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Acrylamide gel electrophoresis of several isozyme systems in 27 *Allium* species and their use in numerical taxonomy

(Elektroforesis gel akrilamida beberapa sistem isozim bagi 27 spesies *Allium* dan kegunaannya dalam taksonomi numerik)

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Key words: acrylamide gel electrophoresis, isozymes, numerical taxonomy

Abstrak

Elektroforesis gel poliakrilamida enam sistem isozim daun bagi 27 spesies *Allium* menunjukkan terdapat 75 gelang elektroforesis, termasuk 23 gelang esterase, 9 asid fosfatase, 15 peroksidase, 10 malat dehidrogenase dan 16 polifenol oksidase dan 2 gelang yang lebar bagi katalase. Data elektroforesis isozim ini telah digunakan dalam analisis taksonomi numerik dengan menggunakan program CLUSTAN. Hasil analisis kluster dan analisis komponen utama menunjukkan hubungkait yang agak rapat antara spesies. Analisis ini secara kasarnya membahagikan spesies-spesies yang diselidiki kepada dua seksi. *Allium tibeticum*, *A. caeruleum* dan *A. azureum* yang buat sementara waktu ditempatkan dalam seksi *Allium* untuk penyelidikan ini didapati tidak mempunyai hubungan sama ada dengan seksi *Cepa* ataupun seksi *Allium* berdasarkan hasil analisis komponen utama.

Abstract

Polyacrylamide gel electrophoresis of six leaf isozyme systems in 27 *Allium* species revealed 75 electrophoretic bands in total which included 23 esterase, 9 acid phosphatase, 15 peroxidase, 10 malate dehydrogenase and 16 polyphenol oxidase bands and 2 broad catalase bands. The isozyme electrophoretic data were used in numerical taxonomic analysis using the CLUSTAN programme. Results of the cluster and principal component analysis indicated fairly good relationships among the species. The analyses broadly divided the taxa into two sections. *Allium tibeticum*, *A. caeruleum* and *A. azureum* tentatively placed in section *Allium* were shown by the principal component analysis to be unrelated to either section *Cepa* or section *Allium*.

Introduction

A large number of electrophoretic variants of enzymes have now been discovered in animals as well as in plants (Shaw 1965). With this knowledge comes the idea that enzymes may exist in the same organism in more than one molecular form. Such multiple molecular forms are designated as isozymes (Markert and Muller 1959).

It is well known that genetic differences are often reflected by alterations in chemical structure and behaviour of particular enzymes (Scandalios 1974). Therefore, the utilization of physico-

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chemical properties of enzymes for taxonomic studies is possible (Boulter et al. 1966).

Acrylamide gel electrophoresis provides a highly reproducible method for resolving plant proteins into several fractions, based upon their electrophoretic mobilities and the molecular sieving action of the gels (Shannon 1968; Katayama and Chern 1973). The ability to separate electrophoretically and stain histochemically the multiple molecular forms of various enzyme systems from extracts of plant materials has proved to be a useful tool in studies dealing with phylogenetic relationships (Torres et al. 1978). Electrophoretic procedures have been employed successfully in several plant genera in genetic and phylogenetical studies (Johnson and Hall 1965; Katayama and Chern 1973).

Changes in zymogram patterns during development have been described for numerous enzymes isolated from a wide spectrum of tissues (Scandalios 1974). This is due to tissue specificity and regulatory action of enzymes. It is therefore necessary to standardize the tissue used for isozyme extractions as well as their developmental stages.

Relatively, little is known of the seed protein or isozyme systems of the genus *Allium*. One of the earliest studies on the isozyme system of *Allium* was that of Makinen (1968). He demonstrated the presence of esterases, acid phosphatase, leucine aminopeptidase (LAP), peroxidases and catalases in onion seedlings by starch gel electrophoresis. Gerbrandy and Verleur (1971), on the other hand, found very low phosphorylase isozyme activity from extracts from different parts of the *Allium* plants.

Etoh and Ogura (1981) surveyed the peroxidase isozymes in the leaves of 93 clones of garlic using thin-layer horizontal polyacrylamide gel electrophoresis. They found a total of seven peroxidase bands distributed over 16 different zymotypes. Nakamura and Tahara (1977) used seed protein, lactate dehydrogenase, glutamate dehydrogenase, α-glycero-phosphate dehydrogenase and carbonic anhydrous to distinguish between four widely divergent *Allium* species i.e. *A. cepa*, *A. fistulosum*, *A. porrum* and *A. tuberosum*. Apart from the studies as above and several others, there are practically very few other investigations on the proteins and isozymes in *Allium* (Klozova et al. 1979).

In this investigation, varieties and species in *Table 1* were analysed with respect to several of their isozyme systems. The zymograms obtained would be used to indicate species relationships using numerical taxonomic methods.

Materials and methods

Isozyme extraction solution and isozyme extraction method

Isozymes were extracted using a solution made up of 0.2 *M* Tris-HCl pH 8.0, 0.1 *M* sucrose and 0.2% cystein hydrochloride. To 500 mL of the above solution was added 10.0 mL of anti-oxidant composed of 1.0 g sodium sulphide and 0.75 g sodium metabisulphide, both dissolved in 100 mL water.

For isozyme extractions, young leaves (first and second leaf) from bulbs were ground in a chilled mortar with the mentioned protein extraction solution. For every 1 g of leaves, 5 mL of the extraction solution was used in the maceration process. The extracts were centrifuged for 20 min at 7 000 rpm at a temperature of 5 °C. The supernatants were used directly for electrophoresis or after storage for a few days in a deep freezer.

For extraction of the polyphenol oxidase isozyme, the anti-oxidant was not added to the extraction solution because of its inhibition effect on polyphenol oxidase activity, which in turn affected staining intensity.

Table 1. Sources of *Allium* species in sections *Cepa* and *Allium* used in the study

Acc. No.	Species	Source/origin
	Section *Cepa*	
261	*A. cepa* cv. The Queen	Dobbies Seed Company, U.K.
203	*A. cepa* cv. White Spartan	Sutton Seed Company, U.K.
7	*A. cepa* var. *viviparum*	Botanisher Garten de Techichen Hochchule, Aachen, Germany. Acc. No. 338
314	*A. fistulosum*	Hortus Botanicus Univ., Budapest, Hungary (HBU). Acc. No. 2335
16	*A. schoenoprasum*	Hortus Botanicus Instituti Scientiarum, Lithuana, U.S.S.R. (HBIS), Acc. No. 153
258	*A. royeli*	Beltsville, U.S.A. Acc. No. C 502
363	*A. galanthum*	National Vegetable Research Station, Wellesbourne, U.K. (NVRS) Acc. No. Do 134
259	*A. vavilovii*	Beltsville, U.S.A. Acc. No. P1405035
14	*A. altaicum*	HBIS, Acc. No. 138
360	*A. sibiricum*	HBU, Acc. No. 2402
319	*A. ledebourianum*	HBU, Acc. No. 2353
365	*A. pskemense*	NVRS, Acc. No. Do 316.
5	*A. ascalonicum*	–
376	*A. chinense*	NVRS
	Section *Allium*	
76	*A. tibeticum*	Berlin
372	*A. ampeloprasum*	Galilee, Israel
297	*A. atroviolaceum*	HBU
207	*A. porrum*	Sutton Seed Company, U.K.
371	*A. babingtonii*	NVRS
338	*A. sativum*	HBU, Acc. No. 2391
2	*A. longicuspis*	HBIS
269	*A. sphaerocephalon*	Marden Nurseries, Kent, U.K.
366	*A. scorodoprasum*	NVRS
243	*A. caeruleum*	Kew, Acc. No. 121–10
273	*A. azureum*	Parkers Bulb Company
370	*A. vineale*	NVRS
310	*A. jailae*	HBU, Acc. No. 2345

Electrophoresis and isozyme staining methods

Electrophoresis was performed in cylindrical 7% acrylamide gels in which 12 running tubes were used. The procedures of Davis (1964) for preparation of small pore gels and the large pore gels were used. Exactly 0.2 mL of isozyme extracts was placed in each tube using small capillary tubes. They were then run electrophoretically after a small amount of reference bromophenol blue stain was placed inside the top reservoir buffer.

Electrophoresis was conducted at 4 °C at about 24 mA (2 mA per tube) for about 15 min and then at 60 mA for about 45 min or until the reference bromophenol blue band had reached almost the bottom of the gel. Tris-glycine (pH 8.7, 0.2 *M*) reservoir buffer was used.

Visualization of enzyme activity after electrophoresis was achieved by histochemically staining the gels. Esterase was stained according to the method of Shaw and Prasad (1970), peroxidase by the method of Graham et al. (1965), malate dehydrogenase by the method of Scandalios

(1969) while catalase and acid phosphatase by the method of Brewbaker et al. (1968). For polyphenol oxidase, the gels were stained for 30 min at 37 °C in the staining solution (0.01 mL-dihydroxyphenyl alanine in 0.05 *M* phosphate buffer, pH 6.8) with vigorous aeration, and then fixed in 7% acetic acid.

Electrophoretic characterization and numerical taxonomic analysis

The migration rates of individual isozyme bands were characterized by their Rf values (after Orf and Hymowitz 1977). In this method, the top surfaces of the small pore gels were arbitrarily designated an Rf value of 0, and the bromophenol blue marker band at the bottom of the gel an Rf value of 1. The Rf value of a particular band was proportional to the distance between the two reference standards.

The data of isozyme bands obtained were then used in numerical analysis. The data were coded in the form of presence or absence of a particular band. The staining intensity and width of a particular band were not taken into consideration.

Numerical analysis was carried out using the CLUSTAN 2 programme. Hierarchical cluster analysis, based on the matrix of similarity values (Euclidean distance), was carried out and the results presented as a dendogram. The dendogram was formed through Wardš (1963) clustering method. Secondly, the data matrix, standardized to zero mean and unit variance as with the clusterings, was used for principal component analysis. Plots of the first against the second eigenvector as well as the third against the fourth eigenvector were made.

Results and discussion

Esterase

A total of 23 different bands was observed for this isozyme system and diagrammatically represented in *Figure 1*. In this figure as in *Figure 2* to *Figure 4*, the staining intensity of the isozyme bands are represented by the three shades of darkness; dark bands are for darkly stained isozyme bands while lightly shaded bands are for lightly stained bands. Band widths are also represented graphically in the figures, there being wide, narrow and intermediate size bands. From this zymogram, it could be seen that there was one band at Rf 0.35 that was usually wide and darkly stained and present in all the accessions analysed. The next most important bands were the two bands occurring below the major band at Rf 0.4 and 0.44.

The upper half of the gels (above Rf 0.35) showed large diffused areas. These could be enzymes that were not completely freed from particulate matter, or were non-specific aggregate of proteins, or were hydrolytic enzymes contained within pocket-like organelles and not large enough to be sedimented during the centrifugation process.

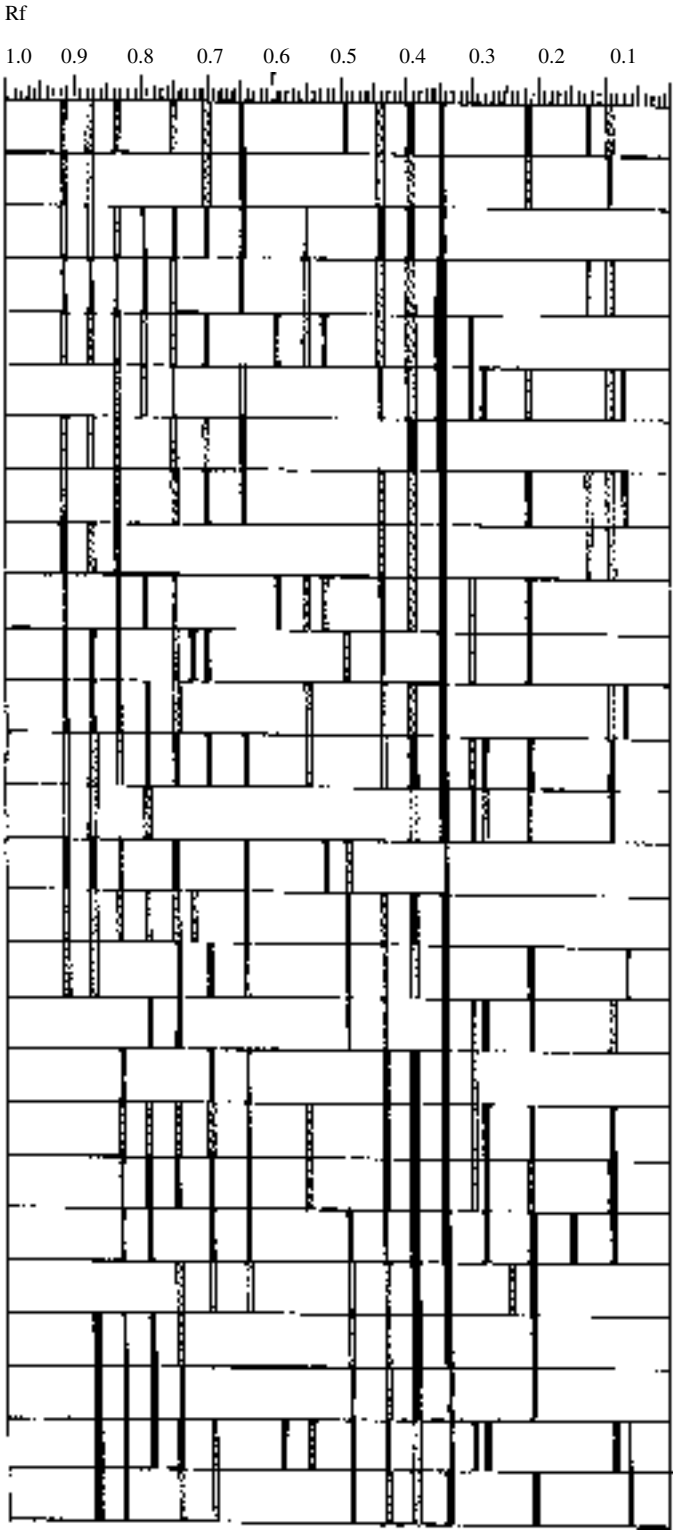
From the examination of the zymogram pattern, it was not possible to assign any specific band to a particular taxon. It was also not possible to assign any specific band pattern characteristic of the two *Allium* sections studied.

Acid phosphatase

A total of nine bands with acid phosphatase activity was observed *(Figure 2)*. A band at Rf 0.235 was thick and darkly stained in all the species. Another band at Rf 0.31 was also present in many of the species studied. Three other bands at Rf 0.59, 0.77 and 0.83 were all important bands occurring in many of the species. Here again it was not possible to identify specific or sectional acid phosphatase band pattern.

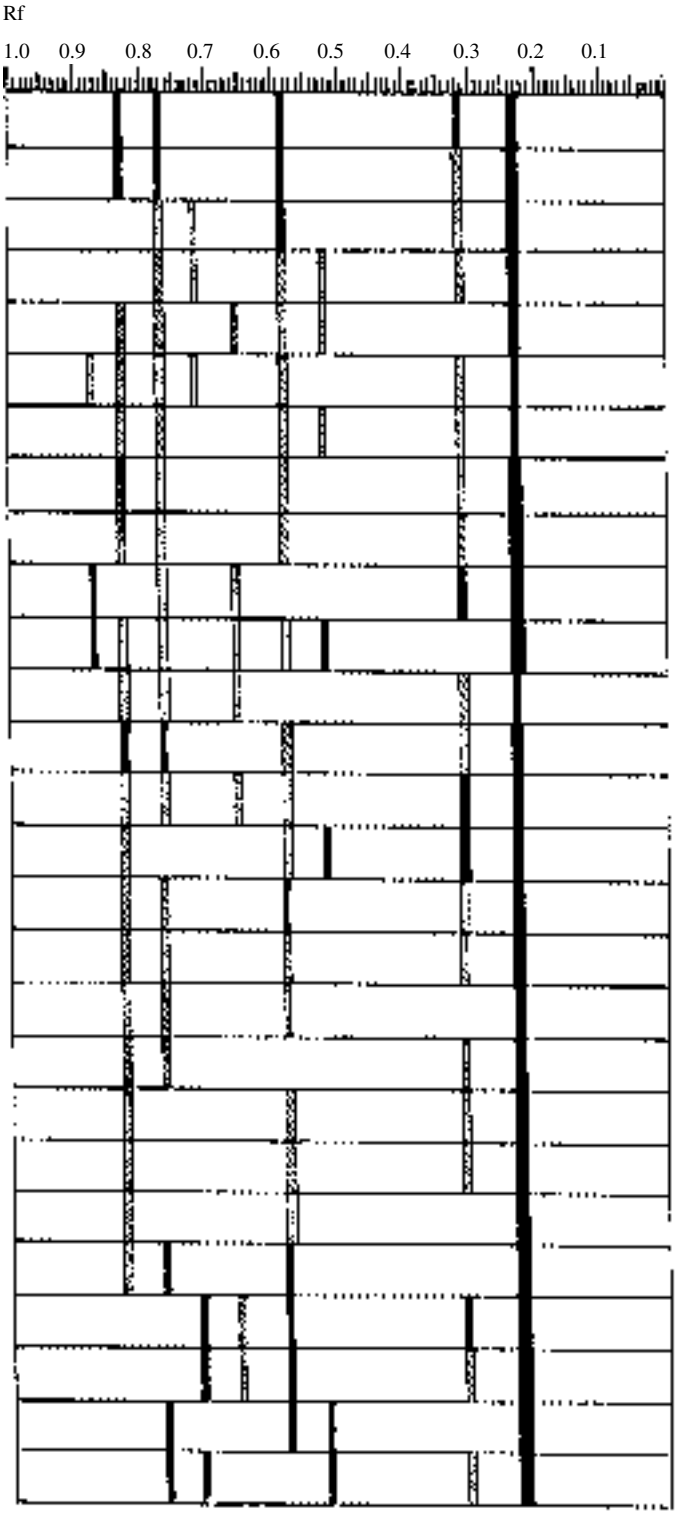
Peroxidase

The peroxidase isozyme bands in *Allium* leaves observed are diagrammatically represented in *Figure 3*. Fifteen bands in total were observed. Etoh and Ogura (1981) found a total of seven observable bands amongst 93 clones of garlic studied. The two accessions of garlic and its close



Acc. No.	Taxon
261	*A. cepa* (2 x)
203	*A. cepa* (2 x)
7	*A. cepa* var. *viviparum* (2 x)
314	*A. fistulosum* (2 x)
16	*A. schoenoprasum* (2 x)
258	*A. royeli* (2 x)
363	*A. galanthum* (2 x)
259	*A. vavilovii* (2 x)
14	*A. altaicum* (2 x)
360	*A. sibiricum* (4 x)
319	*A. ledebourianum* (4 x)
365	*A. pskemense* (2 x)
5	*A. ascalonicum* (2 x)
376	*A. chinense* (3 x)
76	*A. tibeticum* (2 x)
372	*A. ampeloprasum* (6 x)
279	*A. atroviolaceum* (6 x)
207	*A. porrum* (4 x)
371	*A. babingtonii* (6 x)
388	*A. sativum* (2 x)
2	*A. longicuspis* (2 x)
269	*A. sphaerocephalon* (3 x)
366	*A. scorodoprasum* (4 x)
243	*A. caeruleum* (2 x)
273	*A. azureum* (2 x)
370	*A. vineale* (4 x)
310	*A. jailae* (2 x)

Figure 1. Esterase zymogram of Allium leaf extracts



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371	*A. babingtonii* (6 x)
388	*A. sativum* (2 x)
2	*A. longicuspis* (2 x)
269	*A. sphaerocephalon* (3 x)
366	*A. scorodoprasum* (4 x)
243	*A. caeruleum* (2 x)
273	*A. azureum* (2 x)
370	*A. vineale* (4 x)
310	*A. jailae* (2 x)

Figure 2. Acid phosphatase zymogram of Allium leaf extracts

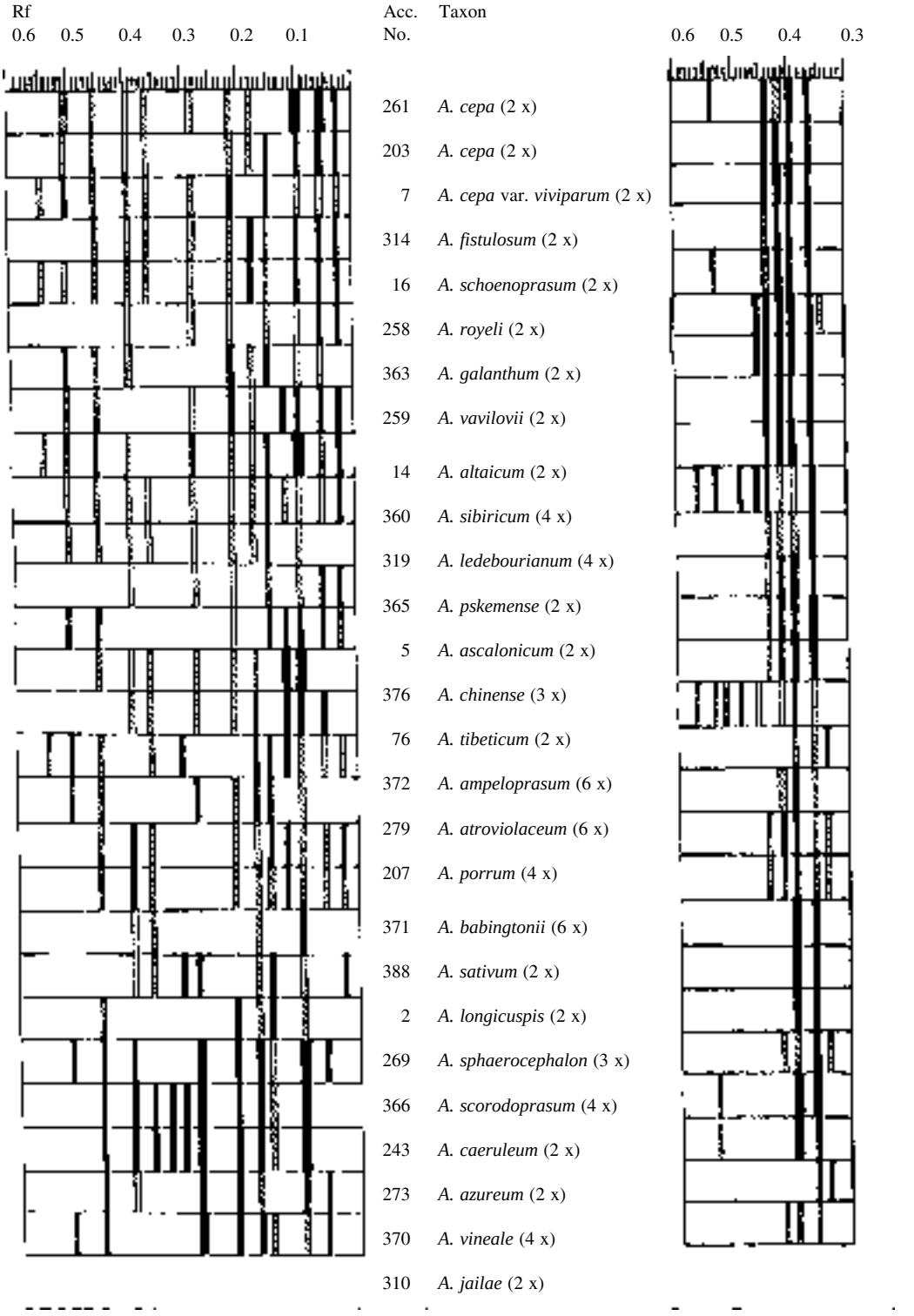
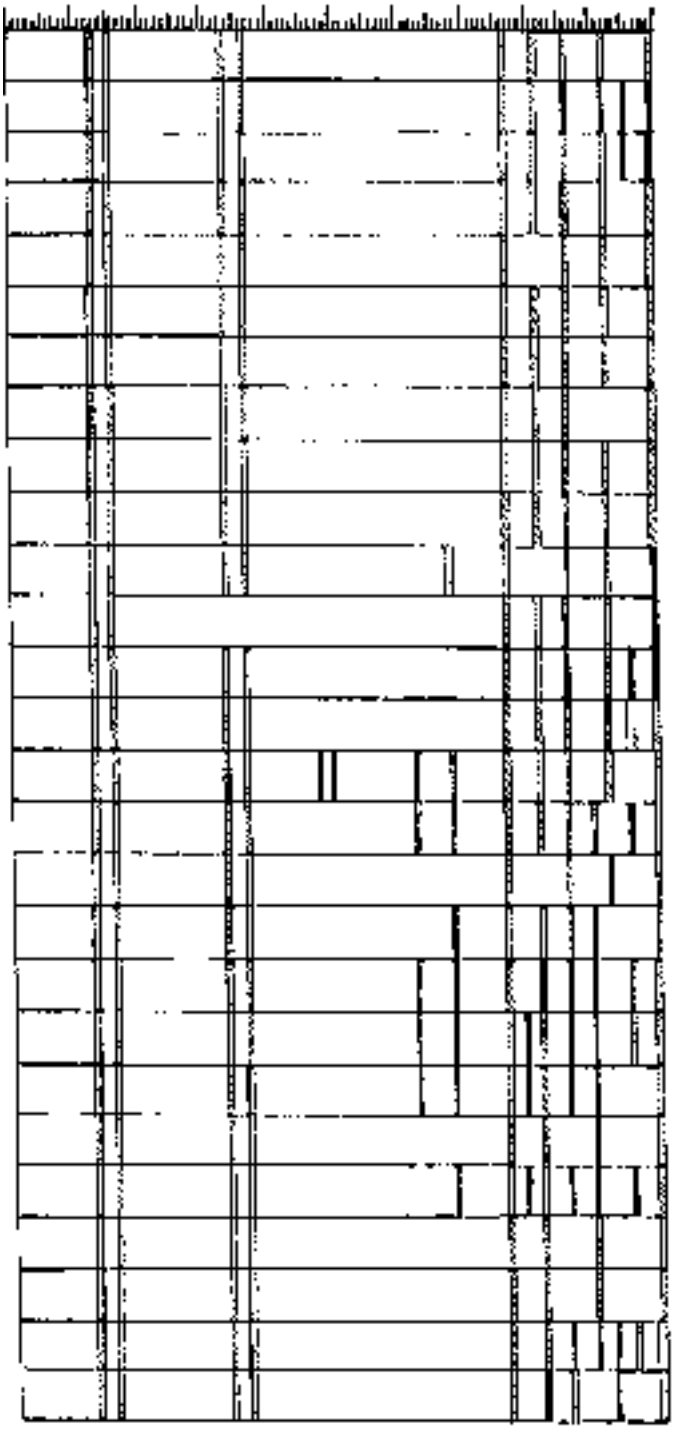


Figure 3. Peroxidase (left) and malate dehydrogenase (right) zymograms of Allium leaf extracts



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370	*A. vineale* (4 x)
310	*A. jailae* (2 x)

Figure 4. Polyphenol oxidase zymogram of Allium leaf extracts

relative, *A. longicuspis* here were found to have 5–7 peroxidase bands each. Makinen (1968) found three dark coloured peroxidase bands using *A. cepa* coleoptile extracts. Nakamura and Tahara (1977), however, found very weak reaction for this isozyme in four different *Allium* species.

From the zymogram, it could be seen that most of the peroxidase bands were concentrated on the upper half of the gel. One band at Rf 0.09 was present in all the species and accessions. As in the two isozyme systems earlier, differences between species and accessions could only be inferred from the presence or absence of bands at the different Rf points in the zymogram.

Malate dehydrogenase

A total of 10 greenish-blue bands were observed *(Figure 3)*. Two bands at Rf 0.415 and 0.445 were homomorphic. All the bands were concentrated near the middle part of the gel between Rf 0.4 and Rf 0.6.

Most of the species studied possessed 3–5 bands, except for accessions of *A. sativum*, *A. longicuspis* and *A. sphaerocephalon* which possessed two thick, densely coloured bands only. The tetraploid *A. sibiricum* and *A. tibeticum*, on the other hand, showed eight bands each.

Nakamura and Tahara (1977) found that the malate dehydrogenase zymograms for *A. cepa*, *A. fistulosum*, *A. porrum* and *A. tuberosum* were similar in having the same number of bands.

Polyphenol oxidase

The polyphenol oxidase isozyme zymogram for the 27 *Allium* species is shown in *Figure 4*. Sixteen bands in total were observed. Four bands, one at the extreme tip of the gels, another at Rf 0.24, as well as those at Rf 0.845 and 0.87 were common for all the accessions studied.

From the zymogram, it could be seen that most of the species shared the same band pattern. Most of the bands were concentrated at the top part of the gels with only two double bands occurring in the lower half of the gels. Four bands occurring between Rf 0.32 and Rf 0.52 were found in the profile of several accessions, particularly those in section *Allium* and in *A. schoenoprasum*. Not all the four bands though occurred together in any accession except in *A. tibeticum*.

Catalase

The pattern of the catalase zymogram was found to be almost similar in all the species studied. The zymogram consisted of a very broad band of catalase activity from Rf 0.1 to Rf 0.31. This broad band could well be made up of two broad bands, as in some runs the broad band was found to be dissected by a thin region of inactivity in some of the accessions. This type of zymogram was also found in barley (Almsgard and Norman 1970). Makinen (1968) also found broad white zones at approximately the same position in the zymogram of *A. cepa*.

Numerical taxonomic analysis of isozyme electrophoretic profiles

The dendogram based on the hierarchical cluster analysis of 75 leaf isozyme electrophoretic band characters *(Figure 5)* showed that there was a split at 12% dissimilarity level. The cluster of species which formed a split at this level consisted of accessions of *A. ampeloprasum*, *A. porrum*, *A. sativum*, *A. longicuspis*, *A. sphaerocephalon*, *A. scorodoprasum* and *A. babingtonii*, all of which are usually included in section *Allium* as well as *A. pskemense* of section *Cepa,* and *A. azureum* and *A. caeruleum* which are usually not included in either section. Within this cluster, *A. sativum* seemed to be closely related to *A. longicuspis*, and *A. ampeloprasum*, *A. babingtonii* and *A. scorodoprasum* were closely related to one another. *A. azureum* and *A. caeruleum* seemed to be distantly related to other taxa in this cluster.

The second split in the dendogram

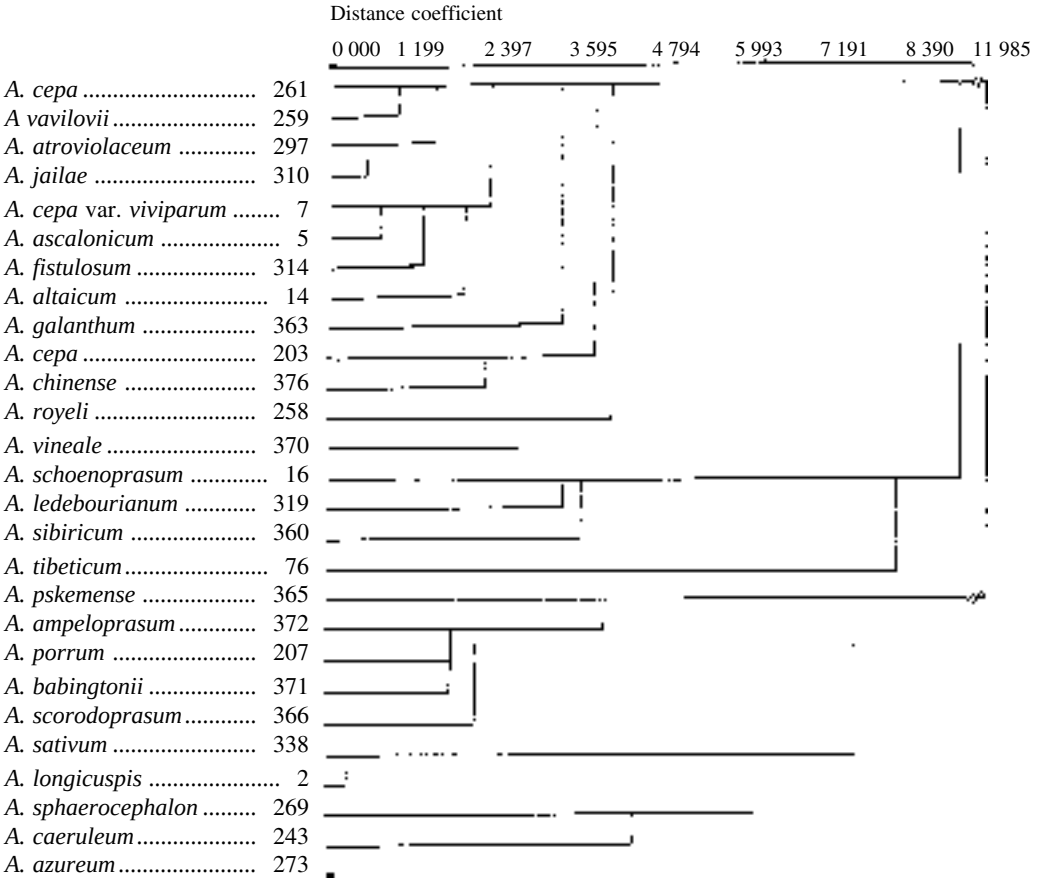

Figure 5. Dendogram showing group average clustering of 27 accessions using Euclidean distances, based on the cluster analysis of leaf isozymes electrophoresis zymograms

occurred at 9% dissimilarity level. The cluster of species which split at this level consisted of species of the '*Schoenoprasum Alliums*', i.e. *A. schoenoprasum*, *A. ledebourianum* and *A. sibiricum*, as well as *A. tibeticum*. Although *A. tibeticum* was a member of this cluster, the dendogram showed that it was distantly related to other members of the cluster.

Results of the principal component analysis *(Table 2)* showed that less than half of the total variance was encompassed within the first four eigenvectors. The plots of eigenvector 1 against eigenvector 2 showed that the taxa were scattered into two broad zones corresponding to the two sections into which the taxa are classified. *A. tibeticum* which clustered together with species in section *Cepa* in the dendogram of *Figure 6* is seen in the scatter to be unrelated to species in section *Cepa* or in section *Allium*. From the scatter, *A. pskemense* is seen to be more closely related to members of section *Cepa* than to species in section *Allium* in contrast to the relationship depicted in the dendogram.

The plot of eigenvector 3 against eigenvector 4 did not reveal any significant taxonomic information, probably due to the small amount of variance encompassed within these two eigenvectors. The only information that could be drawn from the scatter plots is that *A. tibeticum*, *A. caeruleum* and *A. azureum* are distantly related to all other species investigated. This was judged from the distance at which their

Table 2. Eigenvalues 1–10, together with percentage and cumulative variance for the principal component analysis of 75 leaf isozyme electrophoresis band characters

Eigenvector	Eigenvalue	Percentage variance	Cumulative variance
1	9.68	12.90	12.90
2	6.45	8.60	21.51
3	5.16	6.88	28.39
4	4.78	6.37	34.76
5	3.82	5.09	39.85
6	3.55	4.73	44.58
7	2.96	3.94	48.52
8	2.82	3.75	52.27
9	2.45	3.27	55.54
10	2.25	3.00	58.54

scatter points were distributed in relation to the region encompassing all other species.

Results of the cluster and principal component analysis considered together seem to indicate fairly good relationships amongst the species. The analyses broadly divided the taxa into two sections. *A. tibeticum*, *A. caeruleum* and *A. azureum* were shown by the principal component analysis to be unrelated to either section *Cepa* or section *Allium*.

The discrepancies in the results shown by both methods of analyses concerned the close relationships of *A. atroviolaceum*, *A. jailae* and *A. vineale* to species in section *Cepa* than to species in section *Allium* in which they naturally belonged.

Conclusion

Leaf isozyme electrophoresis resulted in the detection of 75 isozyme electrophoretic bands altogether from the six isozyme systems analysed. However, it was not possible to delimit species using a particular isozyme system or by consideration of all the isozyme systems studied. However, results of the cluster and the principal component analysis considered together gave a fairly good picture of relationships among the species. The species studied was broadly divided into two sections. *A. tibeticum*, *A. caeruleum* and *A. azureum*, not normally included in either sections *Cepa* or *Allium,* were shown by the principal component analysis to be unrelated to either sections.

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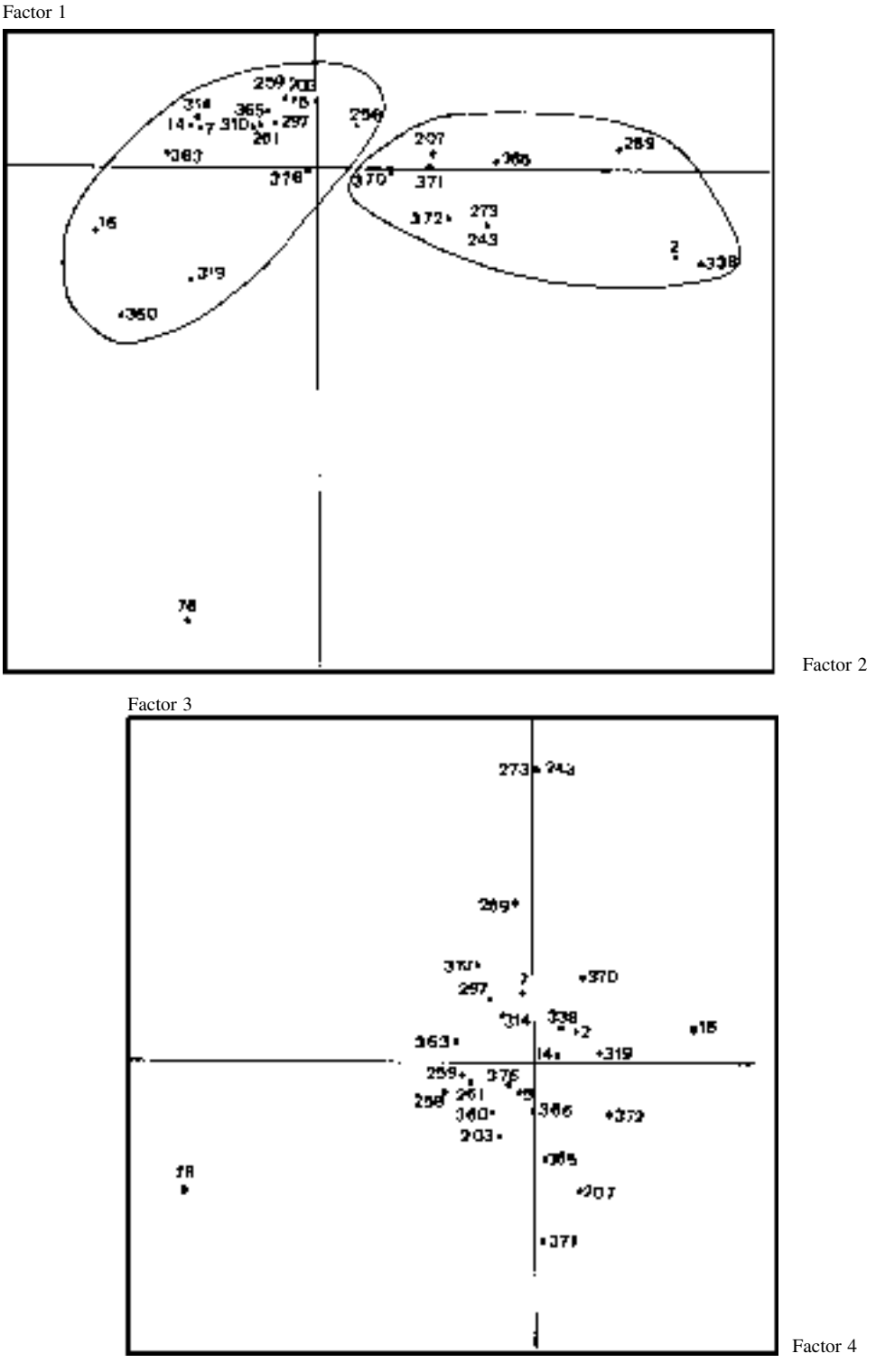

Figure 6. Principal component analysis of leaf isozymes zymograms

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