a habitat which is rare in Scotland), on the lower slopes of Schiehallion, Perthshire (altitude 330m, about 100km inland and 16km north-north-east of the *Ben Lawers* population). All plants were typical of *C. fragilis*.

The Kindrogan population. This population grew along a roadside bank near the Kindrogan Field Centre, Perthshire (altitude 300m, about 70km inland and 45km north-east of the *Ben Lawers* population). All plants were typical of *C. fragilis.*

The Rocky Overhang Spore Bank population. As part of an earlier study (Lindsay, 1995; Dyer & Lindsay, 1996), sporophytes of *Cystopteris* were recovered from the natural soil spore bank at the rocky overhang at Cove. Soil samples taken from this site were cultured in petri dishes in an incubator at 20°C, taking steps at all stages to avoid contamination by airborne spores. When sporelings developed, they were 'potted-on' and raised to maturity in a glasshouse at the RBGE. These *Cystopteris* plants constitute the *Rocky Overhang Spore Bank* population.

The Balerno population. This population was growing at RBGE. The spores from which it was raised were collected from plants, typical of *C. fragilis*, growing in Dyer's garden at Balerno, on the south-west fringe of Edinburgh (altitude 155m, about 50km inland, 100km south-east of the *Ben Lawers* population). The origin of the plants in Dyer's garden was unknown but over the last 30 years they have colonized many new sites in the garden from one small group of plants present in 1968.

Sampling

Frond samples were collected in August and September 1993 during the sporing season. A collecting licence was issued to Lindsay by *Scottish Natural Heritage* for the populations then identified as *C. dickieana*, i.e. the *Cave*, *Rocky Overhang* and *Ben Lawers* populations. The number of plants sampled from each population was as follows: *Cave* – 31; *Rocky Overhang* – 67; *Ben Lawers* – 34; *Schiehallion* – 31; *Kindrogan* – 6; *Rocky Overhang Spore Bank* – 27 and *Balerno* – 30. As most popu-

lations were small, these sample sizes represented a large proportion of the individuals present. One healthy and, if possible, sporulating frond was taken from each plant. These were placed in separate 'ziploc' polyethylene bag and carefully numbered to enable cross-reference to field notes. The bagged fronds were maintained in ice-cooled chests for transport, then in a refrigerator at 4°C. Within 3 days of collection a small piece of each frond was removed and prepared for allozyme analysis; another small piece containing sori was removed and prepared for light and scanning electron microscopy. The remainder of each frond was pressed, dried, and photocopied to maintain a record of frond architecture.

Electrophoresis

Homogenates were prepared by grinding 1–2cm^2 of each sample in the tris-based 'microbuffer' of Werth (1985), pH 7.5 (see also Soltis *et al*., 1983), in a cold porcelain spot plate. Extracts were absorbed onto Whatmann 3MM filter paper wicks. These were either loaded onto 12% starch-gels and electrophoresed while fresh or immediately frozen to −40°C. Frozen samples were electrophoresed within 6 weeks. Frozen replicates of fresh material showed no loss of enzyme activity.

Starch-gel electrophoresis was used to determine allozyme phenotypes for seven enzyme systems. Isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6-PGD), and shikimate dehydrogenase (SKDH) were resolved on the morpholine-citrate system, pH 8.0 (Werth, 1991). Glucose-phosphate isomerase (GPI) and phosphoglucomutase (PGM) were resolved on the sodium borate/tris-citrate discontinuous buffer system #6 of Soltis *et al*. (1983). Hexokinase (HK) was resolved on the lithium borate/tris-citrate discontinuous buffer system #8 of Soltis *et al*. (1983). The lithium and morpholine gels were run at 50 milliamps constant, the #6 gels at 250 volts constant. Iced gels were electrophoresed in a refrigerator at 4°C for c.6h. Staining schedules were similar to Soltis *et al*. (1983) but employed the 'zymecicle' methodology of Werth (1990).

Allozyme band patterns were scored immediately upon development (within 2h of staining for most enzymes; overnight in the refrigerator for HK) after which the gels were photographed with colour print film and discarded. For scoring, the loci were numbered with the most anodal assigned the lowest numbers; within loci, the most anodal bands were assigned the lowest numbers. Scoring of alleles was aligned by reference to two 'standard' plants (a typical *C. fragilis* from the *Balerno* population, and a typical *C. dickieana*, raised from spores collected a few years earlier from the *Cave* population, and in cultivation at RBGE) which were run on each gel. Additionally, at the end of the study, most of the various electromorphs recovered were re-run together on one alignment gel to enhance accurate comparative scoring. Many plants with unusual band patterns or missing bands were re-run to verify their scoring.

Spores

Spores of every fertile frond analysed allozymically were examined with a light microscope while the frond samples were still fresh. A wet mount was prepared after selecting the most mature intact sporangia from at least three sori with some empty sporangia (thereby ensuring that the examined spores were mature). The slide was fully scanned at 100 × so several hundred spores were viewed. In addition to noting the type of surface sculpturing on each spore, five spores were measured with an optical micrometer, and five spores were examined at 400 × to check that there were no significant diagnostic features visible at that magnification that could not be detected at 100 ×. Light micrographs were taken as required. Examples of all spore types detected by light microscopy were also photographed using a scanning electron microscope (a Zeiss DSM962).

RESULTS

Representation of electromorph patterns

The presentation of allozymal data requires consideration of the possible factors contributing to the patterns produced on the gels (Wendel & Weeden, 1989). For polyploids, such as *Cystopteris fragilis* s.l., these factors are more numerous than those for diploids and include the possibility of several genomes expressing alleles from equivalent loci, yielding comigrating bands, fixed heterozygotes, reciprocal gene silencing, etc. These factors may be synergistic. The germane literature is complicated by the various ways workers have presented their data. Werth (1989) has discussed this problem and has suggested a method of designating enzyme loci and alleles of polyploids (his method four), which is utilized here for malate dehydrogenase (MDH). However, in the other enzyme systems the electromorph patterns that we obtained resembled those of diploids, so we have presented them in that way. We recognize that the genetic mechanisms producing the patterns are complex (as noted in the discussion), and that the genetic basis of the patterns cannot be inferred as if the patterns were produced by diploids.

Glucose-phosphate isomerase (GPI). GPI (a dimer) produced banding patterns that were considered indicative of two loci despite the fact that the anodal *Gpi-1* locus was not resolvable. The *Gpi-2* locus was eventually interpreted as having three alleles. At first, it appeared that only single-banded patterns were present in all the populations (Table 1). However, when we analysed the *Balerno* population (from which the *C. fragilis* reference plant was taken), 22 of the plants produced a three-banded pattern and 8 (including the reference plant), exhibited a two-banded pattern with a pale anodal band and a darker cathodal one. As GPI frequently exhibits anodal 'ghost bands' (Kephart, 1990; Werth, 1991), we dismissed the pale anodal band of the *C. fragilis* reference plant as such until we noticed that the homozygous *C. dickieana* reference plant, run repeatedly in the adjacent lane, did not produce a ghost band. Perhaps the two-banded pattern is better explained as a *Gpi-2* hetero-

TABLE 1. Multilocus allozyme phenotypes and associated spore type recovered from Scottish *Cystopteris* populations. KEY: PHEN#, allozyme phenotype number; #IND., number of individuals analysed; S, spore type; E, echinate; R, rugose; Sm, smooth; St, sterile; A, absent; x, absent, possible 'null allele'

Enzyme Systems										
PHEN#	*Gpi-2*	*Hk*	*Mdh-1*$_A$	*Mdh-1*$_B$	*Pgd-1*	*Pgd-2*	*Pgm-2*	*Skdh*	S	#IND.
Cave (*C. dickieana* 'type population')										
1	2	2	1–2	1–2	1	1–2	2–3	2	R	25
2	2	2	1–2	1–2	1	1–2	3	2	R	6
Rocky overhang										
1	2	2	1–2	1–2	1	1–2	2–3	2	R	44
1	2	2	1–2	1–2	1	1–2	2–3	2	St	12
1	2	2	1–2	1–2	1	1–2	2–3	2	Sm	9
2	2	2	1–2	1–2	1	1–2	3	2	R	2
Rocky overhang spore bank										
1	2	2	1–2	1–2	1	1–2	2–3	2	R	19
1	2	2	1–2	1–2	1	1–2	2–3	2	St	7
1	2	2	1–2	1–2	1	1–2	2–3	2	Sm	1
Ben Lawers										
3	2	A	1–2	1–2	1	1–2	3	1	R	4
4	2	2	1–2	1–2	1	1–2	2–3	1	R	1
5	2	1	1–2	1–2	1	1–2	3	1	R	1
6	2	2	1–2	1–2	1	1–2	3	1–2	R	2
7	2	2	1–2	1–2	1	1–2	3	1	R	3
8	3	2	1–2	1–2	1	1–2	3	1	R	2
9	3	1	1–2	1–2	1	1–2	3	1–2	E	4
10	3	1–2	A	1–2	1	1–2	3	1–2	E	8
11	3	1	A	1–2	1	1–2	3	1–2	E	2
12	3	1–2	1–2	1–2	1	1–2	3	1–2	E	3
13	2	1–2	A	1–2	1	1–2	3	1–2	E	1
14	3	1–2	A	1–2	1	1–2	3	1–3	E	2
15	2	1	1–2	1–2	1	1–2	2–3	1–2	E	1
Schiehallion										
3	2	A	1–2	1–2	1	1–2	3	1	E	1
16	2	1	1–2	1–2	1	1–2	3	1–3	E	2
17	2	1	2	1	1	1–2	3	1–3	E	11
18	2	1–2	2	1	1	1–2	3	1–3	E	15
19	2	2	2	1	1	1–2	3	1–3	E	2
Kindrogan										
20	2	1	1–2	1–2	1	1–2	3	1–2	E	6
Balerno										
21	1–2	1	2	1	1–2	2	1–3	1–3	E	22
22	1–x	1	2	1	1–2	2	1–3	1–3	E	8

zygote with a cathodal 'null' allele (Jones *et al*., 1986). In the *Ben Lawers* population, two *Gpi-2* single band patterns are present. These two bands run very close together, so careful alignment was needed to distinguish them.

Hexokinase (HK). HK (a monomer) produced banding patterns that were considered indicative of one locus with two alleles. Most populations are fixed or nearly so for one or the other single band patterns but in the *Ben Lawers* and *Schiehallion* populations, all three band patterns were recovered (Table 1). This system always appeared slowly and produced faint bands. The missing data from the four individuals from the *Ben Lawers* population and the one from *Schiehallion* are more likely to be the result of unresolved bands than gene silencing.

Isocitrate dehydrogenase (IDH). IDH was monomorphic with one band in all plants examined. As this cannot be used for comparison of the populations in this study, IDH is not included in the data set of Table 1.

Malate dehydrogenase (MDH). MDH (a dimer), exhibited variation at several loci. The more anodal complex, (MDH-1), produced banding patterns that were considered indicative of two loci, $Mdh\text{-}1_A$ and $Mdh\text{-}1_B$, which resolved together but did not overlap fully. These two loci together expressed predominantly two patterns of bands (Table 1). In one pattern a broad anodal band (occasionally resolved into three bands when duplicate samples were electrophoresed on separate gels) was seen at $Mdh\text{-}1_A$ and a faint but well resolved three-banded pattern was seen at the cathodal $Mdh\text{-}1_B$ locus. The second band pattern consists of two closely spaced bands (often forming one broad band), with the more anodal running with the broad band in pattern one. Pattern one could arise if both $Mdh\text{-}1_A$ and $Mdh\text{-}1_B$ are heterozygous and the second pattern may result from a slow homozygote of $Mdh\text{-}1_A$ and a fast homozygote of $Mdh\text{-}1_B$.

The two common banding patterns exhibited by MDH-1 were not distributed randomly within the populations (Table 1). The *Rocky Overhang* and *Rocky Overhang Spore Bank* populations, the nearby *Cave* population, and the distant *Kindrogan* population exhibited only the double heterozygote pattern and the *Balerno* population showed only the double homozygote pattern. The *Schiehallion* population had only 3 double heterozygotes of 31 plants examined. The *Ben Lawers* population had 21 of 34 double heterozygotes, but is unusual in that 13 exhibited a third pattern where the anodal $Mdh\text{-}1_A$ band was missing.

A second, more cathodal, MDH-2 group produced evidence of genetic variation (in $Mdh\text{-}2_B$) within the *Rocky Overhang* and *Ben Lawers* populations and between these and some of the others, but the bands appeared close to the origin and were not always clearly resolved. For this reason MDH-2 is not included in the data set of Table 1.

6-Phosphogluconate dehydrogenase (6-PGD). 6-PGD (a dimer) produced two banding patterns that were considered indicative of two loci. One pattern consisted of two bands at the *6-Pgd-1* locus and one band at the *6-Pgd-2* locus. The second

pattern had one band at the *6-Pgd-1* locus and two bands at the *6-Pgd-2* locus. Only the *Balerno* population exhibited the first pattern, for which it was fixed (Table 1).

Phosphoglucomutase (PGM). PGM (a monomer) produced banding patterns that were considered indicative of 2 loci. *Pgm-1* was monomorphic with one band in all plants examined. As this cannot be used for comparison of the populations in this study, *Pgm-1* is not included in the data set of Table 1. *Pgm-2* locus exhibited three different band patterns each of which is fixed in one or more of the populations. In a diploid these could be explained as three alleles in 1–3 and 2–3 heterozygotes and a 3 homozygote.

Shikimate dehydrogenase (SKDH). SKDH (a monomer) produced banding patterns that were considered indicative of one locus with three alleles. Alleles two and three ran close together so alignment at the end of the study was necessary to delimit electromorphs accurately. All populations except that at *Ben Lawers* were fixed (or nearly so), for one band pattern (Table 1).

Frond architecture

The plants comprising the *Cave* population (or *C. dickieana* 'type population') consistently exhibited the distinctive frond architecture considered typical of *C. dickieana* (Fig. 1) as described by Sim (1848) and illustrated by Moore (1859). However, the plants comprising the nearby *Rocky Overhang* population exhibited a much greater range in frond architecture, with some plants resembling those at the cave and other plants resembling *C. fragilis* in having a narrower blade, more space between the pinnae, and narrower ultimate segments (Fig. 2). *C. fragilis* fronds often vary morphologically in response to microclimate, exhibiting slightly broader, more herbaceous ultimate blade segments when growing in moist, shaded places and more compact, coriaceous fronds in open, sunny, habitats. The fronds of all the *C. fragilis*-like plants in the uniformly cool, shaded, moist, rocky overhang were of the former type (Fig. 2). Fronds of plants in the *Ben Lawers* population varied from those resembling shade forms of *C. fragilis*, like those at the *Rocky Overhang*, to the more compact, coriaceous fronds typical of open habitats (Fig. 3). The *Balerno* and *Schiehallion* populations, growing in less shaded conditions, uniformly had more compact, coriaceous fronds (Fig. 4). The fronds of the *Kindrogan* population were similar.

The characteristic differences in frond architecture between the *Cave*, *Ben Lawers* and *Balerno* populations were maintained when the plants were grown for more than two years side by side in Dyer's garden at Balerno (Fig. 5).

Spores

The *Balerno*, *Schiehallion*, and *Kindrogan* populations produced only echinate spores (Table 1, Fig. 6a). The *Cave* population produced only rugose spores (Fig. 6b).

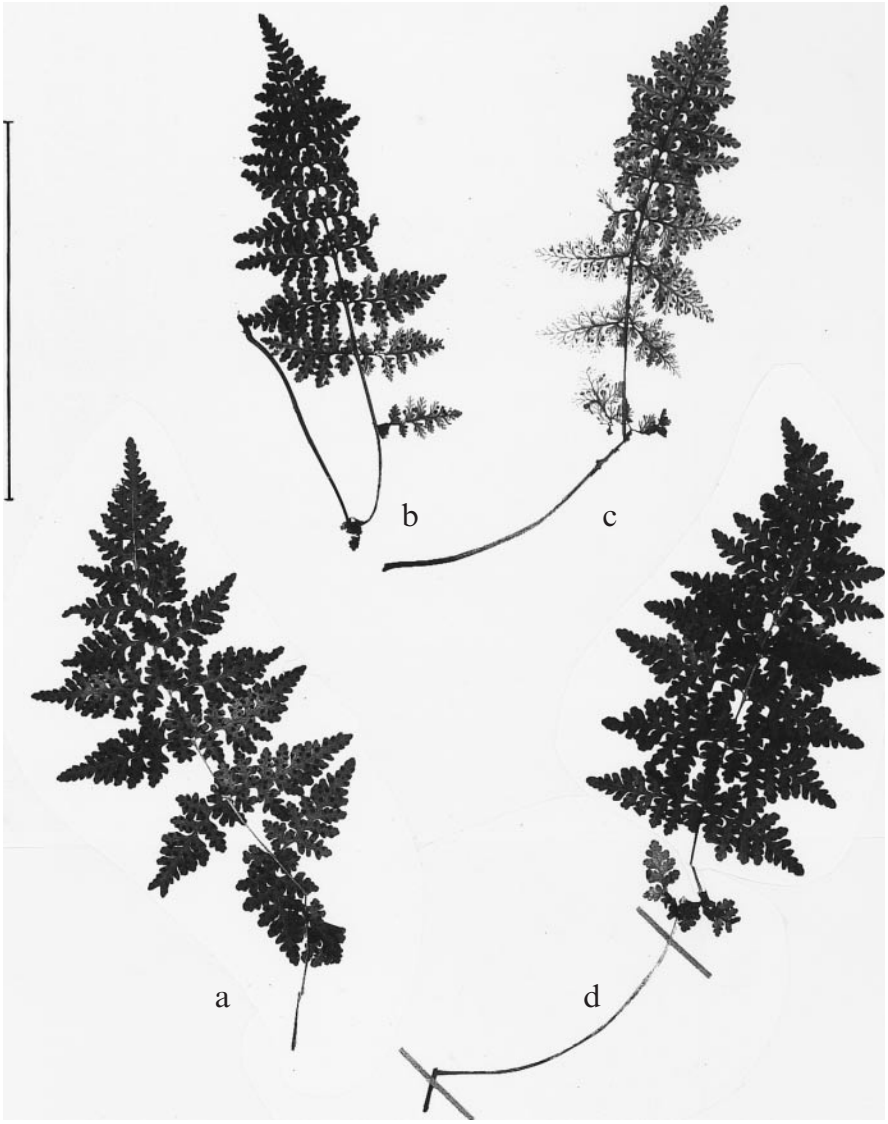

FIG. 1. Silhouettes (photocopies) of fronds collected at the type locality for *C. dickieana* (referred to in the text as the *Cave* population or 'type population'). All have rugose spores. b and c were included in the allozyme analysis. Scale bar = 10cm.

However, in the *Rocky Overhang* population, although most plants had rugose spores, a third spore type (Fig. 6c) was the only one recovered from 9 plants (Table 1). Exhibiting a fairly smooth surface when viewed under a light microscope, this spore type had a slightly granular surface, sometimes with a few threadlike strands, when viewed in a scanning electron microscope. One of the 20 fertile sporophytes in the *Rocky Overhang Spore Bank* population also had this 'smooth' spore type.

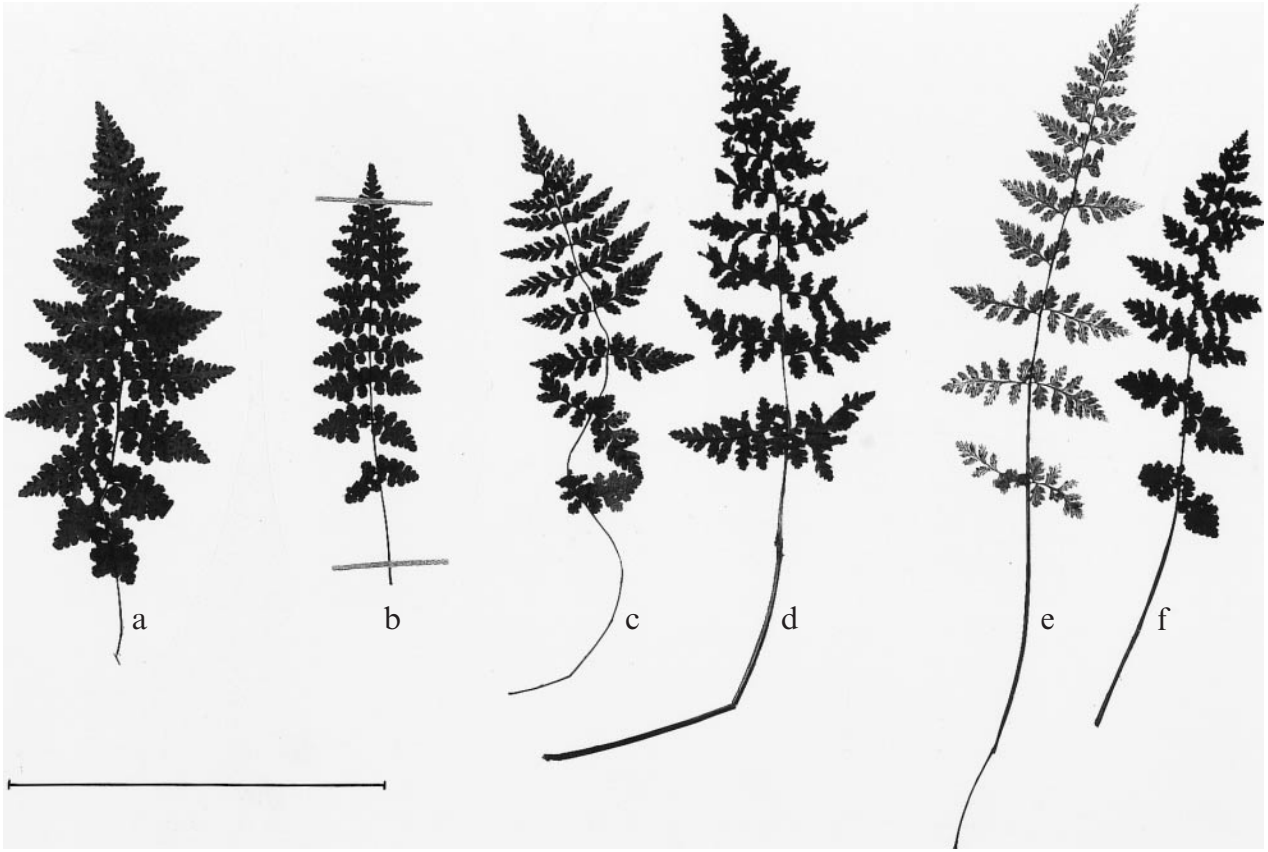

FIG. 2. Silhouettes of fronds collected from the *Rocky Overhang* population: a–d have rugose spores; e and f have smooth spores; c–f were included in the allozyme analysis. Scale bar = 10cm.

FIG. 3. Silhouettes of fronds collected from the *Ben Lawers* population. a–e have echinate spores; f–h have rugose spores. All were included in the allozyme analysis. Scale bar = 10cm.

FIG. 4. Silhouettes of fronds collected from the *Schiehallion* population (a–c) and from the *Balerno* population (plants raised at RBGE from spores collected at *Balerno*) (d–f). All have echinate spores. a–c were included in the allozyme analysis. d–f were fronds produced subsequently by plants previously sampled for allozyme analysis. Scale bar = 10cm.

FIG. 5. Silhouettes of fronds collected on the same day from cultivated plants growing next to each other in Dyer's garden at *Balerno*: a and b, plants raised from spores collected from the *Ben Lawers* population; rugose spores; c and d, plants of the garden population that provided the spores from which the *Balerno* population was raised at RBGE; echinate spores; e and f, plants raised from spores collected from the *Cave* population; rugose spores. Scale bar = 10cm.

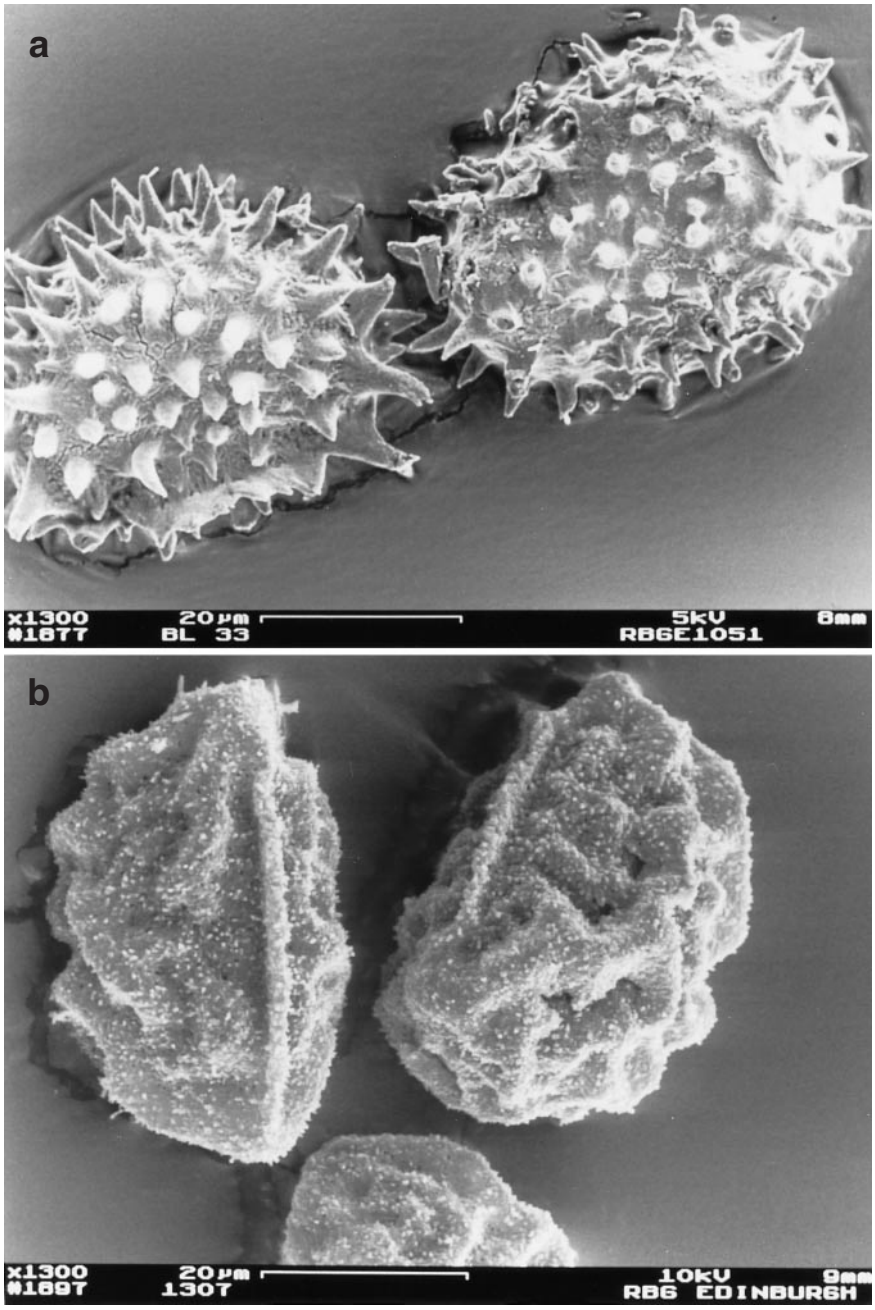

FIG. 6. SEM photographs of spores: a, echinate spores of plant L33 from the *Ben Lawers* population; b, rugose spores from plant L31 from the *Ben Lawers* population; c, smooth spore from plant R46 from the *Rocky Overhang* population.

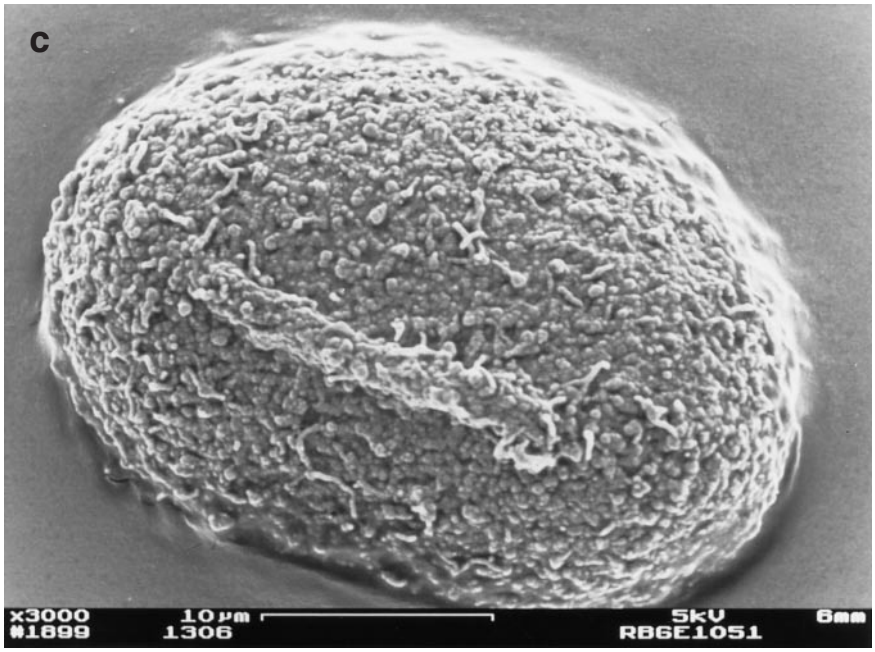

FIG. 6. Continued.

The *Ben Lawers* population yielded 13 plants with rugose spores and 21 with echinate spores. In samples from eight plants, both rugose and echinate spores were seen on the same slide. In all of these, one type was very common and the other type was rare; only the common type was seen inside sporangia. As plants with echinate and rugose spores were often growing in close proximity, it is probable that deposition of a few spores from neighbouring plants explains our results for these eight plants.

Spores averaged 45µm in length which is consistent with published values for tetraploid *Cystopteris* (e.g. Hagenah, 1961; Jermy & Harper, 1971; Pearman, 1976; Haufler *et al*., 1990; Parks & Mosser, 1998).

DISCUSSION

Interpretation of gels

Inferring the alleles present in an individual from the allozyme phenotypes produced by polyploids is complicated by many additional factors beyond those considered when evaluating diploids (Werth, 1989). These may include duplicate loci from the various genomes that produce overlapping or comigrating bands, patterns that, in a diploid would be indicative of a heterozygote but in a polyploid could be a double homozygote of two overlapping loci, and gene silencing (Werth & Windham, 1991). The band patterns produced by monomers may be especially difficult to interpret in polyploids when loci from both parents are expressed and the enzymes are running

close together or overlapping on the gel. For example, two loci, each with two alleles, could yield four types of two-banded patterns, none of which are heterozygotes; rather, they could represent various double homozygous combinations. The apparent 1–3 and 2–3 heterozygote patterns produced by PGM-2 in this study could, in fact, be double homozygotes in a tetraploid (Cai & Chinnappa, 1989). The lack of three- and four-banded patterns (Gottlieb, 1987) suggests that either two loci are completely overlapped or that one locus is silenced. The dimers that we examined yielded patterns consistent with published results in other ferns. For example, we detected four *Mdh* loci. Four scorable *Mdh* loci were found in *Dryopteris* (Werth, 1991), *Asplenium* (Werth *et al*., 1985), and the *Pellaea glabella* complex (Gastony, 1988).

Differential intensity of band staining in apparent heterozygote allozyme phenotypes has been suggested as a way of recognizing unbalanced genotypes in polyploids (Cai & Chinnappa, 1989; Weeden & Wendel, 1989). We observed differential intensity of two bands within some gels stained for SKDH and HK. This would suggest a tetraploid expressing an unbalanced genotype from two loci where the allozymes overlap. However, variation between gel runs made it impossible to score this intensity difference consistently and with confidence.

Except for MDH, the enzymes examined in this study of polyploid members of the *C. fragilis* complex have yielded electromorphs similar to those recovered from diploid *Cystopteris*. This is consistent with Haufler and Windham's (1991) conclusion that *C. fragilis* is an old, rather 'diploidized' polyploid taxon, having undergone much gene silencing. However, allozyme phenotypes of polyploids are often complex and difficult to interpret without comparison with relevant diploid progenitors (Werth, 1989).

The ability to conduct quantitative populational studies utilizing allozymes depends upon accurate determination of the alleles present and their frequencies (Gottlieb, 1977; Wendel & Weeden, 1989). For polyploids, frequently this is not possible. Ranker *et al*. (1994), did a populational genetic analysis of the tetraploid species *Asplenium adiantum-nigrum* after adapting allozymal data for input to the BIOSYS computer program (Swofford & Selander, 1981) which accepts data only from diploids. The assumptions required seem reasonable for *A. adiantum-nigrum*. However, we believe that the allozymal band patterns we recovered and the unavailability of allozymically well-characterized diploid progenitors for the *C. fragilis* complex indicate that such an approach is inappropriate here. Nevertheless, while a populational genetic analysis may not be possible, the electromorphs are genetically based, so direct comparisons of their distributions among the populations, especially when combined with observations on frond architecture and spore sculpturing (spore type) can help to clarify the status of *C. dickieana* within the *C. fragilis* complex.

The populations

The populations in this study are small, geographically isolated, and isozymically differentiated. All populations except *Ben Lawers* have very few multilocus allozyme

phenotypes and most multilocus allozyme phenotypes are unique to one population (Table 1).

The *Cave* and *Rocky Overhang* populations share the same two allozyme phenotypes, which are unique to them and quite distinct from those of the other wild populations. These two populations might have been established by short distance dispersal from one founder. Marren (1984) suggested that recolonization of the cave by spores from neighbouring populations was one explanation for the recovery from the extinctions recorded in about 1860 and 1918. However, a common source for these two populations would not explain the differences between them in the range of frond architectures and spore types. An alternative possibility is that the *Cave* population has resulted from a single colonization event producing a plant with the distinctive frond and spore phenotype which, by inbreeding, at least initially involving intra-gametophytic selfing, has produced and perpetuated a morphologically uniform population. There is evidence from experiments using spores collected at the type locality that 96% of gametophytes yield sporophytes when cultured in isolation, confirming that they are capable of becoming bisexual and undergoing intra-gametophytic selfing (detailed data presented in a poster; for abstract see Prada *et al*., 1993). The two allozyme phenotypes present in the *Cave* population differ by only one band (in *Pgm-2*); they could be the result of a mutation after colonization. Alternatively, initial colonization was by two spores differing only in *Pgm-2*. Perhaps therefore the cave was colonized by only one or two spores from the *Rocky Overhang* population, where identical plants occur among several other frond, spore and allozyme variants. On the other hand, recovery from apparent extinction in the cave could have been by natural regeneration of the same genotypes from a persistent soil spore bank at the site rather than by recolonization.

The plants of the *Rocky Overhang* population varied morphologically (Fig. 2), ranging from the form seen in the nearby cave (Fig. 1) to that seen at *Ben Lawers* (Fig. 3). Allozymically, the *Cave* and *Rocky Overhang* populations shared the same two unique multilocus allozyme phenotypes (Table 1). While all fertile plants in these populations had non-echinate spores, in the *Rocky Overhang* population, nine of 55 fertile plants had a third type of spore; a 'smooth' type (Fig. 6c). [This spore type was also recovered from Scottish *Cystopteris* (identified as *C. dickieana*) collected in Ardnamurchan by Jermy & Harper (1971), who included a fine drawing (their Fig. 3), and was mentioned in the discussion of rugose spores in Pearman's (1976) thorough electron microscopy study of *Cystopteris* spores obtained over a wide geographical range]. The plants with smooth spores were not morphologically distinct (Fig. 2). In fact, there was no correlation between frond architecture, allozyme phenotype or spore type.

The *Balerno* population also exhibited only two allozyme phenotypes, both unique to it. As it is not known how many plants contributed to the spore collection from which this population was raised there are two possible explanations for this result: 1, the original spore collection came from two genetically distinct plants in Dyer's garden; or 2, the sample reflects the situation in the garden population as a whole which has recently established from a small founder inoculum.

Dyer and Lindsay recovered 27 sporophytes from a spore bank taken from the rocky overhang. These plants (comprising the *Rocky Overhang Spore Bank* population) exhibited a similar range in frond architecture as the plants comprising the *Rocky Overhang* population (their presumed progenitors) and they all exhibited the most frequent allozyme phenotype found in that population. It is noteworthy that the smooth spore type was recovered from one of the 20 fertile plants; in the original population nine out of 55 fertile plants had smooth spores. This indicates that fern sporophytes recovered from soil spore banks can reflect the populations that produced them. With respect to spore type, it is further confirmation (in addition to the fact that the spores were taken from fresh sporangia and not dried before examination) that the smooth *and* rugose type spores are genetically based and not an artifact of drying as maintained by Jermy & Harper (1971) and Pearman (1976). It is certainly possible that a population of *Cystopteris fragilis* may be found that is fixed for the smooth spore type.

The *Ben Lawers* population is central to clarifying the status of *C. dickieana*. In this population of *C. fragilis*-like plants we discovered some plants with echinate spores and some plants with rugose spores; we could not predict spore type from the frond architecture in this population. This is consistent with the conclusions of Hagenah (1961) and Haufler & Windham (1991) in North America and Berg (1992) in Norway. After studying over 900 sheets of specimens, Hagenah found that 'Attempts to predict the spore type of herbarium specimens from their leaf architecture were incorrect more times than they were right.'. Haufler and Windham stated that '. . .it is not possible to find a consistent set of sporophytic characteristics that correlates with rugose spores. Isozyme banding patterns of plants with rugose spores did not differ from those of *C. fragilis* having echinate spores.'. Having examined 20 populations of *C. fragilis*-like plants that contained individuals with echinate spores (*C. fragilis*-type) and individuals with rugose spores (*C. dickieana*-type), Berg concluded '. . .gross morphological variation does not support the assumption that those species can be distinguished by means of spore characteristics. Taxonomic maintenance of *C. dickieana* is questioned.'.

Based on his discovery of some plants with aborted spores, Berg (1992) also suggested that populations containing otherwise indistinguishable individuals of both spore types might comprise two morphologically indistinguishable species that hybridize. However, our allozyme data suggests otherwise. A majority of the 21 plants with echinate spores in the *Ben Lawers* population have the combined SKDH '1–2' *and* HK '1–2' band patterns (Table 1), whereas among the 13 plants with rugose spores in the same population this combination of banding patterns is entirely absent. Moreover, none of the six multilocus allozyme phenotypes associated with rugose spores in the *Ben Lawers* population were recovered from the *Cave* population. In other words, within the *Ben Lawers* population, allozyme phenotypes do correlate with spore type but, when either subset of allozyme phenotypes is compared with those of the *C. dickieana* 'type population', no correlation exists (Table 1). When allozyme phenotypes are compared to spore type across all populations, only

the *Hk* slow band is strongly correlated with rugose spores. The *Ben Lawers* plants illustrate the allozymal and spore variation possible in a population of *C. fragilis*.

CONCLUSIONS

In this study of Scottish populations of the *Cystopteris fragilis* complex, including the 'type population' of *C. dickieana*, spore type could not be correlated consistently with either frond architecture or mulitilocus allozyme phenotype. The lack of correlation between spore type and frond architecture, previously reported by Hagenah (1961) and Haufler & Windham (1991) for North American material and Berg (1992) for Norwegian populations, casts further doubt on the validity of using the presence of rugose spores as the single defining character distinguishing *C. dickieana*. We observed considerable allozyme differences among populations, but extending the allozyme analysis to include the 'type population' provided no support for the recognition of *C. dickieana* as a distinct species. This was true whether the taxon was restricted to the type morphology or extended to include rugose-spored plants with *fragilis*-like fronds, referred to by some in the past as *C. baenitzii*. Our results are consistent with the conclusions of Haufler & Windham (1991), based on North American populations, that *C. fragilis* is an old polyploid with several populational variants. However, before reaching a final conclusion on the status of *C. dickieana*, more breeding and hybridization experiments are required. Phenotypic characters reflect past relationships; it is important also to be aware of current relationships and likely future developments. Hybridization studies, testing any viable hybrid progeny for fertility and genome pairing, should be conducted where plants with *fragilis*-like fronds and rugose spores are crossed with typical *C. fragilis* plants from the same mixed population, as in the *Ben Lawers* population described here and those reported from Norway (Berg, 1992) and the USA (Hagenah, 1961; Haufler & Windham, 1991). Even more revealing would be crosses involving both these forms with material fitting the type description of *C. dickieana* (preferably from the 'type population' and not from garden-cultivated material). As our data revealed considerable differentiation among *Cystopteris fragilis* s.l. populations, we would expect any reduction in fertility of hybrids recovered to correlate with populational differences of the parents rather than morphological, allozymal, or spore traits. However, crosses between the different frond and spore types present within the *Rocky Overhang* population would also be of particular interest. Do the plants with frond architecture similar to that of the type of *C. dickieana* freely interbreed with the *fragilis*-like plants (with non-echinate spores) with which they are growing, and how are the spore types inherited? Evidence of barriers to genetic exchange would strengthen the case for taxonomic recognition at some level, though perhaps not as a species.

On the basis of the evidence presented here, there are no grounds for recognizing the type material of *C. dickieana*, or other members of the *C. fragilis* complex that also have rugose spores, as a separate species. Without having access to the type material or other material from the type locality, Blasdell (1963), Berg (1992) and

Haufler & Windham (1991) reached the similar taxonomic conclusion that *C. dickieana* represents a populational variant in the differentiating polyploid *C. fragilis* and should not be recognized as a species. A decision on whether it justifies recognition at the variety or sub-species level will be influenced by the outcome of breeding and hybridization experiments but it might be useful to recognize some of the more distinct morphologies, such as that seen in the cave at Cove, as forms or varieties to encourage their continued study and protection.

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