Symbiosis, 11 (1991) 119–130
Balaban, Philadelphia/Rehovot

Enzyme Similarity as an Indicator of Evolutionary Divergence: *Stereocaulon saxatile* H. Magn.

DIANNE FAHSELT
Department of Plant Sciences, University of Western Ontario
London, Ontario, N6A 5B7, Canada
Tel. 1-519-679-211 ext. 6480, Fax 1-519-661-3292

Received April 18, 1990; Accepted June 6, 1990

Abstract

Two morphological forms of *Stereocaulon saxatile* H. Magn. occur on granite outcrops in the Canadian Shield region east of Georgian Bay in Ontario, Canada. Since multiple enzyme forms are a reflection of the genome, protein extracts of these two forms were subjected to isoelectricfocussing for indications of evolutionary divergence between them. Six enzymes were assayed including three oxoreductases, two hydrolases and one lyase. Of a total of 83 electromorphs, 60 occurred with a frequency of greater than 70% in at least one of the two morphological forms and statistical analyses were based on the distribution of this subset of bands. Of the 60 high-frequency enzyme forms 36 were constant in all samples. However, ordination and clustering of the variable bands indicated that there was a stronger relationship between samples of like morphology than between those which were different. Similarity between the two morphological types expressed by Jaccard's coefficient was much higher than previously observed between populations belonging to different lichen species. Its magnitude suggested that the two forms were interspecific variants rather than entities which had diverged to a greater degree. Since the two forms grow intermingled in the same habitat and niche, morphological differences between them may be accounted for by the involvement of different strains of mycobionts.

Keywords: *Stereocaulon saxatile* H. Magn., multiple enzyme forms, divergence, evolution, morphological forms

0334-5114/91 /$03.50

1. Introduction

One of the current trends in phylogenetic investigations of lichens is the use of more objective characters, including chemical ones (Jahns, 1989). There have been a few attempts to utilize electrophoretically separated general protein bands (Fahselt and Jancey, 1977; Mattson and Kärnefelt, 1986), and the use of multiple forms of specific enzymes has been explored as well (Hageman, 1989). Hageman also provided detailed information on intra- and interspecific variation of enzyme forms which is fundamental to the application of electromorph data in lichens.

Long-standing concerns regarding the use of electrophoretically separated proteins for systematic purposes pertain particularly to lichens. While analysis of mycobionts suggested that the fungal partner of *Cladonia cristatella* Tuck. was generally responsible for lichen enzyme forms (Fahselt, 1985), the respective contributions of symbionts to electromorphic patterns have been little investigated. Different species of algae in culture appear to produce distinctive isozymes (Kilias, 1987), but it is not clear to what extent photobionts affect enzyme patterns in an intact lichen. Of course pure cultures of mycobionts may be grown for enzyme analysis, as recommended by Kilias (1987), but this may not be desirable for evolutionary studies since at least one lichen enzyme is known to be produced only when both symbiontic partners are present (Martin, 1973).

Another consideration is that there is no information regarding the quaternary structure of most protein electromorphs and thus of possible structural relationships between them. There is no knowledge of the genetic basis for lichen enzyme forms, a situation which differs markedly from that in fungi and higher plants. This means that isozymes of lichens cannot provide such precise information about the genome as they do in other organisms. However, they constitute a class of products which reflects genetic character more closely than morphological or anatomical traits or compounds whose production is mediated through a more complex series of processes. Since they have so far been examined very little, they offer a fresh source of insight into evolutionary relationships among lichens.

The same problems prevail with the use of general protein in phylogenetic studies of lichens as in other groups of organisms (Giannasi and Crawford, 1986). Earlier lichen work involving the use of general protein stains (Fahselt and Jancey, 1977; Mattson and Kärnefelt, 1986) was considerably less exacting than that involving particular enzymes. A great many proteins react with a general stain and discrimination among them is difficult even if two-dimensional separation is used. It is especially critical, therefore, both to utilize

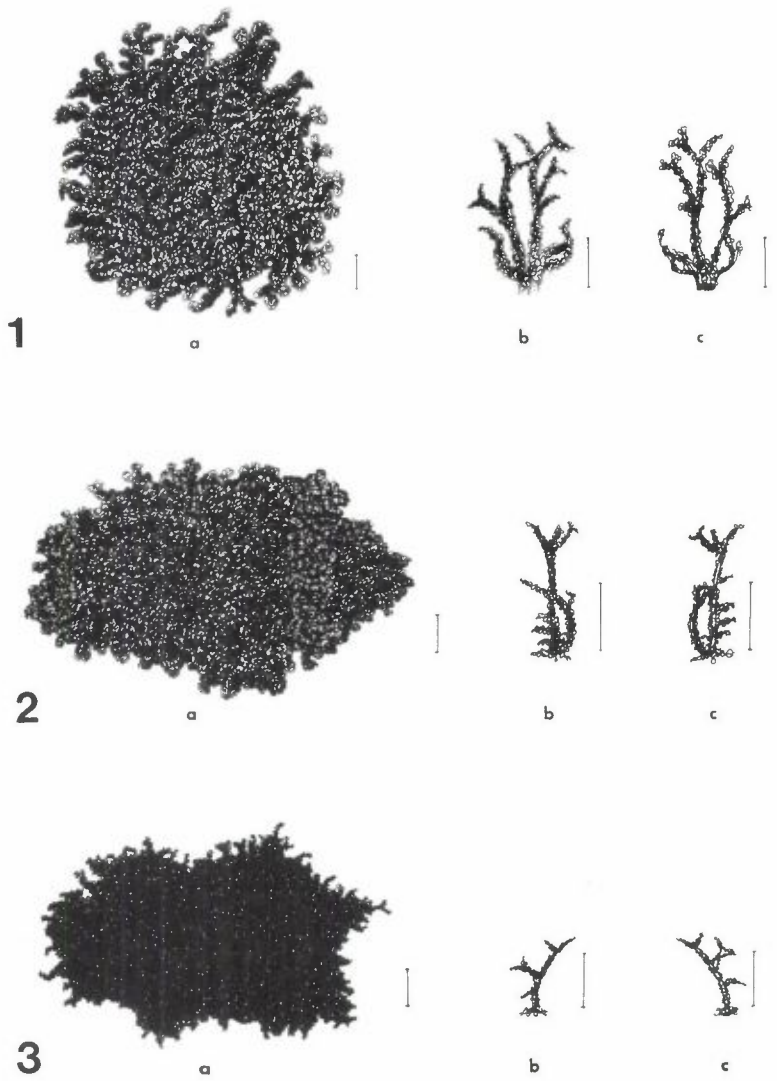
stains which detect specific kinds of enzymatic activity and also to perform simultaneous separations of several test extracts for comparative evaluation of electromorphs. Furthermore, due to the complexity of enzyme complements in lichen thalli, statistical analysis is required for an even-handed interpretation of results.

In earlier papers the sample size was often low. It is now apparent that, though one or two samples may be sufficient to characterize a population with respect to some enzymes, the more polymorphic usually require approximately 10 samples to represent the existing variation (Hageman, 1989). Since esterase is often highly variable, for example, it is difficult to determine whether or not such an enzyme is affected by differing conditions of light and shade (MacFarlane et al., 1983) unless sufficient samples have been analyzed.

Hageman (1989) expressed the level of enzymatic similarity within and between species of the Umbilicariaceae using Jaccard's similarity coefficient (Sj), and found that Sj's between populations of one species are approximately ten times greater than between populations belonging to two different species. Low similarity of enzymes between species suggests evolutionary divergence and indicates a degree of isolation between them. The level of isozyme similarity thus can be applied as one criterion which may be used to evaluate relationships in lichen groups and to document separate or separating entities.

In the field distinctive morphological forms are sometimes encountered which may have become or are in the process of becoming genetically distinct from one another. One example is the many morphological forms of *Cladonia cristatella*. In this species each podetium is small and the habit such that there are not large, discrete, morphologically uniform clumps which lend themselves to exhaustive enzyme analysis, and therefore thalli must be grouped (Fahselt, 1986). *Umbilicaria muhlenbergii* (Ach.) Tuck. is another example of a species with distinct morphological types, but one in which thalli are large enough to be examined individually for enzyme features (Hageman and Fahselt, 1986).

Stereocaulon saxatile H. Magn. has also been described as very variable in morphology, especially in North America (Lamb, 1976), and this lichen has sizeable, distinct cushions or polsters which are ideal for enzyme analysis. Two different growth forms, one in which pseudopodetia are compactly arranged and the other in which they are loosely spreading, co-occur on open granite substrate east of Georgian Bay in the Canadian Shield region of Ontario, Canada. The two forms are easily recognized on the basis of their distinctive appearance and sometimes are found with an intermediate type. In Figs. 1–3, it can be seen that gross morphology depends largely on the length as well as the degree of branching of pseudopodetia. With respect to other aspects of morphology and secondary chemistry, the two forms are the same: loosely adnate



Figures 1–3. Growth forms of *S. saxatile* on granite in the Muskoka region, Ontario, Canada. Figures 1a and 3a represent the most common thallus forms and Fig 2a less frequent type with intermediate appearance. In each case, upper and lower views of the pseudopodetium are illustrated by b and c, respectively. All bar scales are 1.0 cm.

hemispherical polsters or cushions of dorsiventral gray-tomentose pseudopodetia occasionally bearing apothecia and containing lobaric acid and atranorin.

The objectives of this study were, first, to determine whether multiple enzyme forms indicated a genetic distinction between the two morphological types of *S. saxatile* and, second, if a distinction was observed to assess whether it was similar in magnitude to that between separate species.

2. Materials and Methods

All thalli were collected in the Muskoka District, 6 km SW of Gravenhurst, Ontario, Canada (44 54′ N, 79 27′ W) on massive outcrops of granite in a scrub oak forest with a low shrub layer including *Vaccinium* species. In May, 1985, eight samples of each of the two typical growth forms were collected from granite knolls near the south end of Muldrew Lake. The sample size did not permit full enzymatic characterization of the stand, but these initial collections were used to test for enzyme activity. On May 25, 1988, 12 whole cushions of each of the forms were collected about 1 km north of the first collection site, again from granite exposures. Some thalli with intermediate morphology were collected, but material was insufficient for complete enzymatic analysis. Samples were transported to the laboratory immediately, and over a period of approximately 3 days cleaned by removal of dead organic matter, stone fragments, insects, etc., and specimens then were placed in the UWO lichen herbarium. Material was extracted three times with excess acetone on a gyrorotatory shaker for 20 min each time to remove extracellular phenolics and then stored in glass vials at −17°C.

After eight weeks, entire cushions were ground separately in liquid nitrogen. Possibly because a high proportion of older tissues was included, the amount of active protein in the resulting uniform powder was low and it was necessary to use 1.0 g of the pulverized material rather than the usual 0.5 g for each extraction. This amount was homogenized in a ground glass tissue grinder for 5 min at approximately 0°C. Tests for all enzymes in a sample were made on a single extract with replicate gels being subjected to simultaneous isoelectric-focussing on two sets of LKB Multiphor equipment. During the 2.5 hr double runs, remaining extract was sealed and stored at 4°C and then used immediately to prepare the next pair of gels for separation. Other details of methods of staining for the activity of enzymes were those used by Fahselt (1988).

Extracts from the 1985 collections were tested for isocitrate dehydrogenase (IDH, E.C. 1.1.1.41), 6-phosphogluconate dehydrogenase (6PG, E.C. 1.1.1.44), mannitol dehydrogenase (MAN, E.C. 1.1.1.138), glutamate dehydrogenase

(GDH, E.C. 1.4.1.4), one oxidase, esterase (EST, E.C. 3.1.1.1), alkaline phosphatase (ALP, E.C. 3.1.3.1), acid phosphatase (ACP, A.C. 3.1.3.2) and carbonic anhydrase (CAN, E.C. 4.2.1.1), and clear, positive, repeatable results were obtained with 6PG, MAN, GDH, EST, ALP and CAN. The 1988 collections were assayed for these six enzymes, that is, three oxoreductases, two hydrolases and one lyase. On each of the gels extracts of both morphological types were separated simultaneously, and extracts of *Umbilicaria muhlenbergii* were run as reference samples. Stained bands on gels were scored on a presence/absence basis.

The most useful electromorphs for comparative purposes are those which occur dependably, therefore, an arbitrarily chosen minimal level of occurrence was specified for protein bands to be used in analysis. Only those isozymes which were present in at least one of the growth forms with a frequency of 70% or more (Harborne and Turner, 1984) were included in the data matrix that was used to examine group structure.

Electrophoretic results were presented graphically through the use of the complementary techniques of ordination and clustering (Sneath and Sokal, 1973). The program used was SAS (Anonymous, 1986) run under release 5.16 at Tokyo University. Average linkage cluster analysis was used to produce hierarchical groupings of the 24 individual thalli based on a sample X sample covariance matrix. Ordination was performed using an R-type principal components analysis (PCA) based on a correlation matrix between samples. Jaccard's similarity coefficient was determined on the basis of those bands which occurred with a frequency of 70% in one or both of the two morphological forms.

3. Results

A total of 83 different enzyme electromorphs were encountered in the two morphological forms of *S. saxatile* (Table 1). The greatest number, or over half the total, were in the enzymes EST and CAN. However, some of these occurred relatively infrequently, often in only one or two samples. There were nine EST bands which did not achieve a frequency of 70% in either of the morphological forms and seven low-frequency enzyme forms of GDH; MAN and CAN each had a few less frequent bands. Altogether there were 60 multiple enzyme forms with a frequency of 70% or more in at least one of the two morphological types of *S. saxatile*, and 47 of these were found with a frequency of 70% or higher in both. Thus, the Jaccard's coefficient expressing similarity between the two was 0.78. Of the 60 more frequent bands, 24 varied within the sample set while the remainder were invariant and occurred universally in all thalli examined.

Table 1. Numbers of electromorphs in six enzyme systems of two morphological forms of *S. saxatile*. Enzyme names are given in the text and abbreviations here follow those of the International Union of Biochemistry enzyme code.

Category of electromorphs	Enzyme						
	6PG	MAN	GDH	EST	ALP	CAN	Total
Total	10	8	14	23	8	20	83
Frequent +							
In both forms	7	2	6	13	3	16	47
In at least one	10	5	7	14	8	16	60
Constant**	8	0	2	10	2	14	36
Variable*	2	5	5	4	6	2	24

+ Enzyme forms which occurred in at least one of the two morphological types with a frequency of 70% or more.
* Present in some samples, absent in others
** Present in all samples tested

The two main groupings in the dendrogram produced by average linkage clustering (Fig. 4) corresponded exactly to the two thallus types. However, within each type some individuals were a considerable distance from others, e.g., two with the spreading habit were especially different from other thalli with the same morphology and two of the compact type were far removed from others of this type. The thalli which were most different were those lacking several enzyme bands possessed by others. Nevertheless, all joined with individuals of the same morphological form before uniting with others.

The proportions of variation included in the first 5 axes of the principal components analysis were 27.5%, 17.5%, 13.4%, 9.3%, and 7.8%, respectively. Using the scree test (Dillon and Goldstein, 1983) most of the significant variation, including 58% of the total, was judged to be in the first three axes. Eigenvalues of variable enzyme bands are given in Table 2. Isozymes which contributed most to the first axis were one of EST, two of 6PG, one of MAN and three of ALP. Two of EST, two of CAN, three of MAN and one of GDH were important to the second axis and forms of GDH, ALP and MAN all contributed to the third.

Along the first axis, two groups corresponding to the two morphological forms were clearly evident (Fig. 5), a result which was in agreement with the relationships indicated by clustering. Separation according to growth form did not occur on either the second or third axes and each of these indicated enzyme diversity within morphological type. On axis 2 (Fig. 5) thalli of the compact

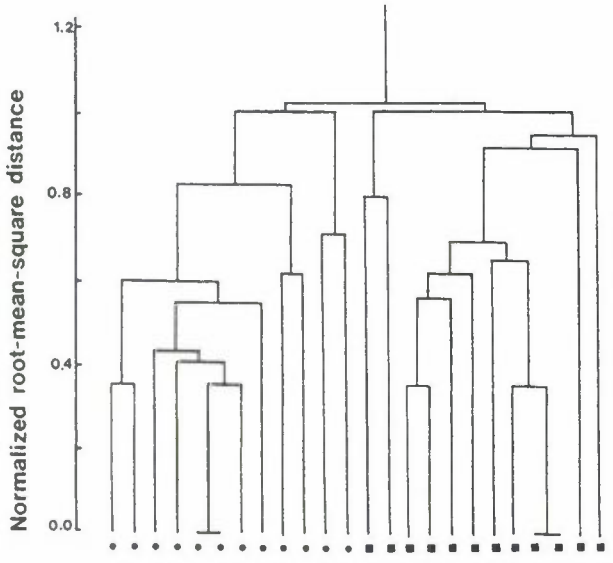


Figure 4. Average linkage clustering of a covariance matrix based on presence/absence of isozyme bands in *S. saxatile* samples from Gravenhurst, Ontario. Circles indicate thalli with a compact growth form and squares those with more loosely-arranged pseudopodetia.

growth form which were outliers in clustering tended to be widely separated from others of the same morphology. The most remote was a thallus in which a number of CAN and EST electromorphs were lacking. On axis 3 (Fig. 6) samples of the compact growth form grouped together but, due to missing GDH and ALP bands in some samples, those with the loosely-spreading habit were more widely dispersed.

4. Discussion

Clustering and ordination both indicated a stronger relationship between *S. saxatile* samples with the same growth form than between those with differing thallus morphologies. This was unlike previously studied morphological forms in the species, *Umbilicaria muhlenbergii*, all of which clustered together within one collection site and exhibited similar enzyme complements (Hageman and Fahselt, 1986). Instead, the level of similarity between the two forms of *S. saxatile*, expressed as Jaccard's similarity coefficient, resembled that between two populations of the same species. The range of Sj values found for over 200 intraspecific comparisons in the Umbilicariaceae ranged between 0.49 and 0.89; in contrast, the maximum Sj for 20 interspecific comparisons was 0.09 (Hageman, 1989). The value of 0.78 found between the

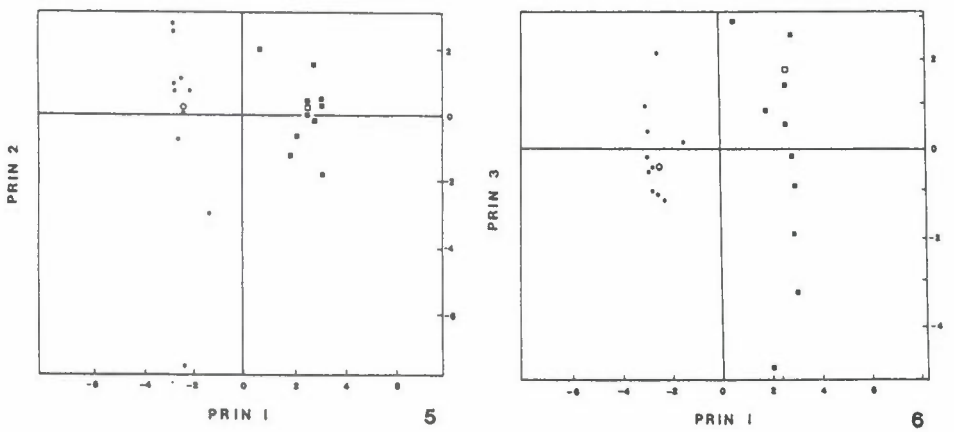
Table 2. Relative contributions of variable enzyme forms to the first 3 axes of PCA. Enzyme abbreviations are those of the International Union of Biochemistry enzyme code.

	Eigenvectors		
Electromorphs	Prin 1	Prin 2	Prin 3
EST – 1	−0.0041	−0.0771	0.1123
EST – 2	0.3175	−0.0014	0.1746
EST – 3	0.0781	0.3825	−0.1423
EST – 4	0.0781	0.3825	−0.1423
CAN – 1	0.0386	0.3605	−0.1287
CAN – 2	0.0892	0.3875	−0.1098
6PG – 1	−0.3358	0.1843	−0.0909
6PG – 2	−0.3358	0.1843	−0.0909
MAN – 1	0.1954	−0.2393	−0.0821
MAN – 2	0.0362	−0.2446	−0.2436
MAN – 3	0.362	−0.2446	−0.2436
MAN – 4	−0.1266	−0.1711	−0.3546
MAN – 5	0.3391	0.0259	0.0029
GDH – 1	0.0402	0.3020	0.0055
GDH – 2	−0.0940	0.0845	0.1268
GDH – 3	0.0248	−0.1701	0.1266
GDH – 4	−0.1574	−0.0016	0.3607
GDH – 5	−0.1756	−0.0643	0.2275
ALP – 1	0.3560	0.0456	0.1593
ALP – 2	−0.0608	0.0508	0.2928
ALP – 3	−0.1545	0.0614	0.4046
ALP – 4	−0.0640	0.0325	0.3248
ALP – 5	0.3560	0.0456	0.1593
ALP – 6	0.3579	0.0569	0.0045
Proportional contributions	27.5%	17.5%	13.4%
Cumulative contributions	27.5%	45.0%	58.4%

two forms of *Stereocaulon*, therefore, was similar to that between conspecific populations rather than populations of two different species. If the enzymes examined reflect evolutionary relationships equally well in umbilicate lichens and in the present study, it would indicate that the two morphological forms of *Stereocaulon* have not diverged to the same extent as at least some lichen species. The occurrence of intermediates in the population also supports the idea that *S. saxatile* is best regarded as one morphologically variable entity which embraces considerable genetic variation.

Lamb (1977) once considered that a separate species was warranted for individuals of *S. saxatile* with loosely-spreading pseudopodetia, but subsequently he came to view it as a variant within the species. Regarding possible genetic distinctiveness of the two Ontario forms from the Muskoka area, it can be likewise concluded that though they differ to a degree, they are probably only as distinct as populations of other lichen species.

An explanation for the enzymatic and morphological differences between the two forms should be considered. Previously, two forms of *Xanthoria elegans* growing in different ecological situations also were determined to be as similar to one another enzymatically as conspecific populations of umbilicate



Figures 5–6. Ordination of 24 thalli of *Stereocaulon saxatile* from one collection site by principal components analysis of enzyme electromorphs. Circles represent compact thalli with smooth appearance and squares those with a loosely-spreading growth form. Open symbols indicate two thalli with identical scores.
Figure 5. Projections of samples on axes 1 and 2.
Figure 6. Projections of samples on axes 1 and 3.

species (Fahselt and Krol, 1989). Enzymatic differences between them were attributed to the fact that collection sites were one or two km distance apart. Their distinct appearances were thought to have been a product of differing environmental influences in the two habitats, one of which was a dry rock surface with moisture derived from annual precipitation of 6 cm/yr and one of which was the bed of a glacial meltwater stream inundated with water for a matter of weeks during the short growing season.

The two forms of *Stereocaulon saxatile* in this study, however, grew side by side in the same habitat on the surface of extensive granite outcroppings. The distance between them was minimal and the seemingly uniform environment was probably not responsible for differences in gross thallus morphology.

Perhaps different strains of mycobiont dominate in the compact and the more spreading forms of *S. saxatile*. While single spores of this species have not been cultured, it may be that each is characterized by distinguishing physiological/anatomical features. Ahmadjian (1973) showed that mycobiont cultures of *Cladonia cristatella* grown from single spores differed from one another in many respects, including growth rate, pigmentation and form. These cultures also produced distinctive enzyme electrophoretic patterns (Fahselt, 1987). Differing fungal components in the two investigated forms of *S. saxatile* might explain the occurrence of two co-existing morphological forms, as well as the enzymatic differences between them.

Acknowledgements

I would like to acknowledge the financial support of the National Science and Engineering Research Council of Canada and the technical assistance of Mr. S. Sweet. I also thank Dr. Y. Hoshino of Tokyo University of Agriculture and Technology for computing assistance.

REFERENCES

Ahmadjian, V. 1964. Further studies on lichenized fungi. *Bryologist* **67:** 87–98.

Anonymous. 1986. *SAS Manual.* SAS Institute Inc., SAS Circle, Cary, NC.

Dillon, W.R. and Goldstein, M. 1984. *Multivariate Analysis: Methods and Applications.* Wiley, New York.

Fahselt, D. 1986. Multiple enzyme forms of morphotypes in a population of *Cladonia cristatella. Bryologist* **89:** 139–143.

Fahselt, D. 1987. Electrophoretic analysis of esterase and alkaline phosphatase enzyme forms in single spore cultures of *Cladonia cristatella. Lichenologist* **19:** 71–75.

Fahselt, D. 1988. Measurement of intrapopulational enzyme variation in five species of epiphytic lichens. *Lichenologist* **20:** 377–384.

Fahselt, D. and Hageman, C. 1986. A comparison of isoenzyme patterns of morphological variants in the lichen *Umbilicaria muhlenbergii* (Ach.) Tuck. *Bryologist* **89:** 285–290.

Fahselt, D. and Krol, M. 1990. Biochemical comparison of two ecologically distinctive forms of *Xanthoria elegans* in the Canadian High Arctic. *Lichenologist* **21:** 135–145.

Giannasi, D.E. and Crawford, D.J. 1986. Biochemical systematics II. A reprise. In: *Evolutionary Biology.* M.K. Hecht, B. Wallace and G.T. Prance, eds. Plenum Press, New York, pp. 25–248.

Hageman, C. 1989. Electromorph variation in the lichen family Umbilicariaceae. Ph.D. Thesis, University of Western Ontario, London, Ontario, Canada.

Harborne, J.B. and Turner, B.L. 1984. *Plant Chemosystematics.* Academic Press, New York.

Jahns, H.M. 1989. III. Systematics, morphology and ecology of lichenized fungi. In: *Progress in Botany.* **50:** 267–283.

Kilias, H. 1987. Protein characters as a taxonomic tool in lichen systematics. In: *Progress and Problems in Lichenology in the Eighties.* Bibliotheca Lichenologica **25:** 445–455.

Lamb, M. 1977. A conspectus of the lichen genus *Stereocaulon* (Schreb.) Hoffm. Hattori Bot. **43:** 191–355.

MacFarlane, J.D., Kershaw, K.A., and Webber, M.R. 1983. Physiological-environmental interactions in lichens. XVII. Phenotypic differences in the seasonal pattern of net photosynthesis in *Cladonia rangiferina. New Phytol.* **94:** 217–233.

Martin, E.J. 1973. Lichen physiology: the invertases of the lichen *Parmelia caperata* (L.) Ach. and its isolated symbionts. Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan.

Mattson, J.E. and Kärnefelt, I. 1986. Protein banding patterns in the *Ramalina siliquosa* group. *Lichenologist* **18:** 231–240.

Sneath, P.H.A. and Sokal, R.R. 1973. *Numerical Taxonomy: the Principles and Practice of Classification.* San Francisco, W.H. Freeman.